Design and Characterization of Nanostructure Topical Gel of Betamethasone Dipropionate for Psoriasis

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ABSTRACT

Psoriasis is a chronic T lymphocyte mediated autoimmune inflammatory disorder that affects the skin, joints, and tendons. Betamethasone dipropionate (BD) has anti-inflammatory, immunosuppressive,and antiproliferative activity. The aim of this study was to investigate and evaluate a nanoemulsion topical gel of betamethasone dipropionate. For the preparation of nanoemulsion eucalyptus oil and babchi oil was taken. Nanomulsions were prepared by aqueous phase-titration method. Pseudoternary phase diagrams were constructed for the identification of nanoemulsion existence zones. Prepared nanoemulsions were subjected to different thermodynamic stability tests and characterized for droplet size, viscosity and refractive index. In vitro skin permeation of betamethasone dipropionate through rat abdominal skin was determined by the Franz diffusion cell. The prepared nanoemulsion gel is a potential vehicle for improved topical delivery of BD for better treatment of psoriasis.

INTRODUCTION

Psoriasis is a chronic; T-lymphocyte mediated autoimmune inflammatory disorder that affects the skin, joints, and tendons in up to 2.5% of the population worldwide (Christophers, 2001). In the United States alone, more than 4.5 million people are affected. Psoriasis is associated with significant healthcare costs, economic losses, and quality of life. The most common areas of involvement are the elbows, knees, gluteal cleft and the scalp (Alam *et al*., 2012). Corticosteroid such as betamethasone has been used extensively in topical therapy for the treatment of mild to moderate psoriasis (Baboota *et al*., 2011; Zulfakar *et al*.,2010). Betamethasone dipropionate (BD) is a

highly potent glucocorticoid receptor agonist which possesses immunosuppressive, anti-inflammatory, and anti-proliferative effects. 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. There are various types of dosage forms available such as ointment, cream, lotion, and foam which is use for the treatment of mild to moderate psoriasis (Zulfakar *et al*., 2010; Simonsen *et al*., 2004). However, the clinical limitation of BD has poor permeability through skin which reduces its therapeutic effectiveness at the target site. The main limitation lies in the barrier function of the skin. Therefore, the major challenges for a topical formulation are to provide a sufficient increase in drug penetration into the skin, without any significant functional and histological change in the skin and also irreversible alteration to the skin barrier function (Baboota *et al*., 2007).

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In recent years, much attention has been focused on lipid-based formulations to improve the permeability and bioavailability of poorly water soluble drug compounds. 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, we select natural oils such as eucalyptus oil which act as a penetration enhancer as well as vehicle for the nanoemulsion. Eucalyptus oil also act as an antiinflamatory and antiseptic which is desirable for psoriasis because some time psoriasis may be due to some microorganism (*Psoriasis vulgaris*).

One more important thing is that we select babchi oil in combination with eucalyptus oil in view of that babchi oil act as a vehicle for nano emulsion and also itself have antipsoriatic activity. One of the most promising techniques for enhancement of skin permeation of drugs is nanoemulsion. Nanoemulsions have several significant advantages including low skin irritation, powerful permeation ability, and high drug-loading capacity for topical delivery when compared with the other carriers. The main objective of this study was to formulate BD nanoemulsion in which babchi oil used as a vehicle in combination with eucalyptus oil in view of that active constituent of babchi oil is psorelean which act as a antipsoriatic (Ali *et al.*, 2008; Maghraby, 2008). BD into nanoemulsion would result in enhancement and sustaining of corticosteroid delivery rate leading to better anti-psoriatic activity. The low viscosity of nanoemulsion restrains its clinical application due to inconvenient use, and therefore hydrogel-thickened nanoemulsion systems were formulated with good stability, powerful permeation ability, and suitable viscosity for the topical delivery which provided longer contact with skin. The present investigation was focused on the preparation and characterization of nanoemulsion with BD, in vitro permeation studies, and in vivo anti-inflammatory activity of the optimized formulation. Furthermore, assessment of permeation of drug into skin was done by histopathological studies. The long-term goal of this work was to develop topical BD formulations for clinical use to increase therapeutic value.

MATERIALS AND METHODS

Materials

BD was obtained as a gift sample from Gaurav Pharma Limited (Delhi, India). Babchi oil was obtained from Mahaveer Enterprises (Rajasthan, India). Eucalyptus oil was obtained from Scientific International (New Delhi). PEG 200, Tween 80, Tween 20, Pleurol oleic, Glycol, Brij35, Propanol and ethanol were purchased from Merck (Merck, India). Labrasol and capryol were obtained as a kind gift samples from Gattefosse (Mumbai, India). All other chemicals used were of analytical grade.

Screening of excipients

The most important criterion for screening of components is the solubility of poorly soluble drug in oils, surfactants and cosurfactants.

Screening of oil for nanoemulsion

To find out the suitable oil that can also provide excellent skin permeation rate of BD, the solubility of BD was determined in different oils, viz. eucalyptus oil, babchi oil, sefsol oil, castor oil, labrafil, IPM, babchi oil: ecalyptus oil (1:1), and babchi oil: eucalyptus oil (1:1.5) mixture. One milliliter of different oils was taken in small vials and excess amount of the drug was added. The vials were tightly stoppered and were continuously stirred for 72 h at 25°C, and after 72 h, samples were centrifuged at 5000 rpm for 20 min. The supernatant was separated, filtered through a 0.45-μm membrane filter, and after appropriate dilution with methanol, solubility of drug in different oils was determined by UV method at 240 nm.

Screening of surfactant and cosurfactant for nanoemulsion

To find out the suitable surfactant and cosurfactant, the solubility of BD was determined in various surfactants including labrasol, Tween 80, Tween 20, Brij35 and a combination of Tween 80: labrasol. The solubility of BD was also checked in cosurfactants including Ethanol, propanol, PEG 200, Glycol, Capryol and Pleurol oleic.

Phase studies

On the basis of solubility studies, babchi oil: eucalyptus oil (1:1) was selected as an oil phase. Tween 20 and ethanol were chosen as surfactant and cosurfactant, respectively. Distilled water was used as an aqueous phase. For the determination of existence zone of nanoemulsion, pseudoternary phase diagrams were constructed using water titration method (spontaneous emulsification method) Surfactant and cosurfactant (S_{mix}) were mixed in different weight ratios (1:0, 1:1, 1:2, 1:3, and 1:4). These S_{mix} were chosen in increasing concentration of cosurfactant with respect to surfactant. For each phase diagram, oil and specific S_{mix} were mixed well in different ratios. Sixteen different combinations of oil and S_{mix} (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. Slow titration with aqueous phase was done for each weight ratio of oil and S_{mix} under moderate stirring, and visual observation was used for transparent and easily flowable nanoemulsion. Gels were claimed for those clear and highly viscous mixtures that did not show a change in the meniscus after being tilted to an angle of 90°. 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 cosurfactant at fixed weight ratio (S_{mix} ratio).

Selection of formulations

From the pseudoternary phase diagrams showing maximum nanoemulsion area, a number of nanoemulsions with different formulas were selected covering the entire range of nanoemulsion occurrence in the phase diagrams with minimum

surfactant and maximum water concentration. 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 formulations were subjected to various physical stability tests.

Physical stability studies

To overcome the problem of metastable formulations, physical thermodynamic stability tests were performed (Shafiq *et al.*, 2007; Bernardi *et al.*, 2011). The selected formulations 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 carried out. Those formulations that were found stable were subjected to a freeze-thaw cycle test. Three such cycles were done for the formulations between -20° C and + 25° 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 min.

Characterization of nanoemulsions
In vitro skin permeation studies

In vitro skin permeation studies were performed on a fabricated Franz diffusion cell with an effective diffusional area of 7.24 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 hair was removed with an electric clipper. The subcutaneous tissue was removed surgically, and the dermis side was wiped with isopropyl alcohol to remove adhering fat (Chen *et al.*, 2004). 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 phosphate buffer (pH 7.4). 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 (0.05 mg/mL BD) 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 0.45-membrane filter, and analyzed for drug content by UV spectrophotometer at λ$_{max}$ of 237 nm

Permeation and distribution data analysis

The cumulative amount of BD permeated through the albino rat skin (Q, μg/cm^2) was plotted as a function of time (t, h)

for each formulation. The permeation rate (flux) at the steady state (J$_{ss}$, μg/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:
$K_p = J_{ss}/C_o$
Enhancement ration (E$_r$) was calculated by dividing the J$_{ss}$ of the respective formulation by the J$_{ss}$ of the control formulation as given below:
$E_r = J_{ss}$ of formulation/J_{ss} of control
In order to determine the drug disposition in the skin, it was weighed, cut into small pieces, and sonicated for 15 min with methanol in order to extract the BD content. The resulting solution was centrifuged and passed through 0.25 μm filter and drug content (μg/mg of skin) was determined spectrophotometerically.

Particle size and polydispersity index

The average size and polydispersity index of the nanoemulsion droplets were determined by photon correlation spectroscopy (Nano ZS90, Malvern Instrument, Worcestershire, UK) at 633 nm 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.

Viscosity, refractive index, conductivity, and pH

Viscosity of nanoemulsion was determined by using Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Middleboro, MA, USA). 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 nanoemulsion by using a calibrated digital pH meter (Mettler Toledo MP 220, Greifensee, Switzerland) in triplicate at room temperature. Conductivity was measured by using a digital thermo conductivity meter (1152, Emcee Electronics, Venice, FL, USA) and current flow was observed.

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 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.

Hydrogel-thickened nanoemulsion

The very low viscosity often exhibited by nanoemulsion is not suitable 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, sodium alginate, ethyl cellulose, and HPMC have been used to prepare the nanoemulsion-based gel for improving the viscosity of nanoemulsion (Xu *et al*., 2008). 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). Because BD nanoemulsion is a type of o/w type, so carbopol 934 was selected for preparation of nanoemulsion gel. For preparation of nanoemulsion gel, initially 3 g of carbopol 934 taken then upto 87 mL of the water added and stirred with magnetic stirrer till homogenous mixture was obtained. Then 12 mL of already prepared nanoemulsion was added drop by drop to make a total volume of 100 mL till homogeneous mixture was obtained. The clear hydrogel-thickened nanoemulsion was evaluated for viscosity, pH, extrudability, homogenecity, and spreadability. Content uniformity was carried out to ascertain that concentration of drug in each portion was uniform. For that an accurately weighed quantity of gel (6 g) from three different portions was taken and extracted with methanol. The extracted drug was analyzed by using UV spectrophotometer. Hydrogel-thickened nanoemulsion was applied gently on stratum corneum and in vitro permeation study was performed.

In vivo anti-inflammatory study

The protocol to carry out *in vivo* anti-inflammatory efficacy studies was approved by the Institutional Animal Ethics Committee Translam Institute of Pharmaceutical Education and Research, Meerut, U.P, 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 180-200 g (Shakeel *et al*., 2009). 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 three groups (control, optimized nanoemulsion gel A2, and marketed formulation) each containing six rats. Both optimized and marketed formulations were applied on the dorsal area of 9 cm^2 gently with the help of micropore adhesive, 0.5 h before carrageenan injection. No formulation was applied to the control. Acute inflammation was produced by injecting 0.1 mL of 1% (w/v) carrageenan suspension in the subplantar region of the left hind paw 0.5 h after treatment with drug. The paw volume was measured at 0, 1, 2, 3, 6, 12, and 24 h. The amount of paw

swelling was determined for 24 h and expressed as percent edema relative to the initial hind paw volume. Percent inhibition of edema was calculated for placebo and drug loaded group with respect to control group using the following formula:

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

Histopathology studies

Abdominal skin of Wistar rats was treated with the optimized BD nanoemulsion gel. After 24 h, the rats were killed and skin samples were taken from untreated (control) and treated areas. Each specimen was stored in 10% formalin solution in phosphate buffer saline (pH 7.4). The specimens were cut into sections vertically. Each section was dehydrated using ethanol embedded in paraffin wax for fixing and stained with hematoxylin and eosin. These samples were then observed under light microscope (Motic, Japan) and compared with control samples (Shakeel *et al*., 2008).

Stability studies as per ICH guidelines

Stability studies on optimized nanoemulsion were performed by keeping the sample at refrigerator temperature (4^oC) and room temperature (25^oC). These studies were performed for the period of 3 months. The droplet size, viscosity and refractive index were determined at 0, 1, 2 and 3 months. Accelerated stability studies were also performed on optimized BD nanoemulsion as per international conference on harmonization (ICH) guidelines. Three batches of optimized formulation were taken in glass vials and were kept at accelerated temperature of 30, 40, 50 and 60^oC at ambient humidity. The samples were withdrawn at regular intervals of 0, 1, 2 and 3 months. A reversed-phase high performance liquid chromatography (RP-HPLC) method was used for determination of betamethasone dipropionate. The HPLC column was the YMC J'sphere ODS-H80, 150 mm × 4.6 mm I.D., 4 μm particle size (YMC, Milford, MA). The mobile phases consisted of *A*: 0.05% (v/v), methanesulfonic acid in water and *B*: 100% acetonitrile in (70/30, v/v). The flow rate was 2.0 mL/min and column temperature was 35 °C. Retention time was found to be 4.4 min. Analysis was carried out at each time interval by taking 100 μl of each formulation and diluting it to 5 ml with methanol and injecting into the HPLC system at 240 nm. The pH of the sample diluent was adjusted to about 2.7 by 1 M hydrochloric acid. The solubility of sample in methanol was 43.6 mg/ml. In addition, samples of pure oil (combination of babchi oil and eucalyptus oil), pure surfactant and cosurfactant (S_{mix}) were run separately to check interference of the excipients used in the formulations (Shou *et al*., 2009). The amount of drug decomposed and the amount remaining (undecomposed drug) at each time interval was calculated. Order of degradation was determined by the graphical method (Alam *et al*., 2012). Degradation rate constant (K) was determined at each temperature. Arrhenius plot was constructed between log K and 1/T to determine the shelf-life of optimized nanoemulsion

formulation. The degradation rate constant at $25^{\circ}C$ (K_{25}) was determined by extrapolating the value of $25^{\circ}C$ from Arrhenius plot. The shelf-life ($T_{0.9}$) for formulation was determined by using the formula:

$$\text{Shelf Life} = \frac{0.1052}{K}$$

Where K is the degradation rate constant at 25°C.

RESULTS AND DISCUSSION

Criteria for excipient selection

The excipients selected were needed to be pharmaceutically acceptable, nonirritating, and nonsensitizing to the skin and to 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 a large amount of surfactants may cause skin irritation. Nonionic surfactants are considered to be less toxic than ionic surfactants and therefore Tween 20 were selected. Another important criterion for selection of the surfactants is that the required hydrophilic lipophilic balance value to form the o/w nanoemulsion should be greater than 10. The right blend of low and high hydrophilic lipophilic balance 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 was seen maximum in case of Babchi oil: Eucalyptus oil mixture (1:1) (Figure1).
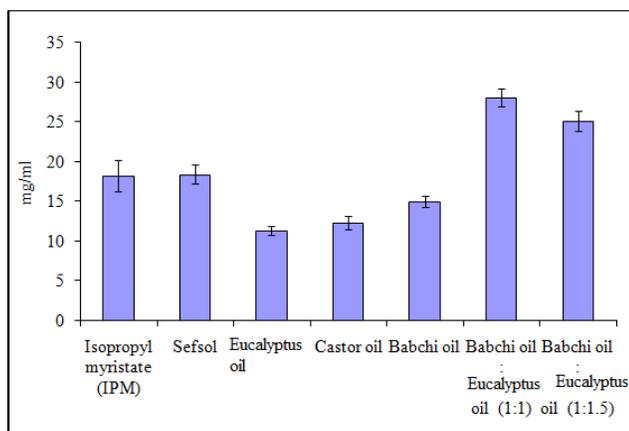
Fig. 1: Solubility of BD in different oils alone and in combination.

Moreover, Eucalyptus oil is a penetration enhancer for transdermal delivery, as it increases the fluidity of the intercellular lipid barriers in the stratum corneum by forming separate domains that interfere with the continuity of the multilamellar stratum corneum and induce highly permeable pathways in the stratum corneum.

Nonionic surfactants are widely used in topical formulations as solubilizing agents, but some recent results indicate that they may affect the skin barrier function. Among the various surfactants evaluated, the maximum solubility of BD was found in Tween 20 (Figure 2) so it was selected as surfactant. The maximum solubility of BD in cosurfactant was found with Ethanol (Figure2). Ethanol was selected as cosurfactant because it is very good penetration enhancer and solubilizing agent. The penetration enhancement of lipophilic drugs by alcohols is due to the higher solubility of the drug substance in the lipophilic area of the stratum corneum because of the presence of alcoholic enhancers. The effect of alcohols on the phase behavior of nonionic nanoemulsion depends on the number of carbons of alcohol. The presence of alcohol overcomes the need for any additional input of energy. These properties make the components useful as vehicles for drug delivery. Alcohols can influence the formation of nanoemulsion by both interfacial and bulk effects. So for the development of pseudoternary phase diagram Babchi oil: Eucalyptus oil (1:1) was selected as the oil phase, Tween 20 as surfactant, Ethanol as cosurfactant, and distilled water as aqueous phase.
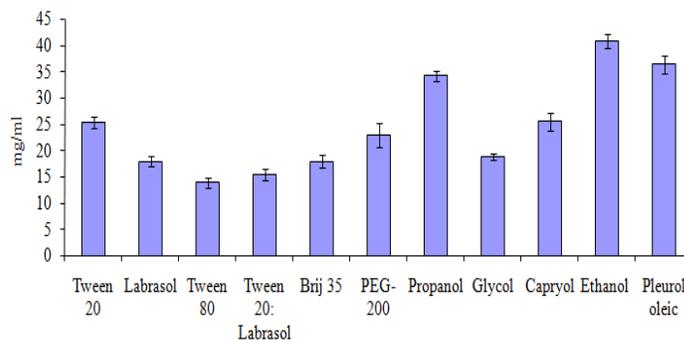
Fig. 2: Solubility of BD in different surfactants and cosurfactants.

Phase studies

The relationship between the phase behavior of a mixture and its composition can be captured with the aid of a phase diagram (Figure 3). The aim of the construction of pseudoternary phase diagram was to find out the existence range of nanoemulsion. Care was taken to ensure that observations are not made on metastable system. Pseudoternary phase diagrams were constructed separately for each S_{mix} ratio for getting o/w nanoemulsion regions. The area of nanoemulsion isotropic region changed slightly as the ratio of surfactant in S_{mix} was increased. In the phase diagrams, the existence of large or small nanoemulsion region depends on the capability of the particular S_{mix} to solubilize the oil phase. The extent of solubilization results in a greater area with the formation of more clear and homogenous solution. The construction of pseudoternary phase diagrams was started using surfactant, i.e., Tween 20 alone (1:0). It was found that the region of nanoemulsion existence was very less and most of the region

was composed of emulsions. Therefore, with surfactant Tween 20, cosurfactant Ethanol was also incorporated in the ratio 1:1 and it was found that region of nanoemulsion existence increased greatly. Increase in the concentration of cosurfactant to (1:2) resulted in even larger area of nanoemulsion existence, along with some emulsion, gels, or nanoemulsion gels area. Increasing cosurfactant 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.
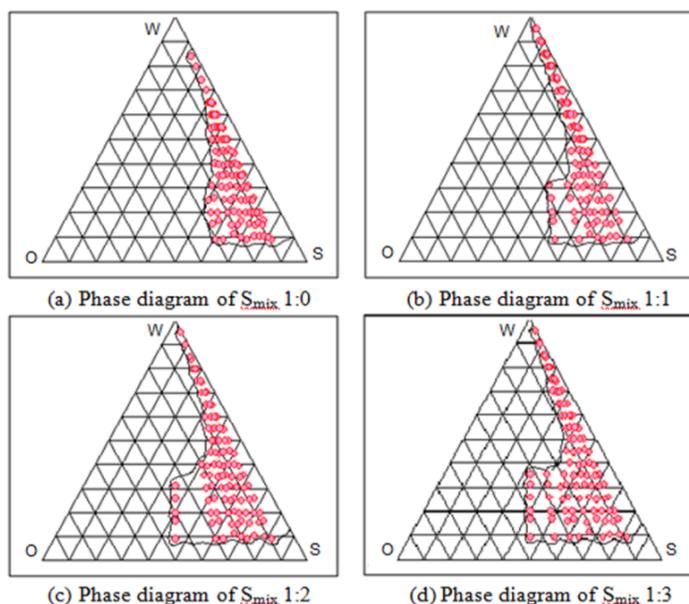
Fig. 3: Pseudoternary phase diagram indicating o/w nanoemulsion region using Babchi oil: Eucalyptus oil (1:1), Tween 20 (surfactant), and Ethanol (cosurfactant) (O = oil, W = water, S = S_{mix}).

The existence of nanoemulsion region wether large or small depends on the capability of that particular surfactant or surfactant mixture to solubilize the oil phase. The extent of solubilization results in a greater area with more of clear, homogenous solution. It was seen that when the surfactant (Tween 20) 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 and 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 cosurfactant 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 cosurfactant concentration (1:3), the interfacial tension of interfacial film increased when compared with above, and more of gel area and less of nanoemulsion area was observed.

Selection of formulation from phase diagram

From the study it is suggested that large amount of surfactant causes skin irritation and toxicity-related problem, and therefore it is preferable to use the minimum amount of surfactant and cosurfactant in the formulation. The surfactant concentration should be selected 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 because high surfactant concentration decreases the thermodynamic activity of the drug in the vehicle, and the affinity of the drug to the vehicle becomes greater. For the preparation of drug-loaded nanoemulsions, 0.05% BD was dissolved in oil phase. Different proportion of oil was taken just to obtained desire quantity of drug from phase diagram in Table 1.

Physical stability studies

Nanoemulsions are considered to be thermodynamically stable systems that are formed at a particular concentration of oil, surfactant, and water, with no phase separation, creaming, or cracking. Selected formulations 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 in some phase separation occurred. One reason of this instability in nanoemulsions may be due to the Ostwald ripening in which molecules move as a monomer and coalescence of small droplets takes place, resulting in the formation of large droplets by diffusion processes driven by the gain in surface free energy. The other reason may be that 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 favored by the change in curvature free energy. Only those formulations, which showed no phase separation, creaming, cracking, coalescence, and phase inversion during stress stability tests, were selected for further studies (Table 1).

Characterization of nanoemulsions

The formulations that passed physical stability test were evaluated for droplet size, polydispersity index, viscosity, pH, and refractive index.

In vitro skin permeation studies

The permeation ability of various BD loaded nanoemulsions was evaluated using the *in vitro* permeation experiments. A steady increase of BD in the receptor chamber with time was observed. The permeation profiles of nanoemulsions were in accordance with the Fick's diffusion equation. On the basis of permeation studies, it was found that the formulation A2 consisting of 15 % oil phase, 35 % ($S_{mix\ 1:1}$) and 50% distilled water exhibited 44.43 of cumulative amount of drug permeated ($\mu g/cm^2$) with the flux of 1.34 ($\mu g/cm^2$/h) after 24 h and highest amount of drug was deposited in skin 32.563 µg/mg.

Table. 1: Selection of formulation and physical stability studies of drug loaded formulations.

Smix Ratio	Formulation code	Oil	Smix	Water	H/C	Cent	Freeze	Result	Drug Conc. (BD)
		Oil used: Babchi oil:Eculaptus oil (1:1) , Surfactant used: Tween 20, Cosurfactant used: Ethanol, External phase: Distilled water							
	A1	10	30	60	√	X	√	Failed	0.05%
1:1	A2	10	33	57	√	√	√	Passed	0.05%
	A3	15	40	45	√	√	√	Passed	0.05%
	B1	10	36	54	X	√	√	Failed	0.05%
1:2	B2	10	40	50	√	√	X	Failed	0.05%
	B3	15	40	45	√	X	√	Failed	0.05%
	C1	10	37	53	√	√	X	Failed	0.05%
1:3	C2	10	40	50	√	√	√	Passed	0.05%
	C3	15	44	41	√	√	√	Passed	0.05%
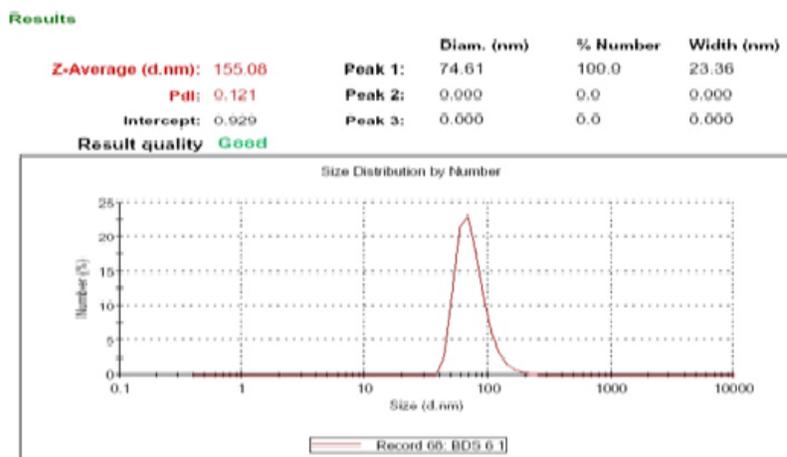
Fig. 4: Droplet size and size distribution of nanoemulsion formulation (A2).

Particle size and polydispersity index

The average size and polydispersity index of the nanoemulsion droplets were determined by photon correlation spectroscopy (Nano ZS90, Malvern Instrument, Worcestershire, UK). The droplets size of all nanoemulsions ranged from 60 to 190 nm. The polydispersity index showed that all the nanoemulsions had narrow size distribution. The average particle size and polydispersity index of the formulation A2 were found to be 155.08 nm (Figure4) and 0.121, respectively, indicating micro range of droplets with minimum variation in particle size.

Refractive index, pH, conductivity, and viscosity of nanoemulsion

Viscosity of the nanoemulsion (A2) formulation was very low (27.35 ± 1.91 mP) as expected for o/w emulsion (Table 2). The low viscosity may be due to presence of low amount of Smix (1:1) also the low concentration of oil. Refractive index is the net value of the components of nanoemulsion and indicates isotropic nature of formulation. Refractive index of nanoemulsion was determined using an Abbes type refractometer (Nirmal International, New Delhi, India) at 25 ± 0.5 °C. The mean value of the refractive index for the formulation A2 was found to be 1.461. The specific conductivity of nanoemulsion A2 was found to be 10^{-4} s cm^{-1}. The apparent pH of the formulation was measured by pH meter (AccumentAB 15, Fisher scientific, USA) in triplicate at 25 ± 1 °C and found to be around 6.7 (Table 2).

Table. 2: Parameters for the optimized formulation A2.

S. No.	Parameters	Result
1.	Particle size (nm)	155.08
2.	Refractive index	1.461
3.	pH ±SD (n=3)	6.7 ± 0.01
4.	Viscosity (mP) ±SD (n=3)	27.35 ± 1.91
5.	Conductivity(s cm^{-1})	10^{-4}

Surface morphology of particle

The TEM studies were carried out to get more insight about the morphology of the nanoemulsion systems. From the results of TEM it was concluded that the particles of optimized formulation were spherical in shape and finely distributed with micron size range between 142 and 170 nm (Figure 5).

Hydrogel-thickened nanoemulsion

To convert o/w BD nanoemulsion into a gel, nanoemulsion gel was prepared using carbopol 934 (3 %) and acetate buffer solution to mimic pH of the skin. A small quantity of gel was pressed between the thumb and index finger, and the consistency and homogeneity of the gel were observed. It was found that there were no coarse particles in the optimized gel formulation. The spreading of carbopol gel was found to be more uniform and the gel spreaded in a circular pattern equally on all sides and it almost reached to 1.32 times of the initial diameter upon application of 44.6 g weight. From the *in vitro* studies it was found that the gel of optimized formulation (A2) shows 32.17

μg/cm^2 cumulative amounts of drug permeated through the abdominal rat skin (Figure 6) and 31.738 μg/cm^2 drug deposited in the skin (Table 3). A decrease in the permeation from nanoemulsion-based gel was seen due to high viscosity of gel. pH of the formulation was found to be 5.7 which is compatible with skin pH. The spreadibility of the formulation was found to be 1.41 times greater than marketed formulation, and drug uniformity was found to be 98.67 ± 1.02 % (Table 4). The gel possessed good spreading properties, comparable to that of the marketed product and suggested that the gel would result in a more uniform and wider spread upon topical application.

Table. 3: *In vitro* permeation profile of prepared nanoemulsion gel and marketed gel.

Formulation	Flux (μg/cm^2/h)	Drug deposition (μg/cm^2)	Permeability constant (K_p)
Nanoemulsion gel (A2)	1.34	31.738	2.68 x 10^{-3}
Marketed gel	1.04	20.139	2.08 x 10^{-3}
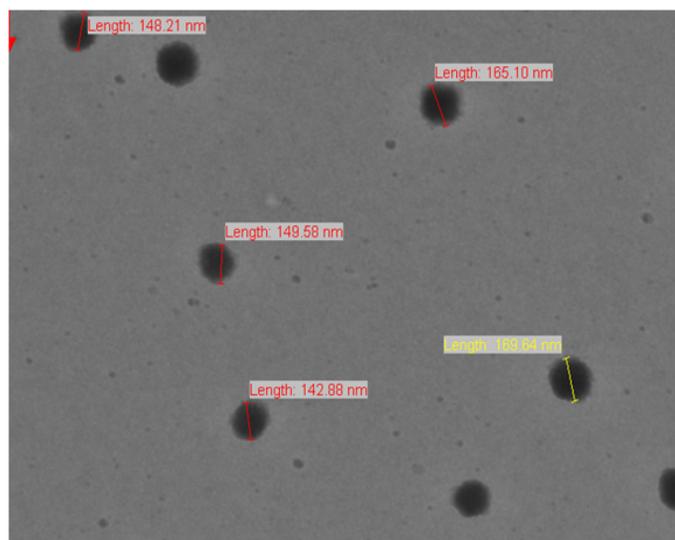
Fig. 5: TEM photograph of particle size of nanoemulsion (A2) drug loaded (betamethasone dipropionate).

Table. 4: Evaluation of nanoemulsion gel parameters.

Viscosity (cps) ±SD (n=3)	pH ±SD (n=3)	Drug content uniformity (%)
4998.75 ± 28.68	5.7 ± 0.182	98.67 ± 1.02

Anti-inflammatory studies

The anti-inflammatory effects of optimized gel formulation were compared with the control and marketed gel formulation. The anti-inflammatory activity of optimized formulation of nanoemulsion gel was evaluated using the carrageenan-induced hind paw edema method using digital Plethysmometer. The rat's left footpad became edematous soon after injection of carrageenan and reached its peak at 3 h (80.2 %). Mean percent edema and % inhibition of inflammation of all the three groups were calculated (Figure 7). Inhibition of edema was found to be highest in the groups in which nanoemulsion gel was applied. The nanoemulsion gel inhibited edema ($P < 0.05$) 77.83 % up to 24 h. Marketed formulation

inhibited the edema 40.97 % up to 24 h. Based on the anti-inflammatory studies, it can be concluded that BD nanoemulsion gel formulation in babchi oil shows maximum inhibition of edema than the marketed formulation and the control.

Histopathology studies

The influence of BD loaded nanoemulsion gel on anatomical structure of the rat skin was assessed with the help of histopathological studies. The photomicrographs of untreated rat skin (control) showed normal skin (Figure 8) with well-defined epidermal and dermal layers. Keratin layer was well formed and lied just adjacent to the topmost layer of the epidermis. Dermis was devoid of any inflammatory cells. When the skin was treated with nanoemulsion formulation, definite changes were observed in the skin morphology. The disruption and extraction of lipid bilayers were clearly evident as distinct voids and empty spaces visible in the epidermal region. The disruption of epidermal layer indicated permeation of BD through stratum corneum.
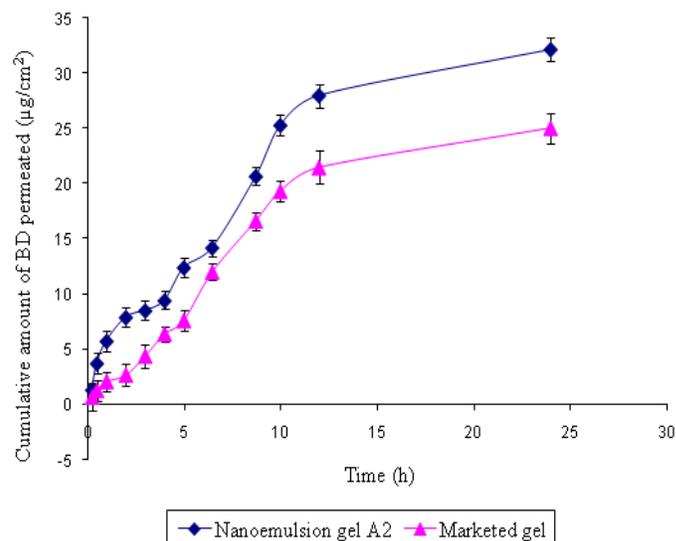
Fig. 6: Comparison of *in vitro* skin permeation of BD from nanoemulsion gel (A2) and marketed formulation gel in phosphate buffer (pH 7.4).

Stability studies as per ICH guidelines

Stability of a drug product refers to the chemical and physical integrity of the dosage unit and when appropriate, the ability of the drug product to maintain protection against microbiological contamination. An ideal drug product must be fully characterized physically, chemically and microbiologically at the start of study and throughout the intended shelf-life period. Therefore optimized nanoemulsion formulation was characterized for droplet size, viscosity and RI for the period of three months. During stability studies droplet size, viscosity and RI were determined at 4 and 25°C. These parameters were determined at 0, 1, 2 and 3 months. It was found that droplet size, viscosity and RI were slightly increased in time at both temperatures (Table 5).

These parameters were compared for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test using Graph Pad In

stat software (GraphPad Software Inc., CA, USA). The changes in these parameters were not statistically significant (P ≥0.05). These results indicated that optimized formulation is stable as there were no significant changes in physical parameters (droplet size, viscosity and RI). For accelerated stability studies, samples were withdrawn at regular intervals of 0, 1, 2, and 3 months. The samples were analyzed for their drug content by HPLC analysis at a wavelength of 240 nm. The degradation of BD was very slow at each temperature which indicated the chemical stability of BD in the nanoemulsion formulation. The optimized nanoemulsion was found to be stable chemically as well as physically, it was concluded that it is suitable for topical delivery.

The degradation of BD was very slow at each temperature which indicated the chemical stability of BD in the nanoemulsion formulation. The optimized nanoemulsion was found to be stable chemically as well as physically, it was concluded that it is suitable for topical delivery of BD. The degraded and remained concentration of BD at different temperatures is shown in Table 6. The order of degradation was determined by graphical method at each temperature. The order of degradation was found to be first order (Figure 9).

In first order degradation, the rate of degradation is independent of the concentration of reacting species. However, the rate of degradation is directly proportional to the first power of the concentration of a single reactant in first order degradation. The correlation coefficients of first order degradation were significant as compared to correlation coefficients of zero order degradation at each temperature as shown in Figure 9 and Figure 10 ($p \leq 0.05$). Therefore for first order degradation, Log % of drug remaining was plotted against time and K was calculated from the slope of the curve at each temperature by following formula:

$$\text{Slope} = -K/2.303$$

Where K is the degradation rate constant.

The values of K at each temperature are given in the Table 7. The log of drug remaining was plotted against time (months). Slope of each line was obtained and K was calculated by the formula. The effect of temperature on the degradation was studied by plotting log K v/s 1/T. (Figure 11). The value of K at 25°C (K_{25}) was obtained by extrapolation of the plot and shelf-life was then calculated by following formula:

$$\text{Shelf life} = 0.1052/K$$

Where K is the degradation rate constant at 25°C. The shelf-life of optimized nanoemulsion formulation was found to be 2.64 years.

Table. 5: Droplet size, viscosity and RI of optimized nanoemulsion A2 during storage.

Time in (months)	Temperature (^{0}C)	Mean droplet size (nm) ± SD (n=3)	Mean Viscosity (mP) ± SD (n=3)	RI ± SD (n=3)
0	4.0 ± 0.5	155.08± 1.71	27.35 ± 1.91	1.461 ± 0.023
1	4.0 ± 0.5	155.33 ± 1.76	27.57 ± 1.51	1.465 ± 0.020
2	4.0 ± 0.5	155.65± 1.81	27.94 ± 1.33	1.467 ± 0.023
3	4.0 ± 0.5	156.05 ± 1.49	28.09 ± 1.11	1.468 ± 0.024
0	25 ± 0.5	155.08± 1.71	27.35 ± 1.91	1.461 ± 0.023
1	25 ± 0.5	155.39 ± 1.53	27.71 ± 1.71	1.467 ± 0.027
2	25 ± 0.5	155.77± 2.11	27.83 ± 1.39	1.470 ± 0.025
3	25 ± 0.5	155.96± 2.44	28.29 ± 1.37	1.473 ± 0.021

Table. 6: Degradation of optimized nanoemulsion A2.

Time (Days)	Temp (ºC)	Drug content (mg)	Drug concentration degraded (mg)	%drug remaining	Log % drug remaining
0	30 ± 0.5	50	0	100	2
30	30 ± 0.5	49.655	0.345	99.31	1.997
60	30 ± 0.5	48.97	1.03	97.94	1.991
90	30 ± 0.5	48.635	1.365	97.27	1.988
0	40 ± 0.5	50	0	100	2
30	40 ± 0.5	49.54	0.46	99.08	1.996
60	40 ± 0.5	48.635	1.365	97.27	1.988
90	40 ± 0.5	47.97	2.03	95.94	1.982
0	50 ± 0.5	50	0	100	2
30	50 ± 0.5	48.97	1.03	97.94	1.991
60	50 ± 0.5	48.525	1.475	97.05	1.987
90	50 ± 0.5	47.53	2.47	95.06	1.978
0	60 ± 0.5	50	0	100	2
30	60 ± 0.5	49.2	0.8	98.4	1.993
60	60 ± 0.5	48.08	1.92	96.16	1.983
90	60 ± 0.5	47.09	2.91	94.18	1.974

Table. 7: Observation table for calculation of shelf life of nanoemulsion A2.

Temperature (ºC)	Slope	K (month^{-1})	Log K	Absolute Temperature (T)	1/T × 10^3
30	-0.00405	0.009333	-2.03	303	3.30033
40	-0.00600	0.013836	-1.859	313	3.19488
50	-0.00734	0.016904	-1.772	323	3.09597
60	-0.00851	0.019771	-1.704	333	3.00300
25		0.004743	-2.324	298	3.35570
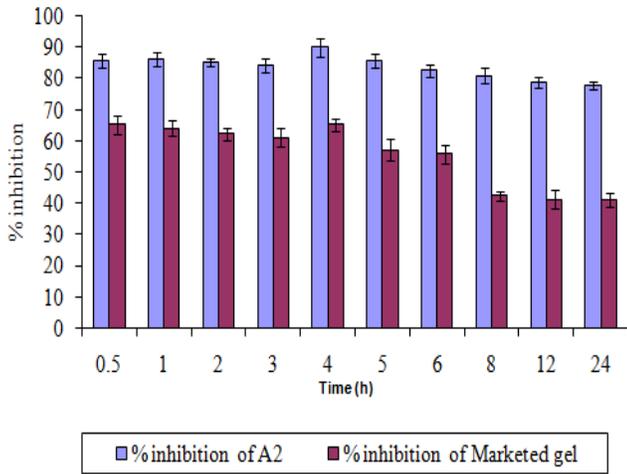
Fig. 7: Comparison of anti-inflammatory activity of marketed product and nanoemulsion gel (A2).
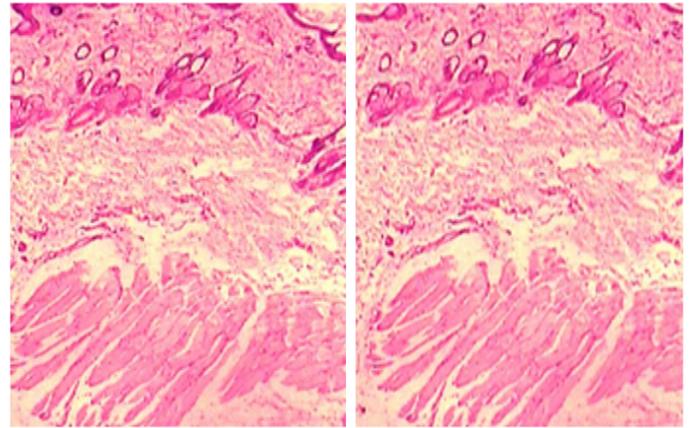
Fig. 8: Light power photomicrograph of (a) Control skin (b) Treated skin.
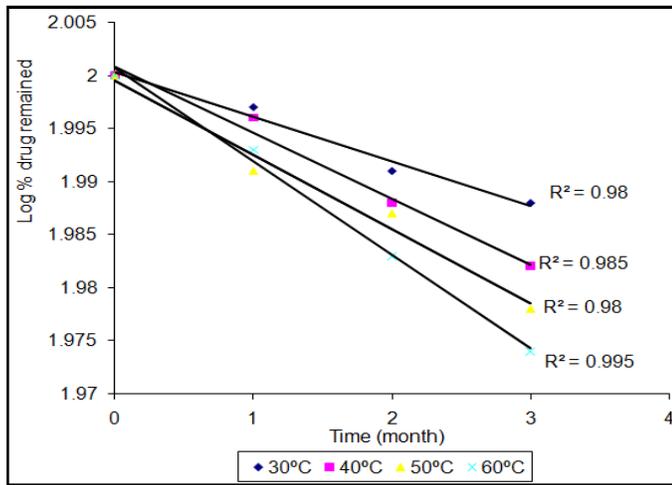
Fig. 9: First order degradation kinetics of BD from nanoemulsion formulation at different temperatures.
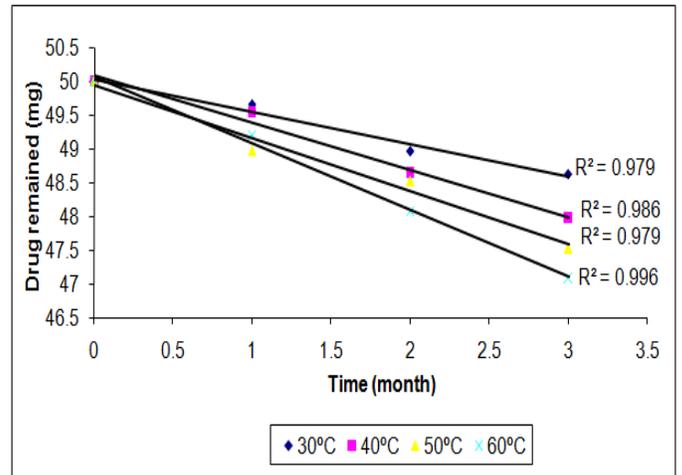
Fig. 10: Zero order degradation kinetics of BD from nanoemulsion formulation at different temperatures.
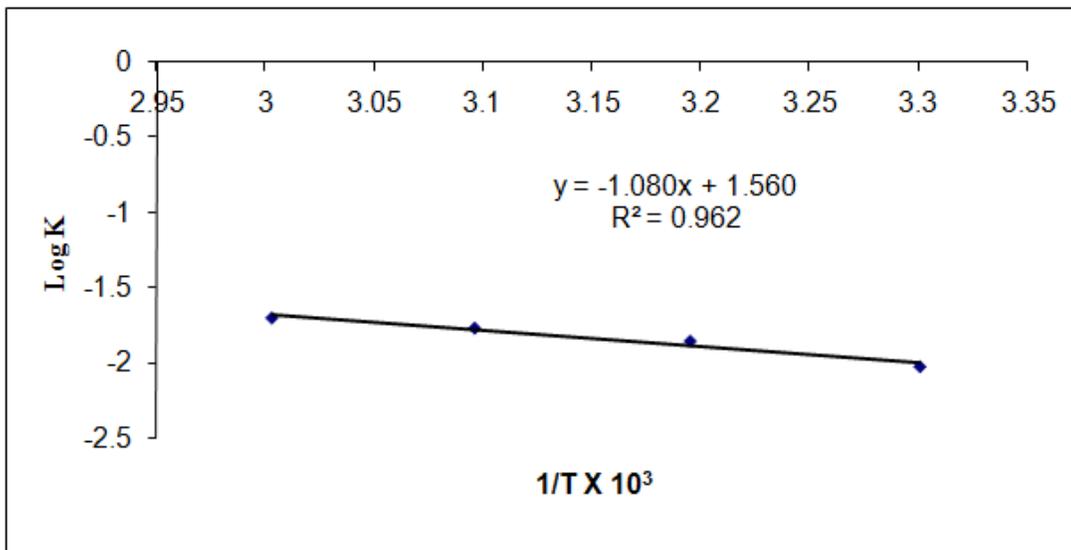
Fig. 11: Arrhenius plot between Log K and 1/T for nanoemulsion formulation A2.

A safe and effective nanoemulsion gel formulation of BD in Babchi oil for the treatment of psoriasis was developed, which provided enhanced permeation of the drug, reduced dosing frequency, and sustained the drug release for the desired period of time and also have improved anti-inflammatory activity. Presence of eucalyptus oil in the formulation has antiseptic action which can prevent microbial infection of skin. The droplet size, viscosity and RI of optimized nanoemulsion formulation were not significantly changed during 3 months of storage suggesting that prepared nanoemulsion was physically stable. The degradation of BD after 3 months of storage was also slowest in the formulation. Slower degradation of BD indicated the chemical stability of BD in nanoemulsion. The shelf-life of nanoemulsion formulation was found to be 2.64 years at room temperature. These results indicated that both physical as well as chemical stability of BD can be enhanced in nanoemulsion formulation using Tween20 as surfactant.

Conflicts of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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