Open Access Contents
Int. J. Drug Dev. & Res.
| April - June 2014 | Vol. 6 | Issue 2 | ISSN 0975-9344 |
www.ijddr.in

Full Length Original Research Paper

Omega 3 – Fatty Acid (Epa and Dha) Rich Salmon Fish Oil Enhance Anti-Psoriatic activity of Glucocorticoid (Betamethasone Dipropionate) in Nano Form

ǂSARFARAZ AHMAD[1]

NITESH KUMAR[2]

[1]Faculty of Health Sciences, Department of Pharmacy, Sri Venkateshwara University, Gajraula, Amroha, [2]Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science & Animal Husbandry, Rewa, India.

Corresponding Authors:
Sarfaraz Ahmad
Email: sarfaraz3030@gmail.com

Abstract:
The aim of the present study was to investigate the potential of nanoemulsion formulation for topical delivery of Betamethasone Dipropionate (BD) using Salmon fish oil (containing omega-3 fatty acids) as the oil phase. BD has anti-inflammatory, immunomodulatory and antiproliferative activity. However, its clinical use is restricted to some extent due to its poor permeability across the skin. Salmon fish oil was used as the oil phase and was also exploited for its anti-inflammatory effect along with BD in the treatment of inflammation associated with psoriasis. Nanoemulsion formulations were prepared by aqueous phase titration method, using Salmon fish oil, tween 80, Transcutol P and water as the oil phase, surfactant, co-surfactant and aqueous phase respectively. Furthermore, different formulations were subjected to evaluate for ex vivo permeation and in vivo anti-inflammatory and irritation study. The optimized nanoemulsion was converted into hydrogel-thickened nanoemulsion system (HTN) using carbopol 971 and had a viscosity of 98.07 ± 0.07 mP. The optimized formulation had small average diameter (129.89 nm) with zeta potential of -36.09 mV which indicated good long-term stability. In vivo anti-inflammatory activity indicated 85.22% and 33.31% inhibition of inflammation for drug loaded and placebo formulation respectively. Anti-inflammatory activity of placebo nanoemulsion reveals that Salmon fish oil having Anti-inflammatory activity and in combination with BD may be useful for psoriasis treatment in future.

Keywords: Salmon fish oil, Anti-inflammatory study, Betamethasone Dipropionate, Irritation study, Nanoemulsion.

Introduction

Psoriasis is a common dermatological condition affecting 2% of the population. It is a chronic (prolonged) inflammation of the skin characterized by erythematous scaly plaques. Only the superficial regions of the skin i.e. epidermis and dermis are affected (1-3). It can cause red, itchy and scaly skin. The disease is more common in women with approximately 1 in 5 women experiencing it at some point during their lives (4). The symptomatic treatment of psoriasis involves the use of topical corticosteroids particularly because of their vasoconstrictive, anti-inflammatory, immunosuppressive and antiproliferative effects (5). Among the various corticosteroids, Betamethasone Dipropionate (BD) is the drug of choice for the reason that it is highly potent (6). It exerts its action by inhibition of phospholipase A_2 which leads to the inhibition of synthesis of arachidonic acid and controls the biosynthesis of prostaglandins and leukotrienes (7). However, there a few drawbacks associated with the use of topical BD such as skin atrophy, steroid acne, telangiectasia, hypopigmentation and poor absorption through skin (8). The main limitation lies in the barrier function of the skin, which is considered one of the most impermeable epithelia of the human body to exogenous substances. Therefore, the major challenges for a topical formulation are to provide a sufficient increase in drug penetration into the skin, without inducing any significant irreversible alteration to

the skin barrier function (9-11). There has been increased interest during recent years in the use of topical vehicle systems that could modify drug permeation through the skin. Many of the dermal vehicles contain chemical enhancers and solvents to achieve these goals. But use of these chemical enhancers may be harmful, especially in chronic application, as many of them are irritants. Therefore, it is desirable to develop a topical vehicle system that does not require the use of chemical enhancers to facilitate drug permeation through the skin. Although several attempts have been made to alleviate the adverse effects and improve the therapeutic efficacy. Various colloidal carriers such as poly(d, l-lactic-co-glycolic acid) (PLGA) microspheres , solid lipid nanoparticles, lipid nanospheres, nanostructured lipid carriers, polymeric nanocapsules and lecithin/chitosan nanoparticles containing BD have been developed to enhance the therapeutic efficacy.

Recent novel nanotechnology based delivery systems offer unique properties and substantially improves applications in topical delivery. Nanoemulsions are submicron oil-in-water (o/w) emulsions with mean droplet diameters ranging from 50 to 500 nm (12). Studies have proved that smaller particle size in nanoemulsions ensure close contact with stratum corneum thereby improving the absorption and therapeutic concentration of poorly water soluble drug in the target tissue. In addition it ensures low skin irritation, high solubilisation capacity for hydrophilic and lipophilic drugs, and high drug-loading capacity for topical delivery (13). Nanoemulsions act as a depot for slow and controlled release and reduce the frequency of drug administration thus diminishing adverse effects (14).

The main aim of the present study was to evaluate the potential of Salmon fish oil which contains DHA and EPA (15) along with BD in the treatment of inflammation observed in psoriasis, in the form of nanoemulsion using Salmon fish oil as the oil phase and other non irritating pharmaceutical acceptable ingredients without using penetration enhancers. Salmon fish oil was used as an excipient as well as an active ingredient. The low viscosity of nanoemulsion restrains its clinical application due to inconvenient use, therefore hydrogel- thickened nanoemulsion (HTN) system were formulated with good stability, powerful permeation ability and suitable viscosity for the topical delivery which provided longer contact with skin. The hydrogel in the formulation will enable close proximity of the formulation with the skin facilitating cutaneous absorption of the active moiety. This will lead to accumulation of drug in the skin and prevent leaching of the drug into systemic circulation. Salicylic acid added in external phase during gel preparation for the removal of superficial dead cell from skin so that skin becomes more permeable for drug (16). The present investigation focused on the preparation and characterization of HTN system with BD, ex vivo permeation studies, in vivo irritation study and in vivo anti-inflammatory activity. The long-term goal of this work was to develop safe topical BD formulations for clinical use to increase the anti-psoriatic activity.

MATERIALS AND METHODS

Materials

BD was obtained as a gift sample from Ranbaxy Research Laboratory (Gurgaon, India). Omega 3 fatty acid enriched Salmon fish Oil was a gift sample from Shandong Yuwang Pharmaceutical

Co., Ltd. China. PEG 400, Tween 80, Tween 20 and ethanol was purchased from Merck (Merck, India). Caprylo caproyl macrogol-6 glycerides (Labrasol), diethyleneg- lycol monoethyl ether (Transcutol P) and Plurol Oleique were obtained as a kind gift sample from Gattefosse (Mumbai, India). All other chemicals were of analytical grade.

Screening of Excipients

An important criterion for screening of components for high loading of drug and more stability in nanoemulsions is the solubility/miscibility of drug in oil, surfactant and co-surfactant. For determination of solubility of BD in Salmon fish oil, an excess amount of BD was added to each 5-mL capacity stopper vial and mixed using a vortex mixer (Nickel- Electro Ltd., Oldmixon Crescent, UK). The mixture vial was then kept at 37 ± 1 °C in an isothermal shaker (Nirmal International, New Delhi, India) for 72 h to get to equilibrium. The equilibrated samples were removed from the shaker and centrifuged at 3000 rpm for 15 minutes. The supernatant was taken and filtered through a 0.45 µm membrane filter. The concentration of BD was determined in Salmon fish oil by UV spectrophotometer (Shimadzu, Kyoto, Japan) at 241 nm. For selection of surfactant and co-surfactant, miscibility of fish oil was done with a number of surfactants like Tween 20, Tween 80, Labrasol and Tween 60 and co-surfactants like Ethanol, Transcutol P, Plurol olequie, PEG 200 and PEG 400, in 1:1 ratio (oil: surfactant/co-surfactant). Observations were done visually for miscibility. The mixtures which were clear/ transparent in a ratio of 1:1 (v/v) were considered for further studies.

Phase Studies

On the basis of solubility/miscibility studies, Salmon fish oil was selected as the oil phase, Tween 20 as a surfactant and Transcutol P as a cosurfactant. Double distilled water was used as an aqueous phase to avoid surface active impurities. Surfactant and co-surfactant were mixed (Smix in different weight ratios (1:0, 1:1, 2:1, 3:1, 4:1, 5:1) with increasing amount of surfactant with respect to co-surfactant. Sixteen different combinations of oil and Smix (1:9,1:8, 1:7, 1:6, 1:5 1:4, 1:3.5, 1:3, 3:7, 1:2, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1) were made so that maximum ratio could be covered for the study to delineate the boundaries of the phases formed precisely in the phase diagrams. For the determination of existing zone of nanoemulsion, pseudoternary phase diagrams were constructed using aqueous phase titration method. Slow titration with the aqueous phase was done for each weight ratio of oil and Smix, and visual observations were made for transparent and easily flowable oil-in-water (o/w) nanoemulsions. The physical state of nanoemulsion was marked on a pseudo three component phase diagram with one axis representing the aqueous phase, second representing oil and the third representing a mixture of surfactant and co-surfactant at fixed weight ratio (Smix ratio) (16).

Selection of Formulations

Among the pseudoternary phase diagrams showing maximum nanoemulsion area, a number of formulations were selected covering the entire range of nanoemulsion occurrence in the phase diagrams with minimum surfactant and maximum water concentration. Exactly 0.05% w/w of BD, which was kept constant in all the selected formulations, was added to the oil phase during the formulation of nanoemulsions. Selected

Covered in Scopus & Embase, Elsevier
Int. J. Drug Dev. & Res., April - June 2014, 6 (2): 61-76

formulations were subjected to various physical stability tests (17-18).

Physical Stability Studies

To overcome the problem of metastable formulations, physical stability tests were performed. The selected nanoemulsions were subjected to centrifugation at 5000 rpm for 30 min. The formulations that did not show any phase separations were taken for the heating and cooling cycle. Six cycles between refrigerator temperature (4°C) and (45°C) with storage at each temperature of not less than 48 h were done. The formulations which were found stable were subjected to a freeze-thaw cycle test. Formulations were kept in deep freezer (Vestfrost, Delhi, India) at 20 °C for 24 h. After 24 h the nanoemulsions were removed and kept at room temperature. The physically stable nanoemulsions returned to their original form within 2-3 minutes, 3 such cycles were repeated (19-20).

Characterization of Nanoemulsions

Particle Size and Zeta Potential

The average size and polydispersity index of the nanoemulsion droplets were determined by photon correlation spectroscopy (Nano ZS90, Malvern Instrument, U.K.) which is based on the principle of dynamic light scattering. The measurements were performed using a He-Ne laser at 633 nm by using Avalanche photo diode detector. Light scattering was monitored at 25°C at a 90° angle. Droplet size distribution studies were performed at refractive index of 1.40 because the refractive index for all formulation was in this range. The viscosity and dielectric constant of the medium were set at 4.55 mPas and 79.4 respectively. Zeta potential was determined by using second generation PALS (Phase Analysis Light Scattering), called M3PALS which measures the particle velocity.

Refractive Index, pH and Viscosity

Viscosity of nanoemulsion was determined by using Brook- field DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Middleboro, MA). Refractive index was determined for different nanoemulsion formulations by using Abbe's refractometer (Nirmal International, Delhi, India) at 25°C in triplicate. The pH was determined for the optimized nanoemulsions by using a calibrated digital pH meter (Mettler Toledo MP 220, Greifensee, Switzerland) in triplicate at room temperature.

Ex Vivo Skin Permeation Studies

Ex vivo skin permeation studies were performed on a fabricated Franz diffusion cell with an effective diffusional area of 3.14 cm^2 and 5 ml of receiver chamber capacity using rat abdominal skin. The full-thickness rat skin was excised from the abdominal region, and hairs were removed with an electric clipper. The subcutaneous tissue was removed surgically, and the dermis side was wiped with isopropyl alcohol to remove adhering fat. The cleaned skin was washed with distilled water and stored in the deep freezer at -21 °C until further use. The skin was brought to room temperature and mounted between the donor and receiver compartment of the Franz diffusion cell, where the stratum corneum side faced the donor compartment and the dermal side faced the receiver compartment. Initially the donor compartment was empty and the receiver chamber was filled with acetate buffer pH 5. The receiver fluid was stirred with a magnetic rotor at a speed of 100 rpm, and the assembled apparatus was placed in the oven and the temperature was maintained at 37 ± 1°C. All the

receiver fluid was replaced every 30 min to stabilize the skin. It was found that the receiver fluid showed negligible absorbance after 4.5 h and beyond, indicating complete stabilization of the skin. After complete stabilization of the skin, 1 ml of nanoemulsion formulation (0.5 mg/ml clobetasol propionate) was placed into each donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular intervals (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 20, 22, and 24 h), filtered through a 0.45µ membrane filter, and analyzed for drug content by UV spectrophotometer at 241 nm (21).

Permeation and Distribution Data Analysis

The cumulative amount of BD permeated through the albino rat skin (Q, g/cm^2 was plotted as a function of time (t, h) for each formulation. The permeation rate (flux) at the steady state (Jss, g/cm^2 /h) and lag time were calculated from the slope and intercept of the straight line obtained by plotting the cumulative amount of BD permeated per unit area of skin versus time at steady state condition respectively. Permeability coefficient (K_p was calculated by dividing the flux by initial drug concentration (C_o in the donor portion of cell as given below:

$Kp = Jss/C_o$

Enhancement ration (E_r was calculated by dividing the Jss of the respective formulation by the Jss of the control formulation as given below:

E_r = Jss of formulation/Jss of control

Surface Morphology by Transmission Electron Microscopy

Morphology and structure of the nanoemulsion were studied using Morgagni 268D transmission electron microscopy (TEM) (FEI, Netherland) operating at 70 KV and capable of point to point resolution. Combination of bright field imaging at increasing magnification and diffraction modes were used to reveal the form and size of nanoemulsion droplets. In order to perform the TEM observations, a drop of nanoemulsion was applied on carbon coated grid with 2% phosphotungstic acid (PTA) and was left for 30 sec. The dried coated grid was taken on a slide and covered with a cover slip. The slide was observed under the electron microscope.

Formulation of Nanoemulsion Gel Containing Salicylic Acid

The very low viscosity often exhibited by nanoemulsion is inappropriate for topical use. The viscosity can be increased by adding thickening agents, which also change the appearance of the system, usually influencing drug release. Recently the gel matrices such as carbopol 934, xanthum gum, carrageenan, sodium alginate, ethyl cellulose and HPMC have been used to prepare the nanoemulsion based gel for improving the viscosity of nanoemulsion. The selection of polymer for preparing gel is normally based on the character of external phase (oil for w/o type and water for o/w type). Since betamethasone dipropionate nanoemulsion is a type of o/w type so ethyl cellulose, sodium alginate, carbopol 934 and HPMC was selected for preparation of nanoemulsion gel. For preparation of nanoemulsion gel initially 3 gram of carbopol 934 and 5gram salicylic acid were added in 87.68 ml of the water and stirred with magnetic stirrer till homogenous mixture was obtained. Then added drop by drop already prepared nanoemulsion of 12.32ml to make a total volume of 100ml till homogeneous mixture was obtained. According to solubility of drug and selected ratio of oil: S_{mix} :water(15:38:47) 2ml of oil was needed to dissolved 50mg drug in 100ml of

total preparation therefore total volume of nanoemulsion was 12.32ml (22).

In vivo Skin irritation test

All the materials used for preparation of nanoemulsion fall under generally regarded as safe (GRAS) category. Concentration of all materials is very critical issue for this formulation. Large amount of surfactants is usually irritant to the skin. Therefore skin irritation test was performed to confirm concentration of materials used for nanoemulsion preparation is safe (23). Mentioned that a value between 0 and 9 indicates that the applied formulation is generally non irritant to human skin (24). Skin irritation test was performed using either sex of wistar rats weighing 180–200 g. Wistar rats were divided into 2 groups (n=6) and applied the following formulations: optimized nanoemulsion and placebo nanoemulsion. The animals were kept under standard laboratory conditions, temperature at 25 ± 1°C and relative humidity (55 ± 5%). The animals were housed in polypropylene cages, six per cage, with free access to standard laboratory diet and water as mention above. A single dose of 10 µl of optimized nanoemulsion, Placebo nanoemulsion and marketed cream were applied to the left ear of the rat and the right ear as a control. The development of erythema was monitored for 14 days using the reported method (25).

In Vivo Anti-Inflammatory Study

The protocol to carry out in vitro permeation studies was approved by the Institutional Animal Ethics Committee S.B.S College of Pharmacy, Patti, Amritsar, Punjab, India.

The committee's guidelines were followed for the studies. The anti-inflammatory and sustaining action of the optimized formulation was evaluated by the carrageenan induced hind paw edema method by using digital plethysmometer (Ugo Basile, Italy) in Wistar rats of either sex weighing 180 to 200 g. A left hind paw of each rat was marked, just below tibiotarsal junction, so that every time the paw was dipped up to the fixed mark to ensure constant paw volume. Animals were randomly divided into 3 groups (control, placebo and formulation treated) each containing 6 rats. The placebo formulation contained only Salmon fish oil whereas the formulation treated were applied HTN formulations containing Salmon fish oil and BD on the dorsal area of 9 cm^2 gently with the help of micropore adhesive, 0.5 h prior to carrageenan injection. Acute inflammation was produced by injecting 0.1 ml of 1% (w/v) carrageenan suspension in the sub plantar region of the left hind paw 0.5 h after treatment with drug. The paw volume was measured at 0, 1, 2, 3, 6 and 12 h. The amount of paw swelling was determined for 12 h and expressed as percent edema relative to the initial hind paw volume (23). Percent inhibition of edema was calculated for placebo and drug loaded group with respect to control group using the following formula:

$$\% \text{ Inhibition} = \frac{\% \text{ Edema (Control)} - \% \text{ Edema (Formulation)}}{\% \text{ Edema (Control)}}$$

Statistical Analysis

Each experiment was conducted in triplicate and data were analyzed using Excel 2007 (Microsoft Office, Microsoft Inc., US) and expressed as a mean ± standard deviation (S.D.). Comparison between the differences of means was performed by using paired t-test for paired comparisons where p-values of 0.05 or less were considered significant.

RESULTS AND DISCUSSION

Criteria for Excipient Selection

The excipients selected need to be pharmaceutically acceptable, nonirritating, and nonsensitizing to the skin and should fall into the GRAS (generally regarded as safe) category. Higher solubility of the drug in the oil phase was another important criterion, as it would help the nanoemulsion to maintain the drug in solubilized form. Safety is a major determining factor in choosing a surfactant, as large amount of surfactant may cause skin irritation. Non-ionic surfactants are considered to be less toxic than ionic surfactants. Another important aspect to be taken into consideration is the selection of surfactants; ideally the hydrophilic lipophilic balance (HLB) value to form the o/w nanoemulsion should be greater than 10. The right blend of low and high HLB surfactants leads to the formation of a stable nanoemulsion formulation. The presence of cosurfactant decreases the bending stress of interface and allows the interfacial film sufficient flexibility to take up different curvatures required to form nanoemulsion over a wide range of composition.

Screening of Excipients

Drug loading per formulation is a very critical design factor in the development of nanoemulsion systems for poorly soluble drugs, which is dependent on the drug solubility in oil phase. Solubility of BD in Salmon fish oil was found to be 18.95 mg/mL which is very good for topical delivery as the dose of BD is very less for topical application. But due to the presence of other fatty acid in Salmon fish oil; emulsification of oil is very difficult. For getting good nanoemulsion region in ternary phase diagram, the miscibility of oil with surfactant and cosurfactant is important. Therefore the miscibility of oil was performed with different surfactants and cosurfactants Table 1.

Another important criterion is the selection of surfactant with proper HLB value. Hydrophilic surfactant and cosurfactant are considered to prefer the interface and to lower the necessary energy to form the nanoemulsion, consequently improving the stability. For example, the required HLB value to form o/w nanoemulsion is greater than 10. So selection of surfactant and cosurfactant with appropriate HLB value is necessary (26).

Table 1: Miscibility of salmon fish oil with surfactants and co-surfactants

Sl. No.	With surfactant	Observation	With surfactant	Co-observation
1	Tween 20	Turbid	Ethanol	Turbid
2	Tween 80	Clear	Transcutol P	Clear
3	Lecithin	turbid	PEG 200	Turbid
4	Unitop 100	Turbid	Pleurol oleique	Turbid

The miscibility of Salmon fish oil was found to be highest with Tween 80 in case of surfactant and Transcutol P in case of co-surfactant in 1:1 ratio. Apart from this, Tween 80 has high HLB value which can provide good emulsification to the salmon fish oil. Transcutol P is very good solubilizing agent which can provide better penetration to the lipophilic drug such as BD by increasing the solubility to the drug in the lipophilic domain of the stratum corneum. So for the development of pseudoternary phase diagram Salmon fish oil was selected as an oil phase, Tween 80 as surfactant and Transcutol P as a co-surfactant (27).

Phase Studies

Constructing a phase diagram is one of the

primary steps and makes a backbone for the nanoemulsion drug delivery system, particularly when the aim is to accurately delineate a phase boundary. Observations are made carefully to separate metastable systems from phase boundary, although the free energy required toform an emulsion is very low, the formation is thermodynamically spontaneous. The relationship between the phase behavior of a mixture and its composition can be selected with the aid of a phase diagram (28). Salmon fish oil, Tween 80(surfactants) and Transcutol P (co surfactant), were used to study the phase diagrams in detail. The systems were observed for visual clarity and flow ability characteristics. Those which did not show a change in the meniscus after tilting to an angle of 90° were classified as nanoemulsion gels a metastable system and it was not selected. After taking observation, pseudo ternary phase diagrams were constructed based on the observations marked during titration. Phase diagrams were constructed separately for each ratio of S_{mix} prepared, so that o/w nanoemulsion regions could be identified. In the phase diagrams (Figure 1, A-G) only o/w nanoemulsion region is shown. After building the backbone of the nanoemulsion delivery system, different formulations were selected at different point from the phase diagram justifying the drug dose (29).

The construction of pseudoternary phase diagrams was started using surfactant i.e. Tween80 alone (1:0). It was found that the region of nanoemulsion existence was very less and most of the region was composed of emulsions. Now with surfactant Tween80, cosurfactant Transcutol P was also incorporated in the ratio 1:1 and pseudoternary phase diagrams were constructed. It was found that region of nanoemulsion existence increased greatly. Increase in the concentration of co-surfactant (1:2), resulted in even larger area of nanoemulsion existence, along with some emulsion, gels or nanoemulsion gels area. Increasing co-surfactant concentration further from 1:2 to 1:3, and 1:4 resulted in the reduction of the nanoemulsion existence area and more area was composed of emulsion and gels.

The existence of nanoemulsion region whether large or small depends on the capability of that particular surfactant or surfactant mixture to solubilize the oil phase. The extent of solubilisation results in a greater area with more of clear, homogenous solution. It was seen that when the surfactant (Tween 80) was used alone, oil phase was solubilized to a lesser extent implying that surfactant alone was not able to reduce the interfacial tension of the oil droplets to sufficiently low level & thus was not able to reduce the free energy of the system to ultra low level desired to produce nanoemulsions. When a cosurfactant was added, the interfacial tension was reduced to much low level and very small free energy was achieved which helped in larger nanoemulsion area existence in phase diagram. With a further increase in co-surfactant from 1:1 to 1:2, a further drop in interfacial tension and free energy was achieved resulting in maximum area of nanoemulsion formation. With a further increase in co-surfactant concentration (1:3), the interfacial tension of interfacial film increased as compared to above and more of gel area and less of nanoemulsion area was observed.

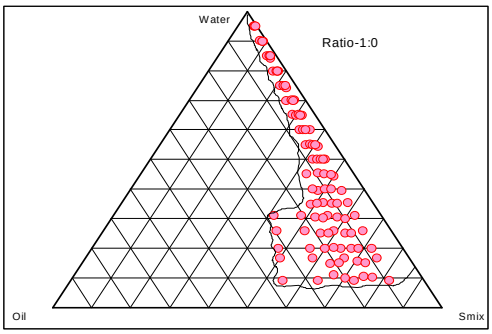

Figure 1 (A): Phase diagram of S_{mix} 1:0

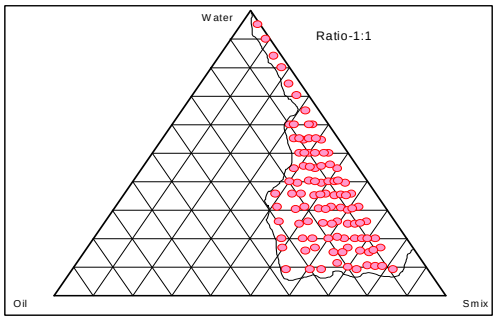

Figure 1 (B): Phase diagram of S_{mix}1:1

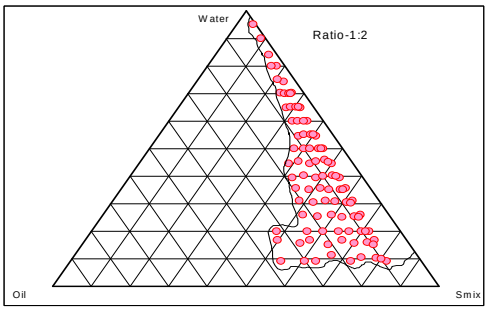

Figure 1 (C) Phase diagram of S_{mix} 1:2

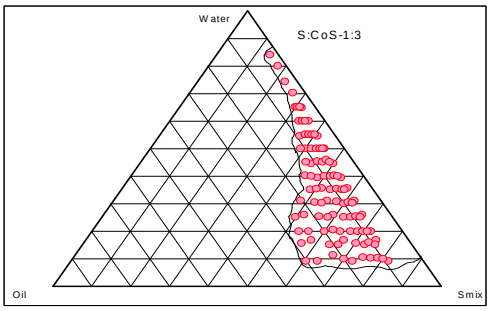

Figure 1 (D) Phase diagram of S_{mix} 1: 3

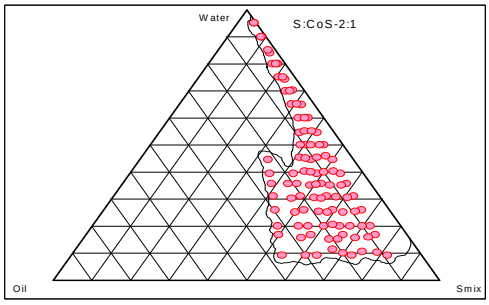

Figure 1 (E) Phase diagram of S_{mix} 2:1

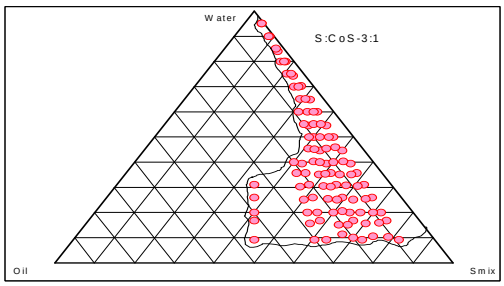

Figure 1 (F) Phase diagram of S_{mix} 3:1

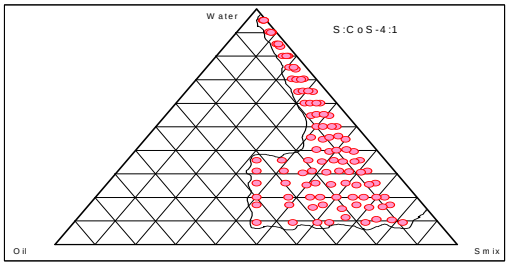

Figure 1 (G) Phase diagram of S_{mix} 4:1

Figure.1 (A-G): Pseudoternary phase diagram of group 1 indicating o/w nanoemulsion region using Salmon fish oil, Tween 80 (Surfactant), Transcutol P (Co-surfactant).

Selection of Formulation from Phase Diagram

It is reported that large amount of surfactant causes skin irritation and toxicity related issues therefore it is important to use minimum amount of surfactant and cosurfactant in the formulation. However, for topical delivery, where enhanced skin permeation is the aim, it is not purposeful to select the lowest surfactant concentration. The surfactant concentration should be chosen so that it gives the maximum flux, which is an

important criterion but its level should not be toxic to cause any irritation to the skin. This is usually not obtained with formulations that contain the highest amount of surfactant since high surfactant concentration decreases the thermodynamic activity of the drug in the vehicle, and the affinity of the drug to the vehicle becomes greater. While going through pseudoternary phase diagram, oil could be solubilised up to the extent of 40% w/w but in such cases the S_{mix} concentration was very high. For the preparation of drug-loaded nanoemulsions, 0.05% BD was dissolved in oil phase. Therefore, from each phase diagram nanoemulsion formulations containing different concentration of oil were selected which contained minimum to maximum S_{mix} concentration (Table **2**).

Table 2: Composition of various BD loaded nanoemulsions

Formulation Code	Salmon fish oil (% w/w)	S_{mix} (% w/w)	Distilled water (% w/w)	BD (% w/w)
A1	7	50	43	0.05
A2	10	37	53	0.05
A3	10	55	35	0.05
B1	5	45	45	0.05
B2	5	40	55	0.05
C1	7	57	36	0.05
C2	9	50	41	0.05
D1	10	51	39	0.05
F1	15	57	28	0.05
F2	20	61	19	0.05

Physical Stability Studies

Nanoemulsions are considered to be kinetically stable systems which are formed at a particular concentration of oil, surfactant and water, with no phase separation, creaming or cracking. Selected formulation from phase diagram were subjected to different stress stability testing like heating cooling cycle, centrifugation and freeze thaw cycle. During physical stability testing some formulations became turbid and some showed phase separation. The most possible explanation of instability in nanoemulsions may be Ostwald ripening, in which molecules move as a monomer and coalescence of smaller droplets takes place, resulting in the formation of large droplets by diffusion processes driven by the gain in surface free energy. Additionally, when temperature quench occurs during stress stability study, instability of nanoemulsion occurs due to separation of oil phase and droplet distribution of smaller size is favoured by the change in curvature free energy. Formulations with negligible phase separation, creaming, cracking, coalescence and phase inversion during stress stability tests, were selected for further studies (Table 3.).

Table 3: Physical stability studies of drug loaded formulations.

Formulation Code	Heating Cooling Cycles	Freeze thaw Cycles	Centrifugation Studies
A1	Passed	Passed	Passed
A2	Passed	Passed	Passed
A3	Failed	Failed	Passed
B1	Passed	Failed	Passed
B2	Failed	Passed	Passed
C1	Passed	Passed	Passed
C2	Passed	Failed	Failed
D1	Passed	Passed	Passed
F1	Passed	Passed	Passed
F2	Failed	Failed	Failed

Characterization of Nanoemulsions

The formulations which passed physical stability test were evaluated for droplet size, polydispersity index, zeta potential, viscosity, pH, conductivity and refractive index.

Particle Size and Zeta Potential

The average droplets size of different nanoemulsions was in the range of 100 to 200 nm.

The polydispersity index is a ratio that gives information about the homogeneity of the particle size distribution in a given system. The polydispersity index (Table **4.**) showed that all the nanoemulsions had narrow size distribution. When the concentration of oil phase was kept constant, it was observed that the decrease in particle size was inversely proportional to the concentration of S_{mix}. However, the droplet size of all the formulations was in the nano range. The average particle size and polydispersity index of the formulation A2 was found to be 1129.89 nm (Figure. **2**) and 0.201respectively indicating nano range of droplets with minimum variation in particle size. The zeta potential depends on both the particle surface and the dispersant. Particles interact according to the magnitude of zeta potential and not their surface charge and therefore zeta potential can be used to predict dispersion stability of the system. Zeta potential for formulation A2 was -36.09 mV which indicated good dispersion stability as it represents significant distance between charged particles in dispersion system.

Table 4: Evaluation of various drug loaded formulations

Formulation code	Average droplet size (nm) ± S.D. (n = 3)	Polydispersity index ± S.D. (n = 3)	Zeta potential ± S.D. (n = 3)	Viscosity (mP) ± S.D. (n = 3)	pH ±S.D (n = 3)	Refractive index ± S.D. (n = 3)
A2	129.89 ±2.12	0.201 ±0.03	-36.09 ±1.03	28.91± 2.03mP	5.73 ±0.05	1.401 ±0.02

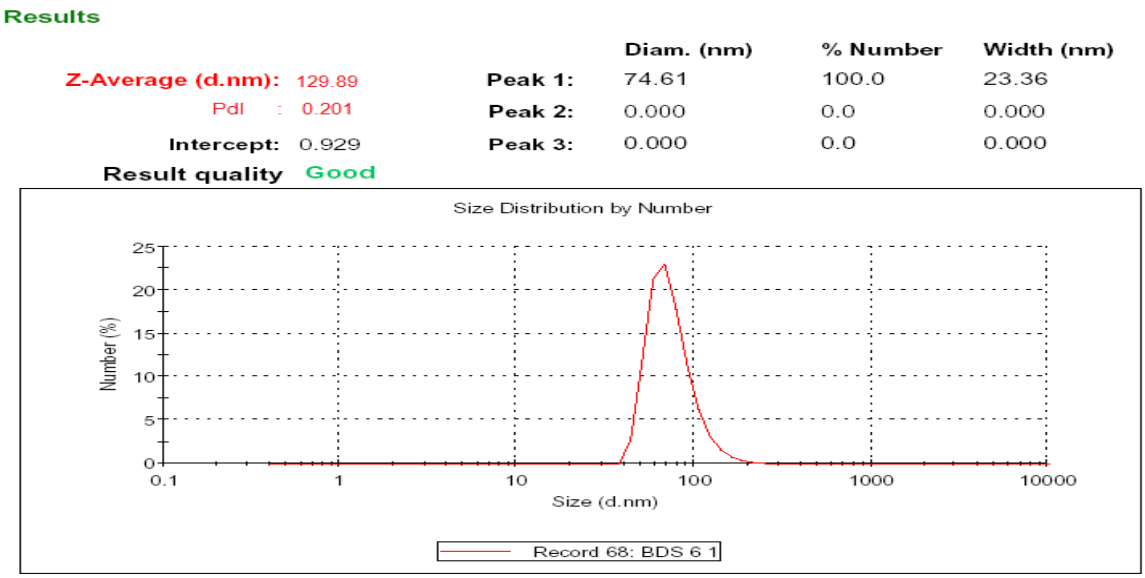

Figure 2: Droplet size distribution of nanoemulsion formulation (A2)

Refractive Index, pH and Viscosity of Nanoemulsion

Refractive index of a formulation indicates isotropic nature of formulation. Refractive index (Table **4.**) of nanoemulsion was measured which basically signify the chemical interaction between drug and excipients. There was no significant difference in the refractive index value of placebo and drug loaded nanoemulsions so it was concluded that the nanoemulsion formulations were chemically stable and remained isotropic thus showing no interaction between excipient and drug. Acceptable pH of the formulation is another important aspect taken

into consideration. Very high or low pH can lead to skin irritation. pH of the formulations were found to be in the range of 5.73 which are very close to skin pH (pH 4.5–6.0). It was found that viscosity of the formulations depends upon amount of surfactant mixture. As the amount of S_{mix} decreased in a formulation, viscosity also decreased.

Ex Vivo Skin Permeation Studies

The permeation ability of the various nanoemulsions loaded BD and control was evaluated using the ex vivo permeation experiments. The ex vivo permeation profiles of BD through excised abdominal skins of rat are shown in (Figure. 3). A steady increase of BD in the receptor chambers with time was observed. The permeation profiles of nanoemulsion were in accordance with the Fick's diffusion equation. Statistical comparison of the flux throughout 24 h showed that the nanoemulsion preparations of BD provided flux (Table **5**.) higher than that of the control (BD suspension) which had low cumulative amount of BD at 24 h after application. Cumulative amount of BD permeated from nanoemulsions was 2.11 times higher that of the control, 24 h post application. The high permeation rate of nanoemulsions might be attributed to several factors. Firstly, the high concentration of BD released in nanoemulsion resulted in high concentration gradient, which might be the main permeation mechanism of BD into the skin from the formulation. Nanoemulsion could act as drug reservoir where drug is released from the inner phase to outer phase and then further into the skin (30). Secondly, due to the small droplet size, droplets settled down and came in close contact with the skin and a large amount of Salmon fish oil in nanoemulsion might have penetrated into the skin. DHA present in Salmon fish oil had strong permeation enhancing effect (31). In addition, due to the small droplet diameters of nanoemulsion, the likely mechanism may also be the permeation of BD directly from the droplets into the stratum corneum without nanoemulsion fusion to the stratum corneum and subsequent permeation enhancement. Drug deposited in skin (µg/cm^2) was found to be 31.06.

Table 5: Ex vivo permeation parameter of BD loaded nanoemulsion formulation and control.

Nanoemulsion	Flux Ratio (µg /cm^2 h^{-1})	Permeability coefficient (K_p)	Enhancement Rate
A2	1.187	2.37*10^{-3}	2.11
Control	0.561	1.12*10^{-3}	1

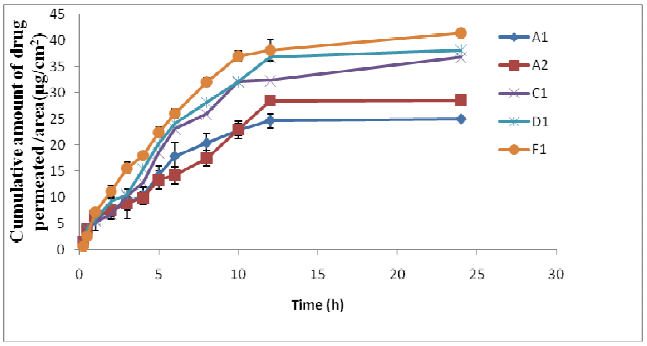

Figure 3: In vitro skin permeation of betamethasone dipropionate from nanoemulsion in phosphate buffer (pH 7.4)

Morphology of Particle

The TEM studies were carried out to get more insight about the morphology of the nanoemulsion systems. When TEM was performed for optimized formulation it was finally concluded that the particles were spherical in shape and finely distributed within nanometer range. Particle size for drug loaded formulation was found between 96–155 nm (Figure. 4) respectively. For BD loaded formulation particle size had increased significantly due to drug entrapment inside the oil droplets. These results

Covered in Scopus & Embase, Elsevier Int. J. Drug Dev. & Res., April - June 2014, 6 (2): 61-76

were in agreements with the droplet size obtained from photon correlation spectroscopy.

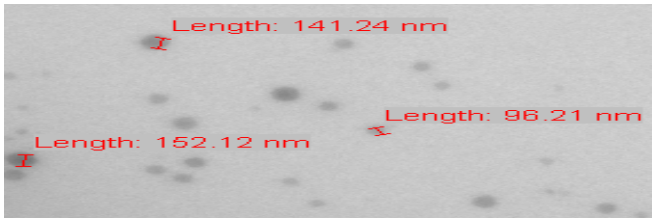

Figure 4: TEM photograph of particle size of nanoemulsion (A2) drug loaded (betamethasone dipropionate)

Hydrogel Thickened Nanoemulsion

Previously, the gel matrix of nanoemulsion has been prepared with carbomer -940, xanthan gum and sodium alginate for improving the rheological behavior of nanoemulsion (38-40). In this work, carbopol-971, carbopol-940, HPMC (15 BDs) and sodium alginate were selected at 1% w/v concentration for the preparation of HTN. However, when carbopol-940 and HPMC were added in nanoemulsion with stirring, only white hydrogel was obtained and the nanoemulsion structure was disturbed. Likewise, when sodium alginate was added in nanoemulsion system it formed hydrogel but after 24 h phase separation occurred. So it was concluded that carbopol-940, sodium alginate and HPMC were not good gel forming polymers for BD loaded nanoemulsion. When carbopol-971 was added in nanoemulsion system a transparent stable hydrogel formed which also maintained nanoemulsion structure of the formulation. As the concentration of polymer was increased its viscosity increased simultaneously. A small quantity of gel was pressed between the thumb and index finger and the consistency and homogeneity of the gel were observed. The HTN showed absence of any coarse particles. Carbopol-971 in HTN resulted in a high viscosity and oily droplets might be distributed in gel network, which might contribute to the enhancement of the stability of droplets in nanoemulsion. The pH value for all three gel formulations was found in the range 5.15–5.55 (Table **6**) which is favorable for topical application. The gels prepared with 0.5 and 0.8% w/v carbopol were not suitable for topical delivery because the consistency of gels was not good. Hydrogel containing 1% carbopol-971 was found to have good viscosity and maximum amount of drug was retained in the skin during permeation study (Table **6**.). 5% salicylic acid was added during gel preparation for each formulation.

Table 6: Evaluation of hydrogel thickened system

S. No.	Carbopol 971 Concentration (%)	Viscosity (mP) ± S.D. (n = 3)	pH ± S.D. (n = 3)	Flux (microgram/cm^2 h^{-1})	Drug retained in skin (**µg/cm^2**)
1	0.4	63.71 ± 0.04	5.14 ± 0.04	1.14	23.16
2	0.8	73.16 ± 0.01	5.46 ± 0.01	1.10	25.05
3	1	98.07 ± 0.03	5.69 ± 0.07	1.09	29.73

Skin irritation test

The mean values of skin irritation score for drug loaded nanoemulsion gel and placebo nanoemulsion gel were found to be 1.66 **±** 0.471 and 2.2 **±** 0.577respectively (Table **7.**). From these results which were based on 14 days test, it can be concluded that optimized nanoemulsion was safe to be used as topical drug delivery system. It clearly indicated that nanoemulsion has more skin irritation potential due to high amount of surfactant in comparison to placebo nanoemulsion because drug itself may has irritation potential. Overall all the formulation

have low irritation score hence it is safe for human use.

Table 7: Skin irritation score of the Placebo nanoemulsion and Drug loaded nanoemulsion

S. No	Group	Score after (days) 1	Score after (days) 2	Score after (days) 3	Score after (days) 4	Score after (days) 7	Score after (days) 14	Mean score±SD
1	Placebo nanoemulsion	2	1	2	2	1	2	1.66±0.471
2	Drug loaded nanoemulsion	2	1	2	3	2	2	.2 ± 0.577

Anti-Inflammatory Studies

The anti-inflammatory effects of BD loaded in an optimized HTN were compared with the placebo (Salmon fish oil) HTN formulations. The rat's left footpad became edematous soon after injection of carrageenan and reached its peak at 3 h (74.54%). Significant ($p < 0.05$) amount of % inhibition (85.22%) (Table 8) was achieved in case of BD loaded HTN formulation which indicated good anti-inflammatory activity at the end of 12 h. Besides, 33.31% inhibition was also achieved in placebo (Salmon fish oil) HTN formulation at the end of 12 h indicating some anti-inflammatory activity of Salmon fish oil which might be due to presence of omega-3 fatty acids. Percentage edema was highest in case of control group and least in case of drug loaded group. This finding revealed synergistic inhibitory effect on inflammation thus suggesting that the optimized formulation could be a promising delivery system for psoriasis treatment.

Table 8: Anti-inflammatory effects of drug loaded and placebo nanoemulsion gel in carrageenan-induced rat paw edema

Group	Formulation	N	Mean Wt ±SD (g)	Time (h)	Mean % Edema± SD	% Inhibition
I	Control (carrageenan only)	6	180.0±12.2	1	28.7±2.3	
				2	41.2±4.11	
				3	74.45±4.33	
				6	58.11±3.21	
				12	39.32±3.37	
II	Drug loaded nanoemulsion	6	190±9.5	1	25.4±2.01	11.49
				2	33.19±2.81	19.44
				3	46.37±4.17	37.71
				6	13.97±1.8	75.95
				12	5.81±1.21	85.22
III	Placebo nanoemulsion	6	200.0±15.5	1	27.5±3.01	4.18
				2	37.01±4.16	10.16
				3	63.87±3.32	14.21
				6	45.01±3.55	22.54
				12	26.22±3.11	33.31

N = Number of rats in each group; SD = Standard deviation

Conclusion

From the physical properties and identification test, it was conclude that the drug samples (betamethasone dipropionate and salicylic acid) were authentic, pure and confirming to the standards. Betamethasone dipropionate exhibited λ_{max} at 239 nm in distilled water, 240 nm in methanol, 228 nm in octanol and 237nm in phosphate buffer (pH 7.4). Salicylic acid exhibited λ_{max} at 296 nm in distilled water, 294 nm in methanol, 296.5 nm in and 295 nm in octanol phosphate buffer (pH 7.4). The partition coefficient of the drugs (betamethasone

Covered in Scopus & Embase, Elsevier Int. J. Drug Dev. & Res., April - June 2014, 6 (2): 61-76

dipropionate and Salicylic acid) were determined in octanol/water distilled water and was found to be 3.39 and 2.09 respectively. Different components were selected for preparation of nanoemulsion. The important criterion for selection of components was that are pharmaceutically acceptable and falls under GRAS category.

Conflict of Interest

The authors state no conflict of interest and have received no payment for this project.

Acknowledgment

We are thankful to SBS College of Pharmacy, Patti, Amritsar, Punjab, India, for providing animal facilities to carry out this work.

References:

1) Alam MS, Ali MS, Alam N, Alam MI, Anwer T, Imam F, Ali MD, Siddiqui MR and Shamim M. Design and Characterization of Nanostructure Topical Gel of Betamethasone Dipropionate for Psoriasis. J Appl Pharm Sci. (2012) 2: 148-158.

2) Alam MS, Babboota S, Ali MS, Ali M,Alam N, Alam MI and Ali J. Accelerated stability testing of betamethasone dipropionate nanoemulsion. Int J Pharm Pharm Sci. (2012) 4: 371-374.

3) Alam MS, Ali MS, Shamim M, Hussain MS, Mujtaba MA and Alam N. Preparartion, characterization and in vitro Irritation study of clobetasol propionate loaded nanoemulsion for psoriasis and atopic dermatitis. World J Pharm Pharm Sci. (2012) 1(4):1189-1208.

4) Alam MS, Babboota S, Ali MS, Ali M, Alam N, Alam MI and Ali J. Accelerated stability testing of betamethasone dipropionate nanoemulsion. Int J Pharm Pharm Sci. (2012) 4:371-374.

5) Ali MS, Alam MS, Alam N, Alam MI, Imam F and Ali MD. Formulation, characterization and in-vivo study of nanoemulsion topical gel of beclomethasone dipropionate for psoriasis. World J Pharm Pharm Sci. (2012) 1(3): 839-857.

6) Arterburn LM, Oken HA, Bailey HE, Hamersley J, Kuratko CN and Hoffman JP. Algal-oil capsules and cooked salmon: nutritionally equivalent sources of docosahexaenoic acid. J Am Diet Assoc. (2008) 108(7): 1204-9.

7) Ashurst PJ, Caldwell IW, Champion RH, Hall-Smith SP and Milne JA. Beclomethasone dipropionate compared with betamethasone-17-valerate in the treatment of inflammatory dermatoses. Br J Clin Pract. (1970) 24(1): 45-47.

8) Babboota S, Alam MS, Sharma S, Sahni JK, Kumar A and Ali J. Nanocarrier-based hydrogel of betamethasone dipropionate and salicylic acid for treatment of psoriasis. Int J Pharm Investig. (2011) 1(3): 139–147.

9) Sharma S, Kumar Anil, Sahni JK, Ali J and Babboota S. Nanoemulsion based hydrogel containing omega-3 fatty acids as a surrogate of betamethasone dipropionate for topical delivery. Adv Sci Lett. (2012) 6: 221-231.

10) Babboota S, Shakeel F, Ahuja A, Ali J and Shafiq S. Design, development and evaluation of novel nanoemulsion formulations for transdermal potential of celecoxib. Acta Pharm. (2007) 57: 315-332.

11) Babboota S, Shakeel F and Kohli K. Formulation and evaluation of once-a-day transdermal gels of diclofenacdiethylamine. Methods Find Exp Clin Pharmacol. (2006) 28(2):109-14.

12) Babboota S, Shakeel F and Kohli K. Formulation and evaluation of once-a-day transdermal gels of diclofenacdiethylamine. Methods Find Exp Clin Pharmacol. (2006) 28(2):109-14.

13) Barbosa H, Slater NK and Marcos JC. Protein quantification in the presence of poly (ethylene glycol) and dextran using the Bradford method. Anal Bio chem. (2009) 395(1): 108-10.

14) Indian J Pharmacol. Dec 2010; 42(6): 338–344. B.N. Vedha Hari, Karuna Priya Chitra, Ramadevi Bhimavarapu, Prabhu Karunakaran,[1] N.

Muthukrishnan, and B. Samyuktha Rani Novel technologies: A weapon against tuberculosis.

15) Arterioscler Thromb. 1991 Jul-Aug; 11(4):903-11. Dietary eicosapentaenoic acid and docosahexaenoic acid from fish oil. Their incorporation into advanced human atherosclerotic plaques. Rapp JH[1], Connor WE, Lin DS, Porter JM.

16) J Drugs Dermatol. 2013 Aug; 12(8):868-73.Comparative evaluation of topical calcipotriol versus coal tar and salicylic acid ointment in chronic plaque psoriasis. Singh P. Gupta S, Abidi A, Krishna A.

17) Brum LM, Lopes LS, Martins NM,Rezer JF, Araujo D, Barbosa GM, Ferrony DA, Roggia I, Trevisan LS, Alves MP and Leal DB. Atividade da NTPDase de LinfócitosnaDermatite de Contato Antes e ApósTratamento com Dexametasona Nanoestruturada. Lat Am J Pharm. (2009) 28(6): 876-84.

18) Date AA, Naik B and Nagarsenker MS. Novel drug delivery systems: potential in improving topical delivery of antiacne agents. Skin Pharmacol Physiol. (2006) 19(1): 2-16.

19) Del R and Do JQ. Combination topical therapy for the treatment of psoriasis. J Drugs Dermato. (2006) 5(3): 232-4.

20) Duckney P, Wong HK, Serrano J, Yaradou D, Oddos T and Stamatas GN. The role of the skin barrier in modulating the effects of common skin microbial species on the inflammation, differentiation and proliferation status of epidermal keratinocytes. BMC Res Notes. (2013) 6(1): 474.

21) Fontana MC, Rezer JFP, Coradini K, Leal DBR and Beck RCR. Improved efficacy in the treatment of contact dermatitis inb rats by a dermatological nanomedicine containing Clobetasol propionate. Eur J Pharm Biopharm. (2011) 79: 241-249.

22) Gittler JK, Krueger JG and Guttman-Yassky E. Atopic dermatitis results in intrinsic barrier and immune abnormalities: implications for contact dermatitis. J Allergy Clin Immunol. (2013) 131(2): 300-13.

23) Kimber I, Basketter DA, Gerberick GF and Dearman RJ. Allergic contact dermatitis. Int Immuno pharmacol. (2002) 2 (2–3): 201–11.

24) Lademann J, Richter H, Teichmann A, Otberg N, Blume-Peytavi U, Luengo J, Weiss B, Schaefer UF, Lehr CM, Wep R and Sterry W. Nanoparticles--an efficient carrier for drug delivery into the hair follicles. Eur J Pharm Biopharm. (2007) 66(2): 159-64.

25) Mei Z, Chen H, Weng T and Yang Y. Solid lipid nanoparticle and microemulsion for topical delivery of triptolide. Eur J Pharm Biopharm. (2003) 56: 189–196.

26) A. A. Date, B. Naik, B. S. Nagarsenkar, Skin Pharmacol Physiol. 19, 2 (2006).

27) R. Mendelsohn, C. R. Flach, and D. J. Moore, Biochimica et, Biophysica Acta. 1758, 923 (2006).

28) Lawrence M.J., Rees G.D. Microemulsion-based media as novel drug delivery systems. Adv Drug Deliv Rev. 2000; 45: 89–121.

29) Narang A.S., Delmarre D., Gao D. Stable drug encapsulation in micelles and microemulsions. Int J Pharm. 2007; 345: 9–25.

30) Barakat N, Fouad E, Elmedany A. Enhancement of skin permeation and anti-inflammatory effect of indomethacin using microemulsion. Asian J Pharm 2011;5:141-9

31) K. Tsutsumi, Y. Obata, K. Takayama, T. Loftsson and T. Nagai, "Effect of cod-liver oil extract on the buccal permeation of ergotamine tartrate", Drug Devel. Ind. Pharm., 24, 757-762 (1998).

Article History: ----------

Date of Submission: 27-04-2014

Date of Acceptance: 15-05-2014

Conflict of Interest: NIL

Source of Support: NONE