Nanosized ethosomes-based hydrogel formulations of methoxsalen for enhanced topical delivery against vitiligo: formulation optimization, in vitro evaluation and preclinical assessment

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Abstract

The present investigation aimed for the development and characterization of ethosomes-based hydrogel formulations of methoxsalen for enhanced topical delivery and effective treatment against vitiligo. The ethosomes were prepared by central composite design (CCD) and characterized for various quality attributes like vesicle shape, size, zeta potential, lamellarity, drug entrapment and drug leaching. The optimized ethosomes were subsequently incorporated into Carbopol® 934 gel and characterized for drug content, rheological behavior, texture profile, in vitro release, ex vivo skin permeation and retention, skin photosensitization and histopathological examination. Ethosomes were found to be spherical and multilamellar in structures having nanometric size range with narrow size distribution, and high encapsulation efficiency. Ethosomal formulations showed significant skin permeation and accumulation in the epidermal and dermal layers. The fluorescence microscopy study using 123 Rhodamine exhibited enhanced permeation of the drug-loaded ethosomes in the deeper layers of skin. Also, the developed formulation showed insignificant phototoxicity and erythema vis-à-vis the conventional cream. The results were cross-validated using histopathological examination of skin segments. In a nutshell, the ethosomes-based hydrogel formulation was found to be a promising drug delivery system demonstrating enhanced percutaneous penetration of methoxsalen with reduced phototoxicity and erythema, thus leading to improved patient compliance for the treatment against vitiligo.

Keywords

Ethosomes, methoxsalen, phospholipid, topical delivery, vitiligo

Introduction

Vitiligo is an acquired idiopathic, de-pigmenting dermatological disorder characterized by well-circumscribed milky white macules devoid of identifiable melanocytes. These asymptomatic white macules can be psychologically extremely damaging, which even may lead to developing suicidal tendency [1]. The exact etiology of vitiligo is unknown, but autoimmune factors and increased sensitivity to oxidative stress are supposed to be responsible for its pathophysiology. The existing approaches for vitiligo treatment include medical, surgical and adjunctive therapy. Medical treatment targets the immune system and control the spread of de-pigmentation, while surgery along with cosmetics is used to achieve re-pigmentation. Adjunctive therapy is generally used with surgical or medical treatments [2]. Despite the availability of these techniques to combat against pain and trauma, the medical treatment remains the most viable option for vitiligo.

Methoxsalen (8-MOP) is a naturally occurring psoralen derivative (8-methoxy psoralen) that induces melanin production on exposure of the skin tissues to ultraviolet light. It is popularly recommended for PUVA therapy, which remains as the “gold standard” for vitiligo treatment [3]. When drug is activated by UVA light, 8-MOP undergoes cyclo-addiction reaction with pyrimidine bases of nucleic acids to form stable cyclo-adducts [4]. Although this mechanism is very effective in treating dermatological disorders, yet some noted side effects have been observed after being exposed to PUVA therapy owing to the formation of psoralen-photo adducts, leading eventually to cause skin cancer and photo-toxicity [3]. However, in order to achieve maximum therapeutic effect of 8-MOP in photo-chemotherapy, the ultraviolet light should be irradiated when the drug levels reach highest concentration in the skin [5]. Besides the pharmacological needs, 8-MOP
suffers from drug delivery challenges like higher side effects after oral administration, low permeability through stratum corneum and poor retenivity in skin.

Therefore, the need was felt to develop dermatological formulation using strategically designed carrier approach which encapsulate the drug, minimize their side effects and improve their therapeutic efficacy. As 8-MOP is a lipophilic drug compound having crystalline needle shape structure, the conventional vesicular carriers, i.e. liposomes and niosomes fail to entrap drug into their core. Also, the sharp needle shaped structure of drug tends to rupture the lamellar structure of the vesicles. One approach has been attempted for topical delivery of 8-MOP via microemulsion for vitiligo treatment [6]. Nevertheless, it is highly desirable to develop drug formulation with improved efficacy and reduced toxicity. This is ostensibly the first study undertaken to minimize the side effects of 8-MOP and improve its absorption and therapeutic concentration at the desired site. By virtue of the unique composition and physicochemical properties, the ethosomes have proved to be an effective means of drug delivery through topical route of administration. Ethosomal systems are primarily composed of phospholipid, ethanol (EtOH) and water [7]. Unlike the conventional liposomes, which tend to deliver drugs to the outer layers of skin, ethosomes have been reported to enhance permeation of the drug through stratum corneum and subsequent retention in the skin layers [8]. More recently, the attempt made for topical delivery of psoralen using ethosomes produced promising results [9].

Development of impeccable vesicular carrier-based systems involves rational blending of a plethora of lipids, surfactants, cosolvents, and at times, edge activators, for yielding maximal therapeutic benefits. The traditional approach of formulation development employing the short-gun approaches like, one factor at time (OFAT) or changing one separate variable at a time (COST), possess limited fruition owing to multiple intricacies like strenuous, uneconomical, unsuitable to plug errors and identify the interactions among the variables for selecting “the best” formulations. Lately, the systematic approach of formulation development employing the quality by design (QbD) paradigms implemented by ICH and USFDA has been highly popularized. Based on the principles of QbD, the paradigms, like quality risk management and design of experiment, furnish critical understanding of the formulation and process variables for identifying the optimized formulation by minimal expenditure of time, effort and developmental cost vis-à-vis the OFAT approach. A score of literature reports have been published in the past a few years vouch higher utility and fruition of QbD approach for developing products by furnishing enhanced understanding of the product quality attributes.

In the present research work, endeavor has been made for systematic QbD-based development of 8-MOP entrapped ethosomal systems by investigating the influence of formulation composition and their characterization for different parameters, namely vesicle size, percentage drug entrapment (PDE), percentage drug leakage (PDL), ex vivo skin permeation and retention, and photosensitivity studies. Besides, the present work also tends to compares (in vitro, ex vivo and in vivo) the ethosomal gel with marketed formulation.

Materials and methods

Materials

Pure sample of 8-MOP was obtained ex-gratis from M/s Gary Pharmaceutical Pvt. Limited, Ludhiana, India. Purified Phospholipon® 90G (PL) (>95% phosphatidycholine content) was obtained as a gift sample from M/s Phospholipids GmbH (Ludwigshafen, Germany), and used without further purification. Ethanol, propylene glycol (PG) and other organic solvents employed during the study were obtained from M/s E. Merck Ltd. (Mumbai, India). Phosphotungstic acid (PTA) and Rodamine 123 were purchased from M/s Sigma Chemical Co. (St Louis, MO). Double-distilled water obtained using an all glass still (Scientific Instruments, New Delhi, India) was used throughout the experiments.

Preformation studies

The solubility studies of 8-MOP were carried out in various solvents to select an appropriate media, which will be utilized as a sink medium in various formulation evaluation studies. Excess amount of drug was added into the various solvent system(s) like hydroalcoholic media containing and shaken horizontally in an incubator bath shaker at 40 ± 2 °C for 48 h. Subsequently, the samples were filtered through a 0.45 μm membrane filter. After appropriate dilutions, the samples were analyzed through liquid chromatography (Shimadzu’s LC-2010 CHT, Tokyo, Japan) as per the in-house developed and validated method. Chromatographic separation was performed using Hibër® Lichrospher 60RP-select B column (250 mm × 4.6 mm, and 5 μm particle size; M/s. Merck KGaA, Darmstadt, Germany) using a UV detector at a wavelength of 254 nm, while data were analyzed using an inbuilt “Class LC solution” software (Tokyo, Japan). The mobile phase, consisting of methanol–water (60:40, v/v), was maintained at a constant flow rate of 1 mLmin⁻¹ with column oven temperature maintained at 40 ± 1 °C [10].

Preparation of 8-MOP ethosomes as per the experimental design

A two-factor, three-level face-centered composite design (FCCD) was employed to optimize varied critical material attributes influencing the response variables and/or critical quality attributes of the ethosomal formulations [11]. The amounts of PL and EtOH were selected as the critical material attributes and employed at three different levels of each factor, namely low (−1), intermediate (0) and high (+1). Amounts of drug (150 mg) and mass of dispersion (100 mg) were kept as constant in all the prepared formulations. A total of 13 experimental runs constituting nine formulations representing the design points and quintuplicate studies on the center point (0, 0). The ethosomes were prepared according to the method reported by Raza et al. [12]. Accurately weighed quantities of PL and 8-MOP were taken in a specially designed, in-house made air-tight borosil glass vessel and dissolved in EtOH/EtOH–PG mixture. The mixture was heated to 30 °C in a water bath. Double-distilled water heated at 30 °C was added slowly to the mixture in a fine stream with constant mixing at 200 rpm in the rotary evaporator for 5 min (Remi Equipment, Mumbai, India). The final milky dispersion of ethosomes was
kept at room temperature for 24 h. Besides, the blank ethosomes were also prepared for encapsulating Rhodamine 123 dye for evaluation of the vesicles through fluorescence microscopy. The prepared formulations were evaluated for various critical quality attributes like, vesicle size, PDE, PDL, permeation flux (J) and skin deposition (Sd).

Characterization of the ethosomes

Vesicle size and zeta potential
The vesicle size and zeta potential measurement was carried out by a dynamic light scattering method using Malvern Mastersizer™ 2000 (London, UK) [13]. A fixed volume of the vesicular dispersion was diluted 100-folds with triple distilled water, mixed thoroughly and particle size was measured as average diameter in nm and zeta potential in mV.

Percent drug entrapment
The amount of 8-MOP entrapped in ethosomal formulation was carried out after obtaining the pellet by centrifugation of vesicular dispersion at 16000 rpm (32,000 g) for 45 min at 4 °C. The supernatant was removed and ethosomal pellet was subjected to 3–4 times washing with the help of hydroalcoholic solution [i.e. pH 7.4, phosphate buffer solution:EtOH (1:1 v/v)] to remove the unentrapped drug. Further, the washed pellet was then digested with the help of 0.1% Triton X-100 solution in phosphate buffer solution (pH 7.4) followed by extraction of drug with the help of hydroalcoholic solution. The drug content was estimated as the amount of entrapped drug with the help of HPLC analysis. The PDE was calculated using Equation (1) as follows:

\[
PDE = \frac{T - C}{T} \times 100, \tag{1}
\]

where \( T \) is the amount of unentrapped drug in the supernatant and \( C \) is the amount of drug detected in the supernatant [14].

Percent drug leakage
The PDL was determined by measuring the amount of drug leach out from the vesicular system after storage at 25 ± 2 °C for a period of four weeks. The vesicular dispersions were kept in 30 mL sealed glass vials after flushing with nitrogen. The samples from each batch were withdrawn at regular intervals and PDL was calculated from the change in the PDE values before and after storage period using Equation (2) as follows:

\[
PDL = \frac{A - B}{A} \times 100 \tag{2}
\]

where \( A \) is the amount of drug entrapped in the vesicular dispersion after storage and \( B \) is the amount of drug entrapped in the vesicular dispersion before storage.

Electron microscopy
The morphology of ethosomal formulation was visualized through transmission electron microscope (TEM) (Philips CM12 Electron Microscope, Eindhoven, Netherlands), with an accelerating voltage of 100 kV. The fixed volume of the ethosomal formulation was diluted 100-folds with triple distilled water. A drop of the sample was placed on to a carbon-coated copper grid to form a thin film and negatively stained with a drop of 1% PTA solution. The grid was allowed to dry in air and samples were viewed under TEM.

Ex vivo animal studies

Ex vivo permeation studies were carried out using shaved skin of Wistar rats procured from Central Animal House, Panjab University, Chandigarh. The experimental protocol was duly approved by Institutional Animal Ethics Committee of Panjab University, Chandigarh.

Preparation of the skin. The animals used for experimental studies were sacrificed by a cervical dislocation method. The hair on the dorsal side of animal was removed with the help of 0.1 mm animal hair clipper. The shaven part of the skin was separated from the animal using surgical blade No. 23. The subcutaneous fat was also removed from the full thickness abdominal part of skin. Dermis part of the skin was wiped 3–4 times with cotton swab and soaked in isopropanol to remove the fatty materials. Subsequently, the excised skin was washed with distilled water and used for permeation studies.

Skin permeation studies. Studies were performed on a Perme Gear’s six station Franz diffusion cell assembly [15]. Prior to permeation experiments, fresh skin tissue of rats was clamped between the donor and receptor compartment of the jacketed vertical Franz diffusion cell (cross-sectional area of 3.14 cm²; capacity 30 mL), while keeping the stratum corneum toward the donor compartment. Hydroalcoholic phosphate buffer solution (pH 7.4)-EtOH (1:1 v/v) was taken as receiver medium and kept at 32 ± 2 °C under constant magnetic stirring for 24 h. Each of the prepared formulation containing drug equivalent to 750 μg of 8-MOP was applied on the donor compartment. The donor chamber and the sampling port were covered by parafilm to prevent evaporation of the EtOH. Aliquots of 0.5 mL samples were withdrawn periodically for a period of 24 h, followed by replacement with an equal volume of fresh medium to maintain the receptor phase volume at a constant level. The samples obtained from the permeation studies were suitably diluted and analyzed by HPLC.

Drug deposition studies. The amount of drug accumulated in the skin layers was evaluated after separating epidermis and dermis by a microwave technique [16]. After permeation studies, the skin mounted on the diffusion cell was removed carefully and the remaining formulation adhering to the skin was scraped off carefully with a spatula. Skin pieces were washed 3–4 with hydroethanolic solution and then wiped with tissue paper to remove residual drug from the surface of the skin. After appropriate dehydration in ambient air, the skin was dried under microwave oven for exposing up to 6–12 s, followed by separation of epidermis from dermis. The skin layers were soaked separately in 10 mL of EtOH for extraction of the drug and subsequently homogenized. Homogenate dispersion was centrifuged for 5 min at 5000 rpm (3913 g), and the supernatant fraction was separated to analyze the drug content by HPLC.
Optimization data analysis and validation studies

The optimization data analysis was carried out using multiple linear regression analysis (MLRA) to fit the data to the second-order quadratic model with added interaction terms. Only the coefficients with significant model terms ($p < 0.05$), see Supplementary Figure S1 were considered for framing the polynomial equation followed by analyzing the model aptness using coefficient of correlation, lack of fit analysis and predicted error sum of squares (PRESS). The response surface analysis was carried out on 3D-response surface plots and 2D-contour plots for mechanistic understanding of the interaction(s) among the CMAs on the studied CQAs. Search for the optimum formulation was carried out by brute force technique through extensive feasibility and grid search along with numerical desirability function by “trading-off” CQAs as per the selected acceptance criteria, i.e. minimization of vesicle size, PDL, and maximization of PDE, permeation flux and skin retention. Validation studies were carried out by evaluating the prognostic ability of the optimization methodology by preparing eight confirmatory check-point formulations and comparing the observed responses with the predicted ones by linear correlation plots. The percent prediction error was also calculated with respect to the observed responses.

Preparation and characterization of the ethosomal hydrogel formulation

The ethosomal hydrogel was prepared by incorporating the optimized ethosomal formulation of 8-MOP into the gel base for convenient topical application. Briefly, Carbopol 934P was dispersed in warm distilled water ($80^\circ$C) with agitation (i.e. 800–1500 rpm) for complete solubilization of the polymer to obtain a homogenous transparent gel, which was further neutralized with triethanolamine. The accurately weighed quantity of ethosomal dispersion was levigated into the hydrogel base in the ratio of 4:1 to obtain the ethosomal hydrogel formulation (OEMOP1-CBP). The prepared gel formulation was visually inspected for color, phase separation, grittiness and presence of crystal particles, if any.

Drug content determination

To determine the drug content in ethosomal hydrogel, accurately weighed quantity of gel containing drug equivalent to 5 mg of 8-MOP was dispersed in EtOH and stirred for 5 min. The dispersion was filtered through a 0.45 μm membrane filter and analyzed through HPLC for the drug concentration, using Equation (3) as follows.

$$\text{Drug content (mg/mL)} = \frac{\text{Absorbance}}{E_{1\text{cm}}^{1\%}} \times \text{dilution factor} \times 10.$$  (3)

Rheological behavior

The rheological behavior of the developed gel formulation was evaluated using cup and bob rheometer (Rheometer HS-143; Rheolab QC, Anton Paar, Vienna, Austria). The gel sample (~5 g) was placed in a concentric cylindrical arrangement equipped with Z4 sensor probe and allowed to rest for 5 min to achieve thermal equilibrium. The shear stress value was increased automatically, linearly from 0.1 to 100 s$^{-1}$. The relationship between shear stress ($\tau$) and shear rate ($\gamma$) of the formulation was determined using the Herschel–Bulkey model, using Equation (4) as follows:

$$\tau = k\gamma^n,$$  (4)

where $k$ is the consistency index (Pa s$^n$), $\tau$ is the yield stress (Pa) and $n$ is the power-law exponent.

Texture profile analysis

The texture analysis of the gel was performed by analyzing the firmness, stickiness and work of adhesion employing Texture AnalyserTM (Stable Microsystems Ltd, Surrey, UK). Formulation was placed into the probe (female cone) of the instrument and pressed to remove any entrapped air. The sample was allowed to equilibrate for 30 min at room temperature before initiating the test. The modes of measurement of sensory properties of gels were force of penetration (g), work of shear (g.s.), force of adhesion (g), work of adhesion (g.s) represented as gel strength, spreadability, extrusion force, stickiness, respectively. All these parameters were measured as max + force, area (+ve), max – force, area (−ve), respectively.

In vitro drug release

Cellophane membrane (HiMedia, Mumbai, India, cut-off size 10 kDa) pieces were boiled for 30 min in distilled water to activate the pore and then equilibrated in the sink media for 1 h before performing the experiment. This study was performed on a Perme Gear’s six station Franz diffusion cell assembly using the procedure described in the “Skin permeation studies” section. At the end of the study, the amount of drug remaining in the donor compartment was quantified by HPLC method described in the “Preformulation studies” section.

Ex vivo skin permeation and drug deposition studies

Ex vivo permeation and drug deposition in different layers of skin after applying the optimized ethosomal formulation gel formulation were studied as per the procedure described in “Skin permeation studies” and “Drug deposition studies” sections. The cumulative amount of drug permeated per unit effective diffusional area and the flux ($J$) were further calculated employing the following equation [17].

$$C_c = C_u + \left( \frac{V_s}{V_i} \right) \sum_{i=1}^{n-1} C_i,$$

where $C_c$ is the corrected concentration for $n$th sample, $C_u$ is the uncorrected concentration of the $n$th sample, $C_i$ is the uncorrected concentration of the previous $n-1$ samples, $V_s$ is the volume of the sample withdrawn and $V_i$ is the total volume of receptor medium. Diffusion curve was constructed by plotting the cumulative amount of drug permeated through total sectional area of the skin against time. Permeation rates (flux, $J$) of drug were calculated from the slope of the regression lines, fitting to the linear portion of the permeability profiles.
In vivo animal studies

Fluorescence microscopy

Fluorescence microscopy study was performed to confirm the skin penetration ability of ethosomes in comparison to the hydroethanolic solution of the drug. The fluorescent marker (Rhodamine 123)-loaded formulations were applied topically to shaved dorsal portion of rats. After 2 h of application, the rats were sacrificed and representative sections obtained from rat skin was cut into small pieces, fixed in 10% formalin and subsequently microtomed [18]. Finally, the stained specimens were observed under fluorescence microscope and skin samples were evaluated for their integrity.

Skin photosensitization and histopathology

For photosensitization studies, the rats were randomly divided into six groups with six rats in each group. Dorsal region of the body was shaved using electric shavers without damaging the skin. The animals in group 1 and group 2 were considered as naïve and sham control group without exposure to UV light, while animals in group 3 were subjected to UV light exposure only without any drug. The animals in groups 4, 5 and 6, however, were subjected to UV light exposure followed by the treatment with MOP-ES, OEMOP1-CBP and MOP-CO formulations, respectively. After grouping, the test formulations were applied topically on the dorsal region of the rat skin followed by exposure to UV radiation after 30 min of the application of formulation. An in-house UV simulator containing UV lamp emitting the full spectrum of UV radiation was used for the experiment. The lamp was attached to snugly fitting hole made in one wall of Styrofoam box with 35 cm × 35 cm × 35 cm in size, while the wall was left open. The animal cages were flush against this open sided box for irradiation to the particular area of skin exposed to the UV irradiation. The distance between the lamp and animals back was kept constant at 35 cm. Skin was examined for photo damage by an observer blinded to the groups allocated of the rats on 35th day. The exposed skin was scored depending on the degree of erythema with ordinal scores such as: 0 (no evidence), 0.5 (minimal or doubtful), 1 (slight redness, spotty and diffuse), 2 (moderate, uniform redness), 3 (strong uniform redness) and 4 (fiery redness). After visual inspection followed by scoring, one rat from each experimental group was anesthetized and sacrificed by cervical dislocation method to collect the targeted area of skin and fixed in 10% formalin. The histopathological examination of the skin was conducted by microtomy followed by staining with hematoxin—eosin dye. The stained specimens were observed under light microscope under 400× resolution and analyzed for the histopathological changes like irregular epidermal or sebaceous hyperplasia, dilation of the dermal blood vessels and tortuosity, in epidermis and dermis, in an observer-blind manner.

Stability studies

Stability studies of the final optimized formulation (i.e. OEMOP1-CBP) were carried out as per the ICH recommended storage conditions, i.e. refrigerated condition (RF; 4 ± 2 °C), room temperature (RT; 25 ± 2 °C/60 ± 5% RH) and accelerated stability conditions (HT; 40 ± 2 °C/75 ± 5% RH) for six months. The prepared formulations were evaluated for size, PDE, PDL and rheological behavior.

Statistical analysis

The statistical data analysis was carried out by Student’s t-test using the GraphPad Prism software version 6.01 (GraphPad Inc., La Jolla, CA). The statistical differences were denoted with $p < 0.05$ and $p < 0.001$ as significant and highly significant results, while $p > 0.05$ was denoted as insignificant.

Results and discussion

Solubility studies

Supplementary Table S1 illustrates the solubility data of 8-MOP in various media. The solubility studies showed that the maximum soluble fraction of 8-MOP was observed in organic solvents, like methanol, EtOH, propanol, PG and chloroform. Besides, the solubility studies performed in various hydroethanolic media to find out the sink medium revealed increase in the solubility of drug with increase in EtOH content. The highest solubility of 8-MOP was observed in PBS with 50% EtOH content (i.e. $2.266 ± 0.041$ mg/mL), thus it was selected for in vitro release and permeation studies.

Characterization of the ethosomes and response surface mapping

Vesicle size

The evaluation of ethosomes prepared as per the experimental design revealed the positive influence of PL and negative influence of EtOH concentration on the vesicle size, as is evident from the 3D- and 2D-plots depicted in Figure 1(A) and (B). The linear escalation in the values of vesicle size indicated higher influence of the EtOH as compared to the PL. The smallest size of the vesicles was observed at lower levels of PL and higher levels of EtOH (Supplementary Figure S1). This can be ascribed to the solvent effect, edge activation and steric stabilization mechanism of the EtOH, which account for decrease in the vesicles size [19]. Increase in the concentration of PL, however, resulted in the larger vesicle size plausibly owing to the formation of highly thickened matrix structure [20].

Percent drug entrapment

Figure 1(C) and (D) depicts the 2D-contour plot with an unambiguous nonlinear trend for the PDE values of the formulations prepared as per the experimental design. The contour plot displays the region of “maximum” at higher levels of PL and intermediate to higher level of EtOH. PDE of vesicles increased with increase in the concentration of EtOH and PL till intermediate levels, followed by a declining pattern. This is conceivable that higher concentration of PL provides enhanced capacity to accommodate the drug in the inner core plausibly owing to higher carrying capacity of the PL molecules and solvent effect [21].

Percent drug leakage

The 3D- and 2D-contour plot portrayed in Figure 1(E) and (F) showed that EtOH exhibited profound effect on the drug
leakage tendencies of the vesicles. Increasing the concentrations of EtOH made the vesicles leakier plausibly owing to partial solubilization of PL and free solubility of 8-MOP in EtOH. Besides, EtOH exhibits fluidizing effect on the vesicle membrane, which causes formation of gap in PL bilayers may leading to higher drug leakage [22]. On the contrary, no significant influence of PL was observed on the drug leakage, as it helps in providing cementing effect to retain the structure of vesicles (REF).

Permeation flux ($J$)

Figure 1(G) and (H) depicts that the 3D- and 2D-plot revealed the effect of PL and EtOH on the flux of drug through skin. Increasing in the concentration of PL resulted in vesicles with controlled drug release property and lower values of flux, whereas increase in EtOH content showed higher values of flux. This can be attributed to the faster permeation of the vesicles across the skin. As PL tend to form lamellar structure, thus increase in PL content revealed formation of increasing number of lamella resulting in controlled drug permeation [23]. Further, solubilization of 8-MOP as well as PL in EtOH may be accountable for increased permeation flux at higher concentrations of EtOH. Apart from this, EtOH helps in attaining faster permeation across the stratum corneum by fluidizing the membrane lipids may be responsible for superior skin penetration ability of ethosomes [24,25].
Equal amount of drug and PG was added, i.e. 150 mg and 20 g, respectively. Finally mass up to 100 g was made up with distilled water.

Skin deposition

Maximum values of Sd for 8-MOP was observed at intermediate levels of the PL and EtOH. Figure (1I) and (J) illustrates the trend for Sd reflecting synergism between the EtOH and PL, which resulted in the formation of supramolecular assemblies with highest Sd at intermediate levels of PL and EtOH. The values of Sd increased with increase in the concentrations of PL and EtOH up to the intermediate levels followed by sharp dip. This can be explained ostensibly owing to the fusion of ethosomal vesicles with skin lipids. Further, increase in PL and EtOH causes disruption of the lipid bilayer to the fusion of ethosomal vesicles with skin lipids. Further, the size of the vesicles obtained was found to be 280 ± 1.2 nm, which coincides well with the size of the vesicles obtained from dynamic light scattering (Figure 3B). Further, the size of the vesicles obtained was found to be 280 ± 1.2 nm, which coincides well with the size of the vesicles obtained from dynamic light scattering technique. Vesicles of this size range have been reported to attain a better skin permeation [26]. Zeta potential evaluation of the ethosomal dispersion was found to be -8.13 ± 0.14 mV, while zeta potential of the 8-MOP-loaded ethosomal formulation showed -2.13 ± 0.14 mV (Supplementary Figure S2). The mild shift in the values of zeta potential toward negative value can be attributed to the incorporation of drug into the ethosomes [7].

Table 1. Design matrix depicting the experimental runs and values of the response variables obtained for experimental runs for MOP-loaded ethosomes conducted as per FCCD.

| Batch no. | Formulation code | Factors | Factors | Response variables |
|-----------|------------------|---------|---------|-------------------|
|           |                  | X₁ Phospholipid (g) | X₁ Ethanol (g) | PDE (%) | Size (nm) | PDL (%) | J (µg/h/cm²) | Sd (µg/cm²) |
| 1         | A(−1, −1)       | 1       | 20      | 47.3     | 245      | 2.67    | 7.21      | 13.89       |
| 2         | B(−1, 0)        | 1       | 30      | 49.6     | 156      | 2.89    | 7.92      | 16.12       |
| 3         | C(−1, +1)       | 1       | 40      | 56.1     | 132      | 4.67    | 8.14      | 11.24       |
| 4         | D(0, −1)        | 2       | 20      | 57.2     | 290      | 2.09    | 5.37      | 27.9        |
| 5         | E(0, 0)         | 2       | 40      | 68.6     | 267      | 2.34    | 6.32      | 29.62       |
| 6         | F(0, +1)        | 3       | 20      | 73.8     | 204      | 4.23    | 7.21      | 22.2        |
| 7         | G(+1, −1)       | 3       | 40      | 54.7     | 335      | 2.03    | 3.46      | 18.23       |
| 8         | H(+1, 0)        | 3       | 30      | 61.5     | 300      | 2.26    | 3.86      | 21.92       |
| 9         | I(+1, +1)       | 4       | 30      | 69.0     | 230      | 4.11    | 4.27      | 16.75       |
| 10        | E'(0, 0)        | 2       | 30      | 66.8     | 267      | 2.34    | 6.32      | 29.62       |
| 11        | E"(0, 0)        | 2       | 30      | 66.8     | 267      | 2.34    | 6.32      | 29.62       |
| 12        | E'3 (0, 0)      | 2       | 30      | 66.8     | 267      | 2.34    | 6.32      | 29.62       |
| 13        | E'4 (0, 0)      | 2       | 30      | 66.8     | 267      | 2.34    | 6.32      | 29.62       |

Optimization data analysis and validation studies

The optimization data analysis was conducted after characterization of the prepared formulations. Table 1 illustrates the responses for the 13 formulations prepared as per FCCD. The obtained data were fitted to the second-order quadratic polynomial model by analyzing the coefficients ranging from the numerical optimization with desirability value closer to unity. Based on the aforesaid criterion, the optimized formulation was embarked upon containing PL 2.16% and EtOH 28.2%, exhibiting vesicle size 281.3 nm, PDE 67.12%, PDL 2.12%, J 5.8 µg/h/cm² and Sd 30.09 µg/cm². The desirable optimal region was also selected in the design space overlay plot followed by demarcation of the optimized formulation in design space (Figure 2). Validation of the experimental methodology was carried out employing linear correlation plots and residual plots with values of “r” ranging between 0.9918 and 0.9965, and overall percent prediction error less than -0.54% ± 0.91% (Supplementary figure S1). This corroborated quite close proximity of the observed responses with the predicted ones for all the checkpoint formulations along with the optimized formulation and ratified high degree of prognostic ability of the selected experimental design (Supplementary Figure S2).

Evaluation of the optimized formulation

The optimized formulation was evaluated for surface morphology, vesicle size and zeta potential. TEM photomicrograph of the optimized ethosomal dispersion (i.e. OEMOP1) showed appearance of ethosomes as the multilamellar vesicles with a predominant spherical shape (Figure 3A). This can be ascribed owing to the effect of EtOH on PL, which helped in providing thinning effect on the outer membrane of vesicles [2]. The multilamellar nature of ethosomes can be explained by the presence of EtOH, which increases the fluidity and flexibility of the phospholipid bilayers to attain a self-assembled structure [27]. Surface morphology evaluation of the ethosomes by SEM also confirmed their 3D confirmation (Figure 3B). Further, the size of the vesicles obtained was found to be 280 ± 1.2 nm, which coincides well with the size of the vesicles obtained from dynamic light scattering technique. Vesicles of this size range have been reported to attain a better skin permeation [26]. Zeta potential evaluation of the ethosomal dispersion was found to be -0.531 ± 0.10 mV, while zeta potential of the 8-MOP-loaded ethosomal formulation showed -2.13 ± 0.14 mV (Supplementary Figure S2). The mild shift in the values of zeta potential toward negative value can be attributed to the incorporation of drug into the ethosomes [7].
Preparation and characterization of optimized ethosomal hydrogel

The ethosomal dispersion possesses low viscosity and practically yield value near to zero indicating lack of patient compliance for convenient application on the skin surface. Thus, considering the suitability for improving the consistency of the final dosage form, a 2% w/w Carbopol® 934 was used for preparing the ethosomal hydrogel of the optimized ethosomal formulation (OEMOP1). The developed ethosomal hydrogel was found to be whitish, translucent and smooth on touch. Microscopic studies did not reveal any evidences of vesicle disruption and revealed that the vesicles were well tolerated the gentle shear during the formation of hydrogel. The pH of the prepared systems was found to be in the range of 5.1 ± 0.6 to 5.7 ± 0.2 indicating skin-friendly and non-irritant nature of the ethosomal hydrogel. The drug content of prepared 8-MOP gel formulation was found to be in the range between 99.14 ± 0.32% to 99.40 ± 0.12%, indicating the minimum loss of drug during gelling process. The above characteristics of ethosomal hydrogel become one of the alternative formulation strategies over the conventional formulation of 8-MOP.

Rheological behavior

The rheological studies revealed that the shear stress was not directly proportional to shear rate (Figure 4A) and the system exhibited a typical non-Newtonian flow behavior. The characteristic bent in the rheogram revealed decrease in viscosity with increase in shear rate indicating a typical pseudoplastic flow behavior of the developed formulation. This can be attributed to the colloidal network structure that aligns itself in the direction of shear, thereby decreasing the viscosity as the shear rate increases [28]. It was observed that with increase in the shear stress, the polymer chains disarranged themselves to align in the direction of flow. This orientation reduces the internal resistance of the material and allows a greater rate of shear at each successive shearing stress. This results in lowering of apparent viscosity. The slope of this graph denotes the value of $N$ greater than 1, indicating non-Newtonian nature of the hydrogel formulation, as is evident from Figure 4(B) [29]. The above rheological characteristics of the developed system fulfilled the ideal requirement of the developed formulations for topical application.

Texture profile analysis

Texture profile analysis is an important aspect of a topical formulation and determines stickiness, ease of application and overall consumer acceptance of a product. Texture profile of OEMOP1-CBP (Figure 5A) was compared with commercial ointment showed that the commercial formulation (Figure 5B) required six-times higher force (560 ± 34.6 g) to penetrate into the skin, while lower value for the optimized ethosomal hydrogel, OEMOP1-CBP (94 ± 6.7 g), vis-à-vis the commercial ointment indicating its ease of application. Gel extrusion studies revealed that the commercial ointment also required approximately five-times (264 ± 22.6 g) higher force than the ethosomal hydrogel (56 ± 12.1 g) to extrude out from the tube. On assessment of stickiness, the commercial ointment was found to be 10-times stickier than the ethosomal hydrogel owing to its higher negative surface area. The results indicated suitability of ethosomal hydrogel formulation for convenient topical application.
**In vitro drug release**

Figure 6 illustrates the cumulative percent release of 8-MOP from OEMOP1-CBP compared to OEMOP1, 8-MOP EtOH solution (MOP-ES), 8-MOP ethosomal hydrogel (MOP-CBP) and 8-MOP commercial ointment (MOP-CO). The drug release from OEMOP1-CBP was significantly ($p < 0.05$) higher (65%) than the marketed product (MOP-CO) (25%). MOP-ES showed nearly complete drug release within 24 h, due to the high amount of EtOH, which increases the vehicle movement freely through the membrane. Analogous release behavior was observed from OEMOP1-CBP and MOP-CBP after 24 h with the values 64.2% and 67.6%, respectively. The drug release studies revealed sustained release nature of the developed ethosomal hydrogel formulation via Fickian-diffusion mechanism.

**Ex vivo skin permeation**

Figure 7(A) portrays the *ex vivo* permeation of 8-MOP from OEMOP1 and OEMOP1-CBP vis-à-vis the OEMOP-CO. This could be attributed to the better entrapment
efficiency of 8-MOP in ethosomes along with enhanced fluidity characteristics of these carriers owing to the synergistic interplay of excipients like, EtOH, PG and PL. In addition to this, the enhanced transdermal efficacy obtained from the ethosomal system could be justified on the basis of dual function performed by EtOH present in the ethosomal formulations, which tend to fluidize both the vesicular lipid bilayers and stratum corneum lipids, thus providing a greater malleability to the vesicles for enhanced permeability through skin [25]. Phospholipids aid in the penetration of the vesicles by mixing well with the skin lipid bilayers and forms opening in the stratum corneum for ease of penetration of the drug molecules. Besides, the lipophilic excipients present in the formulation helps in enhanced retention of the vesicles in the skin layers. On the contrary, the conventional topical formulation shows ionization of the drug free drug at physiological pH of skin, leading to decrease in the permeation rate [25,30]. The above promising characteristics of the developed formulations ratified the enhanced topical drug delivery potential for 8-MOP.

The comparative evaluation of the permeation flux for the developed formulations was found to be in the order of MOP-ES > MOP-CBP > OEMOP1 > OEMOP1-CBP > MOP-CO (Figure 7B). It was observed that the variation in the permeability of different systems (i.e. gelled/no-gelled) can be accounted for the internal 3D structural network and composite nature of the material. The highest drug permeation (i.e. 77.74 ± 0.3%) was observed from MOP-ES within few hours, while MOP-CBP showed higher permeation vis-à-vis their respective OEMOP1 and OEMOP1-CBP. In the simple gel formulation, the drug embedded in the hydrogel
base favors faster escape of the drug into the external micro-environment, thus showed faster drug release and permeation properties. On the other hand, permeation of 8-MOP from ethosomal dispersion and ethosomal hydrogel formulations was retarded by ethosomes. This can be ascribed to the formation of multi-lamellar layers of ethosomes, which act as a barrier for retarding the release of drug from gel microenvironment and prolonged drug permeation [31].

The data from the current studies clearly indicated that ethosomal system was more effective compared to the marketed formulation in delivering the drug into the deeper layers of skin. The permeability of drug through ethosomes into the skin occurs through a series of concomitant processes. The high lipid content and presence of EtOH as edge activator helps in faster permeation of the drug-loaded vesicles through densely packed and highly ordered conformational structure of physiological lipids (REF). After application of ethosomes, EtOH interacts with lipid molecules in the polar head group region, resulting in a reduction in the phase transition temperature \( T_m \) of the stratum corneum lipids owing to the increase in their fluidity. In addition to this, presence of phospholipid contributed for attaining faster permeability of the drug by intercalation with the stratum corneum barrier [32].

**Skin deposition**

The bar diagram depicted in Figure 8 portrays that the ethosomal systems offer better drug deposition in the skin possibly owing to the combined effect of EtOH, PG and PL on skin lipids. On the contrary, conventional systems showed less drug deposition plausibly owing to lower permeability through the stratum corneum and inability to maintain reservoir effect of the drug in the skin layers. The optimized ethosomal formulation, OEMOP1 produced significant \( (p < 0.01) \) accumulation of 8-MOP (i.e. 21.64 ± 0.11 \( \mu \)g/cm\(^2\)) in the deeper dermis layers of skin. In contrast, the formulations like MOP-CO and MOP-CBP showed accumulation only up to 11.33 ± 0.08 \( \mu \)g/cm\(^2\) and 8.9 ± 0.06 \( \mu \)g/cm\(^2\), respectively. High Sd property of the ethosomal vesicles might be explained due to the deformability of ethosomes plausibly by their entanglement with skin lipids, leading to higher chances for retention of them for prolonged periods of time. Furthermore, the ethosomes incorporated in the gel base to prepare OEMOP1-CBP showed higher skin retention ability of the drug (23.17 ± 0.12 \( \mu \)g/cm\(^2\)) within the skin ostensibly owing to the enhancement effect of vesicular gel and transportation of drug through skin appendages may also be responsible for attaining higher permeation efficiency [12]. This inferred that the prepared ethosomal formulations could effectively make the drug accessible within skin layers with enhanced retention of the drug as depot within the skin.

**In vivo studies**

**Fluorescence microscopy**

The extent of penetration and underlying pathways involved during passage of vesicles into the skin was studied after application of hydroethanolic solution and ethosomal hydrogel formulation through fluorescence microscopy studies. The images revealed that the hydroethanolic solution was effective in permeating fluorescence dye though very low intensity vis-à-vis the ethosomal systems (Figure 9). The ethosomal systems increased deeper penetration and consequent fusion of vesicles with skin membrane lipids and exhibited relatively higher fluorescence intensity. This can be explained by enhanced penetration ability of ethosomes across the stratum corneum via formation of channels, disorganization of membrane lipidic barriers and passage through skin appendages involving hair follicles, sebaceous glands and associated structures.

**Skin photosensitization and histopathology**

Skin photosensitization studies after exposure of animals to UV light revealed occurrence of skin erythema in various
treatment groups. The naïve and control group showed no viable changes in the skin appearance without any signs of erythema (score 0). The positive control group exposed to UV light only showed slight erythema (score 1) and wrinkling in three rats (Table 3). However, the animals treated with MOP-ES followed by exposure to UV radiations revealed 83.33% (5 of 6) rats with indication of severe erythema (score 4) and signs of damage on the dorsal region of the skin. The animals treated with MOP-CO formulation also showed marked erythema (score 3) in 66.6% animal population (4 of 6). On the contrary, the animals treated with OEMOP1-CBP showed only slight redness and mild tanning (score 1), affecting only 33.33% (2 of 6) of the animal populations.

Histopathological examination of the skin sections of various groups of animals is depicted in Figure 10. The skin sections of naïve and control group showed quite normal skin physiology without any viable changes. Mild mast cell hyperplasia along with clusters of leukocytes in the superficial keratin layer over the epidermis was observed in the animals treated with UV radiation exposure only. The most striking changes in skin physiology was observed in the animals treated with MOP-ES marked with irregular epidermal hyperplasia, sebaceous hyperplasia, focal spongiosis, dermal blood vessels dilatation and tortuosity. The animals treated with OEMOP1-CBP, however, exhibited minimal damage to the skin with mild generation of mast cells and fibroblastic proliferation in the deeper layers of dermis. On the contrary, the animals treated with conventional marketed gel formulation were found to be quite inferior compared to the ethosomal gel formulation with the presence of mild epidermal thickening and parakeratosis, followed by no signs of neutrophil deposition in the keratinocytes.

Overall, the studies corroborated that the prepared ethosomal systems of 8-MOP exhibited remarkably less phototoxicity on the skin. This could be ascribed owing to the presence of drug entrapped in the supramolecular vesicles, which tend to interact directly with the skin leading to higher concentration of drug in the inner microenvironment. Moreover, the entrapped drug allows slower and sustained delivery of 8-MOP to the targeted tissues, thus minimizing the inflammation and associated events causing changes in the skin physiology upon exposure to the UV radiations. There are many reports showing that drugs entrapped in the lipidic systems interact in a more conducive manner with the affected skin sites with no or very low side effects, while maintaining efficacy of the drugs [33].

The underlying mechanistics for reduced photosensitization properties of 8-MOP loaded ethosomal systems over the free drug can be explained majorly owing to the presence of phospholipids, which interact with 8-MOP and facilitates formation of photo-adducts of non-cyclobutane type upon exposure to UV radiations. These photo-adducts are considered to be less toxic for keratinocytes, thus exhibiting a miniscule phototoxic reactions in the skin [34]. In other words, photoradiation accompanied by isomerization of double bonds in fatty acid molecules, photo-oxidation of phospholipids and psoralen and splitting of fatty acid adducts, all together helps in maintaining the cellular homeostasis in the stratum corneum and deeper layers of dermis [4]. The studies ratified significant importance of developing the phospholipid-based carriers of 8-MOP, which proved to be highly effective in attaining enhanced permeability and biocompatibility properties along with reduced phototoxic adverse affects of drug for long-term therapy.
Stability studies
Stability studies of the prepared formulations did not exhibit significant change in physical appearance, vesicle size, zeta-potential, drug content, PDE and PDL at 4 ± 2°C condition for 6 months. The optimized gelled formulation (OEMOP1-CBP) maintained its pH, viscosity and drug content at this storage temperature for 6 months. At 25 ± 2°C/60 ± 5% RH and 40 ± 2°C/75 ± 5% RH, an increase in the average vesicular size and percent transmittance was observed. After 2 weeks of storage at 40 ± 2°C/75 ± 5% RH, the visible signs of physical instability like vesicle fusion, aggregation, disruption and sedimentation were observed due to the fusion of phospholipid membrane of the ethosomes [35]. After 12 weeks of storage, substantial amount of drug leakage (11.86 ± 0.13%) was observed at 40 ± 2°C/75 ± 5% RH. Significant loss of drug from the vesicles can be assigned to the effect of high temperature on the gel-to-liquid transition of lipid bilayers along with chemical degradation of the phospholipids, leading eventually to the disturbance in membrane packing [36]. Negligible change in the density of the vesicular dispersion was observed at all the temperature conditions, indicating the maintenance of vesicle originality. After 6 months storage of ethosomal hydrogel at 4 ± 2 and 25 ± 2°C/60 ± 5% RH, no significant degradation in the lipid structure was observed. The differences in drug content were statistically significant (p < 0.05) in case of all the formulations stored at 40 ± 2°C/75 ± 5% RH. This may be due to the coagulation of phospholipids at higher temperature leading to formation of aggregate of lipids. The EtOH present in the formulations causes fluidization of the vesicle membrane, leading to higher drug release [37]. Overall, the results showed best storage stability of ethosomal systems at 4 ± 2°C.

Conclusions
The present studies successfully vouch development of 8-MOP-loaded ethosomal hydrogel for enhanced topical delivery against vitiligo. The implementation of QbD approach helped in systematic development of ethosomal system followed by detail understanding of the formulation attributes related to the finished product quality. The prepared formulation not only provided better flux as compared to hydroethanolic formulation and commercial product, but also facilitated higher Sd efficiency of the drug. The fluorescence microscopy also revealed the characteristics of ethosomes supporting permeation of 8-MOP into the deeper skin layers owing to the presence of EtOH on stratum corneum lipids by increasing vesicle fluidity, leading to superior delivery properties. Skin photosensitization and histopathology studies also construed less phototoxic nature of the developed formulation. Overall, the studies report newer therapeutic opportunity for enhanced topical delivery of 8-MOP in the treatment of vitiligo employing ethosomal hydrogels as the promising carriers for the purpose.

Acknowledgements
We are thankful to M/s Gary Pharmaceutical Pvt. Limited, Ludhiana, India and Phospholipids GmbH for generously providing the Gift samples of Methoxsalen and Phospholipon 90 G, respectively. Head, CPBES, PU, Chandigarh is acknowledged for providing the facility to perform fluorescence microscopy. In addition to this, authors are grateful to All India Institute of Medical Sciences, New Delhi, India and CIIL, PU, Chandigarh for providing TEM and SEM facility, respectively.

Declaration of interest
The authors declare no conflict of interest. Authors are thankful to University Grant Commission, New Delhi, and India and Council of Scientific and Industrial Research (CSIR-HRDG) New Delhi, for providing fellowship and grant to carry out the research work.

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Supplementary material available online
Supplementary Figures S1 and S2 and Table S1 and S2