Microemulsions mediated effective delivery of methotrexate hydrogel: more than a tour de force in psoriasis therapeutics

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Abstract

Methotrexate (MTX), a well known drug for the treatment of cancer and rheumatoid arthritis, has gained prominence in the treatment of psoriasis over the period of years. However, the present mode of systemic administration through oral or parenteral route has always been in vogue, full of compromises. The toxicity of drug to the vital organs and physiological environment is the major concern. Also, its poor skin penetration is one major problem. Hence novel system based on lipid carriers has been considered here to overcome the barriers. Microemulsions (MEs) were prepared using pseudo-ternary phase diagram (PTPD) and they were characterized for various parameters such as size, shape (cryo-SEM), PDI, zeta potential, etc. The chosen MEs system (optimized) was then incorporated into secondary vehicles and characterized for rheological behavior, texture profile analysis, in vitro release, ex vivo permeation and drug distribution into different layers of skin. The developed formulations were further evaluated in ex vivo and in vivo such as cell line study, imiquimod-induced psoriatic model, allergic contact dermatitis, rat tail model (% orthokeratosis) and safety test (Draize test).

The MEs based MTX gel has shown its potential in locating the drug at the desired domain of stratum corneum, epidermal and dermal layers of skin and reducing systemic absorption. Our results are suggestive of MEs potential as a novel carrier for topical delivery of MTX in topical therapeutic and safety approaches. In conclusion, developed MEs-based hydrogel has shown promising results in achieving effective delivery of MTX.

Introduction

Psoriasis is a chronic non-infectious, inflammatory autoimmune skin and joint manifestations disorder characterized by well-defined, distinctive erythematous plaques producing adherent silvery white and red scales on the top layer of epidermis. It is frequently seen at the extensor surfaces of the elbow and knees, scalp and sacral areas. It occurs when immune system misguides skin cells such as a pathogen and transmits faulty signals that facilitate growth and division of skin cells resulting in psoriasis [1,2]. The first-line therapeutic approaches developed for the treatment of psoriasis are often less effective due to the poor penetration, cosmetically unacceptable, inconvenient for long term use and/or associated with cytotoxicity [1].

Methotrexate (MTX) is one of the oldest and widely used chemotherapeutic agent, not only in cancer therapy [1], but also in the treatment of autoimmune diseases [1,3]. MTX is a first line treatment for severe stage of plaque psoriasis and oral as well as parenteral forms are available with a weekly recommended dosage between 10 and 25 mg for long periods of time approved by FDA [4]. MTX primarily acts as dihydrofolate reductase inhibitor, and subsequently results in hindered DNA synthesis which in turn inhibits cell proliferation. The drug competes with reduced folates [4] for transport and polyglutamation and also suppresses the autoimmune system and regulates the undesired cell proliferation. Despite of unequivocal advantages of MTX, a few obvious disadvantages such as short plasma half-life, poor absorption, unpredictable bioavailability and clinical usage have been seen. In addition, higher dosage showed non-specific toxicity on normal proliferating cells. The patients receiving long term therapy has alarmed gaining MTX resistance and it needs to be objectively addressed [3]. The topical administration of MTX is recommended to overcome these problems. Several formulation strategies including transcutaneous delivery [5], liposomes [6] and deformable liposomes [1], various surfactants with hydrophilic gel [7], nanogel [8,9], nanostructured lipid carriers (NLCs) [2], passive and iontophoretic transdermal delivery [10], solid in oil nano-carriers [4], ufasomes [11] have been attempted for greater efficacy and to minimize side effects. Despite enormous efforts, no effective and
efficacious topical dosage forms of MTX are commercially available yet.

The pH dependent hydro-solubility, dissociation at physiological pH and high molecular weight are few obvious constraints with MTX for the development of topical formulation. This is due to the limitation of probability of passive diffusion of MTX. In addition, effective penetration of MTX through stratum corneum is a major challenge in transdermal delivery. The presence of lipid in stratum corneum represents lipophilic barrier that restricts permeation of molecules.

Microemulsions (MEs) have distinct hydrophilic and lipophilic domains, allowing dissolution of both water-soluble and water-insoluble drugs [12]. MEs are dispersions of oil and water stabilized with a surface active film composed of surfactant and co-surfactant. They are of special importance due to their spontaneous formation, thermodynamic stability and optical transparency [13]. We selected ME as delivery system based on its unique composition that provides biphasic solubility at molecular level, partitioning into skin tissue for improving topical delivery, and their complete and uniform association with drug molecules.

The novel dermatological products based on phospholipid-carrier-based approach have already been developed in our laboratory and the technology has been successfully transferred to pharma industry. These products are Psoriosome™ (liposomal dithranol) [14], Lipotar™ S (coal tar salicylic acid) and Lipotar™ SS (coal tar salicylic acid and sulfur). Psoriosome™ was a first liposomal product developed for psoriasis in the world and it is used in plaque psoriasis. While Lipotar™ S and Lipotar™ SS are used in wide range of dermatological disorder viz. Seborrheic dermatitis, eczema, psoriasis and dandruff. However, due to increasing attention towards methotrexate (MTX) molecule in the treatment of psoriasis [15,16], further opportunities in the topical dosage form development needs to be explored. Hence, there is a dire need to develop a novel topical formulation to cater the unmet medical needs. The current study was designed to develop and explore the delivery potential of ME based hydrogel for targeted and sustained MTX delivery to stratum corneum, epidermis and dermis of psoriatic site.

Materials and methods

Materials

Methotrexate was obtained as gift sample from M/s Ipca Pvt Ltd, Mumbai. Polysorbate 80 and n-octane (free of olefins) procured from Sigma-Aldrich, India. Hela cell line was procured from the National Center for Cell Sciences (NCCS), Pune, India. Minimum essential medium (MEM), fetal bovine serum, penicillin, streptomycin, amphotericin B, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), dimethyl sulfoxide, cyclophosphamide, glycerol monostearate (GMS) and all other chemicals were used high grade.

Microemulsion preparation

Pseudo-ternary phase

Raw materials were selected based on their drug solubilization capacity and ME-region was searched with the help of pseudo-phase diagrams. ME region was prepared by titration method in a branched series with serial changes in the compositions (Figure 1). Upon titration, phase boundary points were obtained and these points were presented using Gibbs phase triangle that showed changes in phase behavior.
with changes in the weight fractions of water-oil-surfactant mix. To find out the ME-region, at 30°C and room pressure, a mixture of known amount of $S_{\text{max}}$ and oil (i.e. 10:0 to 0:10 w/w) taken and subsequently titrated with aqueous phase (i.e. aqueous titration where aqueous phase acts as titrant) and vice-versa with the help of microsyringe. After each addition, mixture was gently stirred and then placed in a temperature controlled water bath. The resulting mixture was characterized for isotropic/anisotropic phase by visual observation and through crossed Polaroid microscope after allowing sufficient time (typically 3–4 h) to achieving equilibrium at specific temperature. Titration was continued until persistent turbidity of samples was seen or vice versa. Having obtained the final weight of titrant for particular $S_{\text{max}}$ and oil or aqueous ratio, weighed percentage was calculated and plotted in Gibbs phase triangle as boundary points. The entire phase diagrams were mapped in this manner by oil titration too (oil acts as titrant).

**Preparation of microemulsion**

Microemulsion (ME) was prepared by selecting the ratio of all the phases (lipid, water and $S_{\text{mix}}$) from the ME region of ternary phases diagram of C2 (Figure 1). Briefly, weighed quantity of phospholipid 90G was dissolved in co-surfactant (ethanol) using magnetic stirrer and Tween 80 was then mixed, together to form $S_{\text{mix}}$. Isopropyl palmitate (IPP) was added slowly under continuous stirring followed by mixing aqueous phase (PBS 7.4) in a thin stream stirring constantly. The system was further stirred for next 5–10 min for complete mixing of all the phases to attain transparent ME. For incorporating the drug in ME, weighed amount of MTX was added in the above mixture and stirring was continued till drug completely dissolved in it.

**Characterization**

**Size and zeta potential**

Globular size is a characteristics feature of the ME that differentiates it from emulsion. Dynamic light scattering (DLS) technique, also known as Photon Correlation Spectroscopy, was used to determine globular size and its polydispersity [17]. The measurements were carried out at a scattering angle of 90° at 300°C. The zeta potential of MEs was measured by laser-based multiple angle particle electrophoresis analyzer. Delsa Nano particle analyzer (Beckman Coulter Inc., San Jose, CA) was used to analyze mean globular size, PDI and surface potential (i.e. zeta potential) [18].

**TEM and Cryo-SEM**

The morphology of MTX loaded MEs was examined using transmission electron microscopy (FEI Morgagni 268 D). One drop of diluted samples was deposited on a film-coated copper grid and later stained with one drop of 1% aqueous solution of phosphotungstic acid (PTA) and examined under the electron microscope. However, using only this method is not right approach to visualize the nano structures in the case of ME because most of the aqueous content lost during sample processing and during observation. And, any changes in the ratio of pseudoternary phase would lead to transformation in the internal structure. Hence, Cryo-SEM was also performed.

Cryo-SEM was performed on the FIELD Emission Gun (FEG) Scanning Electron Microscopy (SEM; JSM-7600F) attached with JEOL CRYO unit (PP3000T) by Quorum in the facility of IIT Mumbai, India. In brief, a drop of sample was placed on sandwich holder and the sample assembly was rapidly frozen with pool of liquid N2 to avoid ice crystals. Sandwich sampler was then cryo-transferred into the SEM and held on a cryo-holder within the SEM. Frozen sample was fractured by separating the sandwich. A platinum carbon film deposited on the fractured surface was shadowed at 45° angle with electron beam evaporation. Then a support layer of carbon is evaporated into shadowed layer that gave its replica. Same was viewed under SEM after the removing the residual samples.

**Refractive index, transmittance, viscosity and conductivity analysis**

Refractive index (RI) of MEs were checked by refractometer (Shijiazhuang Optical Instrument Factory, China), planar micro lenses with two refracting interfaces facing each other are fabricated with a planar light wave circuit technology. Sample was filled between the two refractive surfaces and the width of the beam passing through the lens was measured. The RI of the liquid could be determined accurately comparing the measured beam width with the analytical results.

Transmittance of the light through the clear medium is expressed as percent transmittance. Here, UV spectrophotometer (Shimadzu UV-1601) was used and developed ME was measured at 550 nm against water as a blank.

The viscosities of MEs were measured at 25°C, using the Rheolab QC, Anton Paar, Germany.

Electrical conductivity was measured using a PICO digital conductivity meter operating at 50 Hz from Labindia Instruments. Electrodes were inserted in a double-walled thermostatic glass cell containing ME. Based on electrical conductivity, phase systems of the MEs were determined.

**Drug loading**

Liquid–lipid extraction technique was used to analyze MTX in the ME samples. Ten folds isopropyl alcohol was added in ME and vortexed to dissolve it. The whole mixture was optically checked for drug particles. The mixture was then suitably diluted with HPLC mobile phase and charged in HPLC for analysis after filtering it through 0.22 μm membrane filter.

**High performance liquid chromatography analysis**

Samples were analyzed using the HPLC system (Shimadzu LC-2010 CHT) equipped with a system controller, quaternary gradient pump, solvent delivery module, online degasser, auto-samples with cooler, auto injector (injection volume ranging between 5 and 100μL) and UV–Vis detector. Reversed phase C18 column (5 μm, 4.6 mm × 250 mm, Waters) was used for chromatographic separation. The mobile phase was a mixture buffer (pH 6.0) and acetonitrile in the ratio of 9:1. The buffer constituted with 0.126 M disodium hydrogen orthophosphate and 0.037 M citric acid.
Samples were analyzed at 1.2 mL/min flow rate and detected at 302 nm. For each analysis, 10 μL sample was charged onto column temperature 30 °C.

**Secondary vehicles for microemulsion system**

The secondary vehicle was selected from various hydrophilic and lipophilic systems to develop efficient ME system from rheological standpoint for topical application.

Brief, hydrophilic gels were prepared initially in higher concentration and weighed amount of ME was gently levigated to prepare final hydrogel to obtain 0.25% w/w methotrexate-loaded microemulsion (M-ME) in different gel concentration of Poloxamer 407 (5–30%). In contrast to hydrophilic gels, gelling material and ME system was heated up to 60 °C while preparing lipophilic gels. Then, M-ME was poured into melted gelling material and mixed gently till the temperature of gel is reached at room temperature.

Gelling systems were initially screened on the basis of their capability to hold the ME i.e. overall physical stability. Two tests were performed in this regard. In first test, gelled systems were stored at 40 and 60 °C for 1 month and observed any kind of separation or changes. In second test, the formulation was centrifuged three times at 2026 × g for 10 min with a gap of 30 min in between.

**Rheology study**

Rheological behavior of formulation was determined by Rheolab QC HS 143 and MCR 302–Rheometer, Anton Paar, Germany [19]. Sufficient amount of the sample was placed in sample holder, fitted with probe assembly, attached with main instrument and allowed to equilibrate at 25 ± 0.1 °C for 30 min. All tests were replicated three times and mean values were used for analysis. The shear rate value was increased automatically linearly from 0.1 to 100 s⁻¹.

The relationship between shear stress (τ) and shear rate (γ) of each formulation was determined using Herschel–Bulkey model.

\[ \tau = k \gamma^n \]

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

**Texture analysis**

The textural properties (i.e. firmness, spreadability, stickiness, etc.) play a crucial role in patient acceptability for an optimal topical formulation. Texture profile was based on the measurement of the force (penetration and detachment force in grams) and the work (work of shear and adhesion in g s) was done by the male cone to penetrate and detach from the test formulation present in the female cone. These properties were determined by employing TTC spreadability rig fitted on Texture Analyzer™ [19]. The experiment was then started under controlled software program (i.e. Exponent 32™).

**In vitro drug diffusion**

Cellophane membrane (12 kDa, Himedia, Mumbai, India) was used to assess drug diffusion pattern from the vehicle. Study was performed on a Perme Gear’s six station Franz diffusion cell assembly. The total capacity of the receptor compartment was 30 mL containing 75% v/v methanol:DMF (10:1) in PBS (pH 5.6). The receptor medium was stirred throughout the study at 500 rpm employing an in-built magnetic stirrer. The temperature of the receptor medium was maintained at 37 ± 0.2 °C by circulating warm water in the outer jacket of the cells employing a thermostatically controlled water circulator. Cellophane membrane was mounted in between the donor and receptor compartment and was allowed to equilibrate with the sink medium. Test formulation was applied uniformly in the donor compartment and aliquots of 1 mL were withdrawn with equal replacement at definite time intervals [20]. At the end of this study, remaining drug in the donor compartment was quantified after extraction using HPLC method, as described in the ‘‘High performance liquid chromatography analysis’’ section.

**Ex vivo study**

**Preparation of skin**

Non-heat treated pig ear pinna (a vestigial part) was collected from the government slaughter house, Industrial phase, Chandigarh, India for ex vivo permeation studies. Outer skin of the ear was separated from the ear cartilage by scalpel. Same scalpel was used to trim the hair. The hypodermis including blood vessels and fat layer was removed. To remove residual fat, dermis side of the skin was wiped 3–4 times with cotton soaked in 50% iso-propanol and then washed thoroughly with saline water. Skin was then wrapped in aluminum foil after whipping with absorbent paper, to remove excess surface water. The skin was stored at −20 °C for maximum of 2 weeks and thawed 12 h before commencing experiments. Skin was carefully checked using microscope and magnifying glass, before using it for permeation study, to check any surface irregularities such as tiny holes, etc [21].

**Permeation study**

Franz diffusion cell with an effective diffusion area of 3.14 cm² was used for in vitro permeation study. The skin was mounted carefully, in between the donor and receiver compartment, on diffusion cells with the stratum corneum side up. All the active comparator viz. M-ME, gelled ME (M-ME Polox-20), drug dispersion in gel (M-Polox-20), drug suspension, conventional cream, equivalent to 1000 μg MTX were applied on the donor compartment. All other conditions were same as used in in vitro diffusion study [22]. All samples were filtered through a 0.22 μm pore size membrane filter and analyzed by HPLC.

**Skin retention study**

The skin mounted on the diffusion cell was removed carefully followed by the permeation study. The remaining formulation, adhering to the skin in the donor compartment, was carefully scrapped off with a spatula and analyzed subsequently for the drug content. The clean skin tissue was washed thrice with saline and dried using lint-free cotton swab and let it dried. Further, epidermal and dermal layers were manually separated using tweezers. Later on, the skin was cut in round shape, acquired while permeation study and chopped into
small pieces. These pieces were then suspended in 2.5 mL trypsin, 0.05% (w/v), at 30°C for 12 h with intermittent sonication using bath sonicator. After due time, 2.5 mL methanol/ethanol was added and again placed at 30°C for 12 h with intermittent sonication under bath sonicator. Whole sample was centrifuged and supernatant was suitably diluted with respective mobile phases. The obtained solution was then filtered through a membrane filter (0.22 μm) and analyzed for drug by HPLC [22,23].

Psoriatic cell lines maintenance and cytotoxicity assay

Hela cells were maintained and propagated in MEM complete medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL amphotericin B in CO2 incubator at 37°C for 48 h.

The anti-proliferative activity of formulations was assessed on cell lines (Hela cells) following MTT assay as earlier reported by Garg et al. [18]. Cell monolayer was washed with PBS (pH 7.4) without Ca2+/Mg2+ and subsequently trypsin/EDTA was added. Then cells were scrutinized and resuspended in a fresh MEM and incubated for 24 h. After incubation, total of 5 × 104 cells in 200 μL of MEM medium were plated per well in a 96 well plate. Medium alone was taken as blank whereas cells with medium as control.

A volume of 50 μL of various drug formulations (in strength of 0.01–1000.0 μM) was plated as per scheme prepared, in their assigned wells. The 96-well plates were incubated for 24 h in a humidified incubator at 37°C with CO2 for 3–4 h. After 24 h, 25 μL of MTT solution (5 mg/mL in PBS pH 7.4) was plated in each well, and the plate was placed on a shaking table for 5 min in order to mix the MTT into the medium. The 96-well plates were further incubated at 37°C with 5% CO2 for 3–4 h. After incubation, 200 μL of medium was discarded from each well and were dried. Formazan thus produced, was resuspended in a 2.5 mL DMSO and placed on a shaking table for 5 min to thoroughly mix the formazan into the solvent. The plate cover was removed and absorbance was measured in each well at 570 nm in micro-plate reader. The percentage cell survival and percentage cell inhibition were calculated.

Animal study

Imiquimod-induced psoriatic model

C57BL/6 mice (8–11 weeks old) were housed in pathogen-free conditions and imiquimod (IMQ) was used to induce psoriasis [24,25]. Topical application of its suspension was applied for 5 consecutive days on shaved back of mice. The dose of IMQ applied (4 mg/day) was optimized based on the induction of skin inflammation. To the inflamed skin area, anti-inflammatory formulations were applied topically every day for 5 days and dexamethasone 0.1% (w/w) was used as a positive control (PC) in the study. Severity of inflammation was daily monitored using standard Psoriasis Area and Severity Index (PASI) [26] scoring system. In PASI, erythema, scaling and thickening was scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The rank order scoring was performed every 24 h for 5 days. Six animals per group were deployed in the study to evaluate immune response.

Allergic contact dermatitis

The C57BL/6 mice were sensitized for 4 consecutive days by applying 25 μL of 0.5% (v/v) 2,4-dinitrofluorobenzene (DNFB) in acetone:olive oil (4:1) on the shaved abdomen [27] area before induction of ACD response. On the fifth day, mice were challenged by epicutaneous application of 25 μL of 0.2% DNFB in acetone:olive oil (4:1) on the right ear to induce allergic contact dermatitis (ACD) response. The left ears were treated with DNFB vehicle alone (acetone:olive oil: 4:1) and served as an internal control. The ACD response was determined by the extent of ear swelling compared with that of the ear before DNFB challenge. Increase in ear thickness was measured with a vernier caliper (Fraction + Digital Fractional Caliper, General Tools and Inst. Co., LLC, New York, NY) at 0, 24, 48 and 72 h. After 2 h of antigen challenge, developed formulations were applied topically on right ear three times a day for 3 days. Dexamethasone (0.5 mM solution in ethanol–PEG-400 mixture) and Protopic® (0.1% w/w Tacrolimus) were used as positive controls. Ear swelling was measured before and after application of anti-inflammatory formulations.

Rat tail model

Wistar rat of either sex weighing 220–250 g were used in the study. Six animals per group were deployed for the study, first group did not receive any treatment and served as control. M-ME Polox-20 and its placebo gel (Polox-20) were applied in second and third group, respectively. In each group, formulations were applied once a day for 2 consecutive weeks on to the proximal part of the tail and a plastic cylinder was fixed with adhesive tape for 2 h. At last day of study, animals were sacrificed by spinal dislocation after 24 h of the last application and the tail skin was cut and stripped from the underlying cartilage. Tissue was fixed in 5% formalin followed by paraffin embedding, and longitudinal sections of approx 5 μm were sliced and stained with Hematoxylin–Eosin and histological evaluation was performed [20]. Skin samples were microscopically examined for the presence of granular layer in the scale regions and epidermal thickness. Induction of orthokeratosis in those parts of the adult rat tail, which have normally a parakeratotic differentiation, was quantified by the following equation:

Percent orthokeratosis = (a/b) × 100

where a = length of the granular layer per scale and b = whole scale length.

Whole scale length represents the length of the scale lying between two adjacent hair follicles. Ten sequential scales per animal were measured and results given in percent orthokeratosis per scale.

Skin safety test (Draize test)

Male albino rabbits (1.5–2.5 kg) were used to judge irritancy of different formulations, as described by Draize et al. [46]. Rabbits were housed in standard housing condition at standard diet. Back hairs were trimmed 24 h prior to the study. Three squares were drawn on back side of each rabbit. Each square was divided into six sub-groups each. The first
group did not receive any treatment and acted as a sham control, second group received PBS (pH 7.4) and acted as a control and third group received 20% SLS solution acting as a positive control. Rest three groups received MTX in PBS (pH 7.4), M-ME Polox-20 and Polox-20, respectively.

Stability studies
Optimized formulation was subjected to stability studies at 5 ± 3°C, 30 ± 2°C/65% ± 5% RH, 40 ± 2°C/75% ± 5% RH for 12 months. The formulation was filled (n = 3) in the collapsible tubes and ungelled formulations were filled in glass containers, sealed and stored at prescribed conditions in the stability chambers. Drug content, globular size, separation, phospholipid content and percent transmittance were considered stability indicators [21].

Statistical analysis
The results were expressed as mean ± SD and the statistical analysis was done by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison post-test using GraphPad InStat™ software (GraphPad Software Inc., San Diego, CA). Statistical differences are denoted as *p < 0.05, **p < 0.01 and ***p < 0.001, respectively. NS = not significant (p > 0.05).

Results and discussion
Construction of pseudo-ternary phase diagram (PTPD)
Based on the solubility study (Supplemental Table S1); IPP was selected as oil phase, PBS as a water phase, Tween 80 as surfactant and mixture of phospholipids and ethanol were used co-surfactant to prepare the PTPDs to get the maximum emulsification area. Changes in the ratio of surfactant:co-surfactant (changes in the internal ratio of $S_{mix}$) and ethanol:phospholipid (changes in the internal ratio of co-surfactant) were made along with the changes in the ratio of $S_{mix}$ with aqueous and oil phase. It was observed that at constant surfactant–co-surfactant weight ratio ME region increased with decreasing ratio of ethanol to phospholipid from 10:1 to 4:1 (Code A–C). On the other hand, further reducing the ratio to 2:1 (Code D) slight reduction in emulsification area was seen. Hence, 4:1 weight ratio of ethanol to phospholipid was selected for further modification in the ratio of surfactant–co-surfactant (Figure 1 for Code 2C) and maximum emulsification area was found with C2 when changes were made in ratios. Consistent to the study reported earlier [28], we obtained the similar results with increasing the weight percent of co-surfactant and C2 ME region was selected for further study.

Preparation and characterization of optimized microemulsion
Microemulsions (MEs) were prepared from C2 ME area with different ratio of oil, water and $S_{mix}$ and characterized them (Table 1), and optimized ME 4 (M-ME) was selected for further study. ME 4 has negative charge which will help better in penetrating the affected skin cells. Also, it has pH closer to skin pH, and therefore it will be more skin friendly and should not cause any skin irritation. It does have balanced

| Formulation code | Refractive index | S$_{mix}$: Water: Oil | Density | Viscosity [cP] at 30°C | Conductivity (mS) | pH | Transmittance (%) | Conductivity (mS) | Zeta Potential (mV) |
|------------------|------------------|------------------------|---------|------------------------|-------------------|----|-------------------|-------------------|-------------------|
| ME1              | 1.459 ± 0.031    | 80:39:20:10            | 0.898 ± 0.01 | 2.36 ± 0.02 | 0.114 ± 0.005 | 4.3 | 62.1 ± 0.005      | -1.02 ± 0.02      | 0.45 ± 0.03       |
| ME2              | 1.399 ± 0.031    | 80:37:20:13            | 0.895 ± 0.01 | 2.57 ± 0.02 | 0.423 ± 0.005 | 5.6 | 72.3 ± 0.005      | -0.92 ± 0.02      | 0.31 ± 0.03       |
| ME3              | 1.398 ± 0.031    | 70:40:20:10            | 0.897 ± 0.01 | 2.73 ± 0.02 | 0.383 ± 0.005 | 5.8 | 79.7 ± 0.005      | -1.02 ± 0.02      | 0.45 ± 0.03       |
| ME4              | 1.398 ± 0.031    | 60:40:20:10            | 0.895 ± 0.01 | 2.79 ± 0.02 | 0.283 ± 0.005 | 6.0 | 76.8 ± 0.005      | -1.02 ± 0.02      | 0.31 ± 0.03       |
| ME5              | 1.398 ± 0.031    | 50:50:20:10            | 0.895 ± 0.01 | 2.85 ± 0.02 | 0.193 ± 0.005 | 6.2 | 74.9 ± 0.005      | -1.02 ± 0.02      | 0.31 ± 0.03       |
| ME6              | 1.398 ± 0.031    | 40:60:20:10            | 0.895 ± 0.01 | 2.91 ± 0.02 | 0.093 ± 0.005 | 6.4 | 72.9 ± 0.005      | -1.02 ± 0.02      | 0.31 ± 0.03       |
composition of surfactant, oil and water. Besides, ME 4 was found more stable as compared to other. The optimized formulation (M-ME) was observed under compound research microscope (Nikon eclipse 80i, M/s. Nikon Corporation, Japan) at 100× and 1000× magnification. No particular structures such as globules or any sort of aggregation or undissolved drug particle in plain as well as drug, loaded ME was observed (Figure 2). To confirm the isotropic behavior of ME, formulation was observed under polarized microscope (LV 100 POL attached with LTS-420, M/s. Nikon, Japan and Linkam, UK). No light was crossed (complete darkness was observed) through the sample under cross polarization, indicating its isotropic behavior [29].

Formulation (M-ME) was observed with TEM to localize the internal structure of the system. The internal phase, i.e. oil globules identified as dark spots found to be in nano-scale range. In Cryo-SEM image of ME, internal oil globules indicated by white arrows (Figure 2C). The average diameter and zeta potential of M-ME was found to be $48.23 \pm 5.8$ nm and $-3.3 \pm 2.9$ mV, respectively, whereas zeta potential for M-ME Polox-20 and commercial cream was found to be $21.1 \pm 1.8$ and $24.1 \pm 1.8$ mV respectively. The slightly negative zeta potential of particle may be attributed to unentrapped MTX on ME surface. Other physicochemical characters of the M-ME are tabulated in Table 1. The results of conductivity (2562 μS) showed that the ME was O/W and the drug content of ME was found to be $2.032 \pm 0.12$ mg/mL.

Secondary vehicle

The optimized ME (M-ME) formulation was incorporated into secondary vehicles (composed of various gelling agents) to facilitate its application through topical route. On screening two systems, viz. M-ME-GMS 10 and M-ME Polox-20, were found better and therefore based upon their physical stability, selected for further screening for in vitro diffusion study. The stability of these systems may be attributed to the surface active nature of the gelling agents to find favorable interactions with the ME structures.

Rheological studies

This study revealed shear thinning nature of the gel formulations, as the graphic (Figure 3A and C) relationship between viscosity and shear stress has confirmed it with the value of the coefficient (power-law exponential) “n” was less than 1. The consistency index and viscosity at zero shear rate ($\eta_0$) indicate that GMS based gel molecular chains are more entangled and system followed a non-Newtonian behavior of flow. The characteristic bent of the rheogram towards the shear stress axis and the decrease in viscosity with increasing
Figure 3. Rheological behavior and texture profile of M-ME-Polox-20 and M-ME-GMS 10.
rate of shear indicated that the developed formulation exhibited pseudoplasticity. This pseudoplasticity results from a colloidal network structure that aligns itself in the direction of shear, thereby decreasing the viscosity as the shear rate increases. As the shearing stress is increased, the normally disarranged molecules begin to align their long axis 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. Despite the high consistency of GMS based gel, its shear thinning behavior may be accounted for changes in the gel-network, made up of long chains of fatty acids on increasing the shear rate.

**Texture profile analysis**

The results of TPA are depicted in Figure 3, show the value of the peak force was taken as a measurement of gel strength; the higher the value better is the strength of gel network. The area of the curve up to this point was taken as a measurement of work of shear, reflecting the work of spreadability of the sample. The negative region of the graph, produced on probe return, was a result of the weight of sample which is lifted primarily on the upper surface of the male cone on return. This is due to back movement and hence, provides an indication of adhesion or resistance to flow off the disc. The maximum negative value is the force of adhesion for the gel, representing the force required to extrude gel from tube. The area of the negative region of the curve was taken as work of adhesion or stickiness. The curve of TP shows uniformity, which confirmed the smoothness and absence of any grittiness or lumps in the formulation and absence of particulate matter and grittiness. TPA exhibited good gel strength to maintain its structure on prolonged storage, extrudable from tube, ease of spreadability, which has been considered favorable for topical application [30,31] and also the systems have shown good bio-adhesion or adequate cohesiveness and would facilitate its longer availability on the application site. Based on the release behavior, rheology and TPA, M-ME Polox-20 was selected for further study under *ex vivo* permeation study.

**In vitro drug diffusion study**

Selected gel formulations (i.e. M-ME-GMS 10 and M-ME-Polox-20) were compared with M-ME, MTX suspension in PBS (pH 7.4; MTX-Sus) and MTX conventional cream (MTX-CC). Methanol and DMF was added in PBS due to higher solubility of Methotrexate in both solvent. It was observed that 88.78 ± 8.12% drug released from M-ME, while 67.60 ± 6.24% drug was released from M-ME Polox-20 (Figure 4). Almost similar percent drug release behavior was observed from M-ME and M-ME Polox-20 at two different concentrations, i.e. 0.25 and 0.125% w/w (Supplemental Figure S1), which indicates that concentration could not change the release and indicating concentration independent release behavior, inferred as passive diffusion as reported earlier [32].

On fitting the various release models, it was observed that the mechanism of release of MTX from ME-based systems did not follow a particular release-kinetic model (Table 2). Invariably, the order of release was near to zero-order. The zero-order release kinetics is the most sought after release pattern in the drug delivery systems and MEs were able to provide the same.

After gelling of M-ME, drug release was hampered up to certain folds changes in the percent cumulative release on log scale (Supplemental Figure S2) and superiority of poloxamer based gels was observed over GMS based gels. This might be because the poloxamer gel provides water channels [33] that might better guide the hydrophilic drug [34], i.e. MTX

![Figure 4. In vitro release plot between percent cumulative release versus time (n = 6).](image-url)

| Formulation code | Zero order | First order | Higuchi | Peppas | Hixon |
|------------------|------------|-------------|---------|--------|-------|
| M-ME             | 0.956      | 0.959       | 0.980   | 0.632  | 0.940 |
| M-ME-GMS 10      | 0.994      | 0.994       | 0.959   | 0.959  | 0.919 |
| M-ME Polox-20    | 0.985      | 0.987       | 0.941   | 0.771  | 0.904 |
| MTX-Sus          | 0.979      | 0.908       | 0.967   | 0.341  | 0.770 |
| MTX-CC           | 0.986      | 0.957       | 0.959   | 0.803  | 0.935 |

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MEs mediated effective delivery of MTX hydrogel
towards aqueous sink media. However, in the case of GMS, complex gel structure, as reported by El Laithy et al. based on its rheological profile [35] create trouble in free drug movement. Although, GMS based gels are characterized by compact structure, its meshes do allow flow of liquid phase. However, the internal molecular ordering and size of network mesh may influence the release rate of drug dissolved in liquid phase by mechanically hindering the free diffusion [36].

**Ex vivo drug diffusion study**

Figure 5(A) shows the permeation behavior from the selected formulation M-ME-Polox-20 and compared its permeation behavior with M-ME, M-Polox-20, MTX-Sus and MTX-CC. The results for varied permeation characteristic viz. cumulative drug permeated in 48 h (Q₄₈), permeability coefficient (P), permeation flux (J) and enhancement ratio (ER) have been listed in Table 3. ME systems (dispersion in hydrogel) offers better drug permeation into the skin possibly due to the combined effect of EtOH and PL on skin lipids. In contrast, 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.

On analyzing these characteristics, it is evident that M-ME system has shown better performance at all level of assessment with 18.43 as ER, 0.682 μg/cm² h as $J_{ss}$, 6.68E–04 cm² h⁻¹ as P and 50.46 μg as $Q_{48}$. The enhanced permeation may be accounted to the alterations in the barrier property of stratum corneum on both, aqueous and lipid pathways [37].

On gelling of M-ME with Poloxamer (20% w/w), i.e. M-ME-Polox-20, cumulative drug permeated decreased from 50.46 to 38.14 μg (in 48 h), with a decrease in permeation flux from 0.682 to 0.496 (μg/cm² h). This indicates slight decline
in drug permeation after gelling but still it showed 3.2-, 8.27- and 13.41-fold higher flux to that of M-Polox-20, MTX-Sus and conventional cream, respectively.

The enhanced permeation could be accounted to the favorable alteration in the lipid and the aqueous polar pathways to allow the penetration of drug. The hydrophilic domain of the ME plays important role by hydrating subcutaneous (s.c.). When its aqueous part enters in the polar pathway of SC, it increases the inter-lamellar volume of the s.c. lipid bilayer that leads to the disruption of its interfacial structures [35]. Corneocyte, a protein available in skin attached covalently with lipid chain, gets hydrated with aqueous part and leads to the alteration in the lipid bilayer. In such circumstances, polar head group of the surfactant molecules and of the disordered lipid molecules facing aqueous region form an additional polar route [35] for hydrophilic drugs like MTX and similar results were reported [38].

In addition to this, the enhanced permeation efficacy obtained from the ME system could be justified based on the dual function performed by ethanol present in the ethosomal formulations, which tend to fluidize both the ME lipid and stratum corneum lipids, thus providing a greater malleability to the MEs and enhancing permeability of the skin [39]. Phospholipids aid in the penetration of the MEs 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. In contrast, the conventional topical formulation shows ionization of the drug free drug at physiological pH of skin, leading to decrease in the permeation rate [39,40]. The above promising characteristics of the developed formulations ratified the enhanced topical drug delivery potential.

Skin drug retention study

The results showed that higher drug concentration (>15-fold) was present in both layers (epidermis and dermis) with developed formulation (M-ME-Polox-20) vis-à-vis the conventional systems (i.e. MTX-Sus and MTX-CC; Table 3). Overall, more than 97% of drug was recovered during ex vivo studies to account for permeation and retention both. This was due to enhanced permeation and retentivity of drug, wherein the ME vehicle played an important role and changes are brought at the cellular and physiological ambience in the particular domain. Also, the effect may be ascribed to the formation of micro-reservoir of drug in to the interiors of skin so as to affect its retention. The enhancement effect of gel may also be related to the benefits of hydrogel which could provide an additional advantageous hydrating atmosphere to the MEs. Thus, it can be inferred that the prepared formulations could effectively make the drug molecules accessible within skin layers (epidermis and dermis), retaining drug within the closed vicinity of the target site. This level of retention is considered to be efficient as drug concentration is sufficient to inhibit the growth of keratinocytes [41]. The latter is a group of key cells involved in continuous proliferation and differentiation, and controlled by a complex network of growth factors and cytokines [42] in the dermis of skin. Thus, control of keratinocytes growth and cytokines (Th1 & Th2 suppression) limits the further growth of severe stage of plaque psoriatic and would help in healing [42].

Cell line studies

The MTT assay was performed to evaluate inhibitory efficiency of MTX in M-ME and M-ME-Polox-20 compared with plain MTX-Sus and conventional cream against Hela cell lines (Figure 5B). The results indicated that M-ME and M-ME-Polox-20 are significantly (p < 0.01) effective against the studied cell lines compare to other formulations. The cell line inhibition was concentration dependent and about 60% of cell line growth was inhibited at 10 µM for M-ME and M-ME-Polox-20, while below 50% cell inhibition was observed for MTX-Sus and conventional cream at same concentration. It was noteworthy that M-ME is slightly more cytotoxicity compare to M-ME-Polox-20, this is ostensibly due to direct interaction between cell membrane and lipid as well as surfactant content of the ME. Thereby it results in efficient drug internalization ultimately resulting into higher cytotoxic response. We have already observed in in vitro release that Poloxamer gel slightly hampered the release MTX from M-ME-Polox-20 and might be due to this it was slightly less effective as compared to M-ME.

Animal study

Imiquimod induced psoriatic model

The model involves the induction of psoriasis at treated as well as remote sites in mice which was serendipitously found. The model, in serendipity, has based on the clinical observation during the treatment of actinic keratoses and superficial basal cell carcinomas with Imiquimod (IMQ) [43]. Hence, in this model, potent immune activator was used to induce psoriasis in C57BL/6 mice. These are genetically modified mice most widely used as models of human diseases like psoriasis. The criteria for evaluation was fixed as PASI and used to score the affected skin of mice.

All the observations are graphically shown in Figure 5(C). Graph showed highly significant (p < 0.001) reduction in PASI score from third day onwards in M-ME Polox-20 group, whereas no cure was observed either in control and placebo group. However, dexamethasone, the positive controls (PC) showed a statistically most significant (p < 0.001) anti-psoriatic effect from second day onwards.

Allergic contact dermatitis (ACD) model

This mice model pertains to the eruption of allergic contact dermatitis which is a T-cell mediated inflammatory skin disease triggered by various allergens. In it, Th1 and Th2 cytokine induced inflammation was correlated with pathogenesis of psoriasis and measured in terms of change in the thickness of the body area, where the allergen was applied. The mechanism of action of MTX in its antipsoriatic activity involves the suppression of these cytokines [44]. In this study, the varied treatments were given to assess their anti-inflammatory activity. Results are shown graphically in Figure 5(D). M-ME Polox-20 has shown statistically highly significant reduction (p < 0.001) in inflammation in comparison to control group as well as to Polox-20. The improved inhibitory
effect of M-ME Polox-20 may be associated with its efficacy in delivery characteristics which can be correlated with its *in vitro* findings. Here, dexamethasone was taken as absolute standard and used as positive control which showed strong anti-inflammatory effect in ACD model.

**Rat tail model**

Histopathological examination of the skin sections of various groups of animals is depicted in Figure 6(A). The skin sections of animals in control group showed quite normal skin physiology without any viable changes. The results reflect the efficacy of M-ME Polox-20 as it showed remarkable reduction in parakeratotic cells after its treatment (Figure 6A), while in the control group as well as in the placebo group (Figure 6A), these cells were found in abundance. By morphometric evaluation, orthokeratosis was quantified [45] in terms of percent orthokeratosis. For M-ME Polox-20 treated group, percent orthokeratosis was found to be 62.12 ± 4.26%, that is much higher than the corresponding control group (19.70 ± 1.25%) and Polox-20 (19.82 ± 1.62%). This indicates significant induction (*p* < 0.001) of a granular layer on topical application of M-ME Polox-20 and confirmed its superior anti-psoriatic efficacy. This confirmed our earlier findings (above) pointing towards the possibility of improvement by way of improved delivery aspects.

**Safety test (Draize test)**

Skin safety is an important concern for topical formulations, as it not only affects the patient’s comfort but can also worsen the disease condition to a great extent. Male albino rabbits were used to check skin safety on topical application of the developed formulations using Draize test [46]. Skin safety was adjudged in terms of irritation potential, which is defined as a non-immunological local inflammatory reaction. The latter is usually reversible, and characterized by erythema and edema [47]. Study was performed for 72 h after the topical application, and photographs were taken before the formulation application on day 1 (Figure 6B I) and after study on day 3 (Figure 6B II).

M-ME Polox-20 was found to be non-irritant with a mean of 0.16 for erythema and 0 for edema (on third day) (Figure 6B II: e), whereas, MTX-Sus showed only a little irritation (i.e. 0.66 score) and edema (i.e. 0.33 score) in same time duration (Figure 6B II: d). The study showed remarkable skin irritation (i.e. 1.33) and edema (i.e. 3.0) with SLS (20% w/w) (Figure 6C). Both control and sham control group did not show any signs of adverse effects (Figure 6B II: a and b). The study concluded that the formulation was topically safe. The compliance may be attributed to the biocompatible and biodegradable nature of components like phospholipids.

**Stability studies**

The physical and chemical stability studies for the optimized formulations of both selected formulations containing MTX were conducted as per the ICH guidelines. Physical stability of M-ME over the studied temperature range is shown in Supplemental Table S2. Neither separation nor any significant changes in average globular size have been noted even after

Figure 6. (A) Histology of rat tail of control, Polox-20 and M-ME-Polox-20 group at (a) 100× and (b and c) 400×. (B) Draize Test: (I) Rabbit skin before formulation application on day one. (a): Control; (b): Sham control; (c): SLS (20%, w/w); (d): MTX-Sus; (e): M-ME-Polox-20; (f): Polox-20. (II) Rabbit skin after formulation application on day three. (a): Control; (b): Sham control; (c): SLS (20%, w/w); (d): MTX-Sus; (e): M-ME-Polox20; (f): Placebo ME gel.
12 months of storage at all three conditions. The PDI remained within the desired limits. In further confirmation of physical stability, its optical clarity, as revealed though the percentage transmittance, was found to be quite supportive. However, the physical stability in terms of viscosity of the M-ME Polox-20 was greatly influenced (i.e. reduced) at the lower temperature. Whereas no significant ($p > 0.05$) change was observed at higher temperature. Plausible reason behind the reduction in the viscosity at lower temperature could be attributed to the thermo-sensitive nature of the Poloxamer gel [48]. The molecules of Poloxamers were subjected to sol to gel transformation and vice versa after subjecting these to temperature changes. Chemical stability of the M-ME Polox-20 in terms of drug and phospholipid contents have been shown in Supplemental Table S3. The M-ME Polox-20 was found to remained stable at all the temperature conditions. However, it showed minimum loss at 5°C and this loss was increased with temperature, but remained within limit of ±5% (w/w). Phospholipid content also remained within limit of ±5% (w/w).

Conclusions

Microemulsion based MTX hydrogel was prepared and characterized with different characteristic properties and compared with MTX-sus and conventional cream. Optimized formulation (M-ME Polox-20) attained maximum cumulative drug permeation with maximum permeation coefficient and permeation flux, and MTX was retained in all layers of skin (stratum corneum, epidermis and dermis). This was due to the effect of ethanol and PL on stratum corneum lipids and on MEs fluidity as well as dynamic interaction between MEs and the stratum corneum, these all attributes contribute to the superior delivery properties. Formulation showed significant antiproliferative activity and was found to be more effective against Hela cell lines. Antipsoriatic activity was further validated by animal studies viz. imiquimod induced psoriatic model, allergic contact dermatitis model and rat tail model (% Ortokeratosis) and results were found in agreement of cell line study. Skin safety and organ distribution study were also performed using Draize test and biodistribution study. The developed formulation was found to be quite safe for topical application. In nutshell, developed formulation showed appreciable antipsoriatic activity via might be dual mechanism viz inhibiting psoriatic cell in the stratum corneum and epidermis and could also be due to immune suppressor (Th1 and Th2) activity in the dermis with minimal dose. Formulation was stable in all conditions and followed ICH guidelines. The developed formulations provide novel and exciting opportunities to treat severe stage of plaque psoriasis. This will prove to be an important example for the advancement in drug delivery and treating psoriasis.

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Declaration of interest

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Supplementary material available online
Supplementary Tables S1–S3 and Figures S1 and S2