RESEARCH ARTICLE

Lipid Nanoemulgel Therapy for the Treatment of Keratoconjunctivitis Sicca: Cyclosporine Formulation Characterization and *In-vitro* Evaluation

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ABSTRACT

Nanoparticulate carrier systems play a significant part in enhancing ocular bioavailability. Emulgels are a potential new delivery method for the administration of lipophilic drugs like cyclosporine A (CsA). In the present research work, CsA, the immunomodulatory drug with a wide safety profile, has been formulated into nanoemulgel for treating vision-threatening ocular surface disorder *viz*. Keratoconjunctivitis sicca. The objective of this study was to prepare lipid-based nanoemulsion gel of CsA aiming to prolong corneal residence time and increase ocular bioavailability. Ophthalmic nanoemulgel was prepared using suitable vehicles, preservatives, hydrophilic and lipophilic emulsifiers *viz* croduret 40, poloxamers, spans, polyethylene glycol, polyoxyethylene 40 stearate, etc., by probe sonication method. It was evaluated for physical appearance, particle size, pH, viscosity, drug release, assay, and stability, etc. using appropriate methods. The pH and osmolarity of transparent ocular liquigel were 7.2 and 155 mOsm/L respectively. The particle size analysis and cryo scanning electron microscopic images of ocular nanoemulgel showed a monodisperse system containing a globule mean size of 190.2 ± 8.93 nm. The drug content was 99.5% w/w and the *in-vitro* diffusion studies showed that the medication was released for 6 hours; followed first-order kinetics with a high regression coefficient (r2) of 0.982. The lipid carrier-based topical cyclosporine ophthalmic liquigel was developed and evaluated. The *in-vitro* study has shown uniform, smooth-surfaced globules of size < 200 nm and prolonged drug release up to 6 hours; thus indicating increased corneal residence by nanocarrier-based gel system.

Keywords: Cyclosporine, Dry eye disease, Ophthalmic delivery, Lipid nanoemulsion gel, Drug delivery systems, Ocular surface disorder, Hydrophilic, Lipophilic surfactants.

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INTRODUCTION

Nanoemulsions have been used mainly for optimizing the formulation's lipophilic-hydrophilic properties, especially in poorly water-soluble drugs.[1,2] Nanoemulsions are transparent to translucent O/W or W/O emulsions with mean droplet sizes between 50 and 1000 nm (the typical range is 100–500 nm) and possess improved shelf stability against gravitational-driven creaming.[3,4] Ocular surface disorders are associated with varied symptoms *viz*. ocular discomfort, loss of corneal transparency, dysfunction of the meibomian gland, and vision loss, affecting the quality of the patient's life.[5-7] In 2003, the Food and Drug Administration (FDA) authorized the use of cyclosporine A for treating dry eyes through topical administration in humans. (Restasis by Allergan- 0.05% eye drop), however, it is also recommended by ophthalmologists for various other indications.[8,9]

A neutral, hydrophobic, and cyclic peptide, cyclosporine A (CsA) is derived from various species of the fungus *Tolypocladium inflatum*. Its molecular weight is 1202.64 Da and its chemical formula.[10,11] is $C_{12}H_{111}N_{11}O_{12}$. It falls under the class IV biopharmaceutics classification system (BCS) category due to its very low bioavailability and poor solubility in water.[12] Lipid nano emulsion type of delivery system is preferred because of its high lipophilicity. Literature supports that topical administration of CsA selectively prevents the release of interleukin-2 (IL-2) by activating T-cells and thus the mechanism of local immunosuppression is useful in treating autoimmune uveitis, rejection of corneal graft, and ocular surface disorders such as dry eyes disease.[13,14] In the present research work, a lipid carrier-based nanoemulsion gel of CsA was prepared aiming to prolong corneal residence time and increase ocular bioavailability, for treating ocular disorder *viz*. keratoconjunctivitis sicca (dry eye disease).

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MATERIALS AND METHODS

Materials

Cyclosporine A was procured from RPG Life Sciences, Mumbai as a gift sample. Polyoxyethylene 40 stearate was obtained from Croda (I) company Pvt Ltd., cremophor EL, croduret 40, poloxamer 188, were obtained as gift samples from BASF chemicals (I) Ltd., span 80, polyethylene glycol, propylene glycol, etc were purchased from Research Lab Fine Chem Industry. Transcutol was received from Gattefosse (I) Pvt. Ltd. as a gift sample.

Methods

CsA's solubility in various vegetable oils was assessed during pre-formulation studies. The shake flask method was utilized with vehicles purified water, ethyl alcohol, and propane-2-ol. Fatty alcohols, fatty esters and oily fatty acids viz. oleic acid and linoleic acid were also used as oil vehicles for solubility determination. Oleic acid was utilized as an oil phase at a concentration of 1% w/w based on data on cyclosporine A's solubility in various oils as well as surfactants that are shown in Table 1 along with low concentration of surfactants mixture to maintain ocular safety.[15,16]

Table 1: Solubility of cyclosporine in different oils and surfactants.[15,16]

Component	*Solubility mean (mg/mL) ± SD (n = 3)*	*Component*	*Solubility mean (mg/mL) ± SD (n = 3)*
Olive oil	12.87 ± 0.15	Transcutol	5.87 ± 0.12
Soyabean oil	2.34 ± 0.11	PEG 400	2.56 ± 0.23
Capmul MCM	3.56 ± 0.21	PEG 200	2.22 ± 0.32
Labrafac	5.32 ± 0.05	Cremophore EL	1.21± 0.22
Oleic acid	22.50 ± 0.76	Tween 80	6.19 ± 0.05
Triacetin	1.34 ± 0.12	Span 20	0.83 ± 0.12
Tween 20	1.33 ± 0.11	Propylene glycol	1.28 ± 0.10

During the formulation of trial batches, surfactant concentration was optimized using 3^2 experimental designs where 2 factors hydrophilic and lipophilic surfactant concentrations were evaluated at 3 different coded levels (-1, 0, +1) representing real values of 2.5, 3.0, 3.5 % concentrations of surfactants. The combinations of hydrophilic surfactants *viz*. polyoxyethylene 40 stearate, croduret 40, poloxamer 188, cremophor EL, and lipophilic surfactants viz. span 80, polyethylene glycol, transcutol were used. The effect of surfactants on emulsion stability was studied for different combinations of hydrophilic and lipophilic surfactants. The conventional and novel surfactants were used in research work, at desired safe concentrations. Cosurfactants have been used to reduce the effective HLB value of high HLB surfactants.[17,18]

A two-step process was followed for the preparation of nanoemulsion. The coarse emulsions containing a 1:1 ratio (Smix) of oil and surfactants were prepared initially using a conventional technique. The hydrophobic surfactant and the lipophilic drug were dispersed in the selected oil phase. This oil phase was then dispersed to the aqueous phase containing suitable hydrophilic surfactant while being stirred, and the two were quickly emulsified using a mechanical stirrer at 800 to 1000 rpm for 20 minutes. Butylated hydroxyl anisole (BHA) at 0.05% was added as an antioxidant to a small amount of water and then dispersed in an emulsion with stirring.

Emulsions were subjected to centrifugation using a Remi centrifuge (4C model) for half an hour at a speed of 3000 rpm. Thermodynamic studies were then performed for emulsions without any phase separation. These formulations were exposed to 15 cycles of heating (R.T.) and cooling (-20°C, refrigerator Samsung 318l RT 34 Model) in alternate 2 days. The coarse emulsions that withstand temperature excursions were selected and converted to nanoemulsions using ultrasonication, the high energy emulsification (Ultra probe sonicator SM 120 PS) technique. Surfactant combinations leading to stable and elegant dispersions were finalized and submicron emulsions were prepared using suitable excipients *viz*. citrate phosphate buffer, and benzalkonium chloride. Lower permitted concentrations of buffer and preservatives have been commonly used in ocular formulations for stability and safety. The emulsions were evaluated for physicochemical characteristics and *in-vitro* diffusion studies. Two compositions containing croduret 40, cremophor as hydrophilic surfactant and propylene glycol, transcutol as lipophilic surfactants were translucent. These compositions were also thermodynamically stable for 6 cycles without any signs of creaming or cracking.

The optimization of type of surfactant and concentration based on particle size as a dependent variable was performed for selected formulation 9A. For different ratios of HS: LS (Cremophor EL: Propylene glycol), factorial randomized design (2^2 full) using Design Expert Software (Version 12) displayed nine potential combinations. The ANOVA results for the selected factorial model for particle size response are given in Tables 2 and 3. Viscosity modifiers HPMC K4M and Carbopol 974P were used in appropriate ratios as gelling agents for the preparation of ocular gel from nanoemulsion. Differential scanning calorimetry (DSC) was performed (Mettler DSC 20) for optimized formulation 9A-gel and placebo across the temperature limits 30 to 300° and at a heating rate of 10° to study excipient interference.

Characterization of CsA lipid Nanoemulsion Gel

Optimized nanoemulsion gel was evaluated for organoleptic properties, phase separation, pH, refractive index, viscosity, osmolarity, mean globule size, total drug content, surface morphology, and *in-vitro* diffusion studies (Table 4).

The instruments used include a digital pH meter (Mettler, Toledo), a Brooke field viscometer (LVDV 1 M), and an Osmometer (K-7000, Germany). The LC Solutions System (Shimadzu, Japan -model LC 2010) was used for the evaluation of drug content using a validated reverse phase HPLC analytical method. The principle of DLS Nano ZS90, Malvern, UK) was utilized to measure particle size (Figure 1). Cryo-SEM was employed (JEOL JSM 7600F model) with a magnification of 40,000 at 5kv, to visualize morphology and shape of CsA nanoemulgel (Figure 2).

Table 2: ANOVA for a selected factorial model for particle size response

Source (ANOVA statistics)	*F-value*	*p-value*	*Data evaluation*
Model	494.87	< 0.0001	Significant
Hydrophilic surfactant	969.57	< 0.0001	-
Hydrophobic surfactant	20.18	0.0064	-
Lack of fit	0.948	0.385	Non-significant

Table 3: Fit statistics for particle size response

Standard deviation	*18.29*	*Adjusted R^2*	*0.9930*
Predicted residual error sum of squares (PRESS)	1672.26	Predicted R^2	0.9782
R^2	0.9950	Adequate precision	53.444

Table 4: Characterization of CsA lipid nanoemulgel RPG and *in-vitro* drug release. All values are expressed in ± SD (n = 3)

Test Parameters	*Results*		
Colour	Colourless to translucid		
pH	6.6–7.4		
Viscosity	Emulsion- 0.887 cps, Liquigel- 60–70 cps		
Osmolarity	Emulsion- 185 mOsm / L		
Total drug content	99.5%		
Mean oil droplet size	190.2 ± 8.93 nm (Desired- < 250 nm) PDI- 0.198 ± 0.08 m (limit 0.1–0.7)		
In-vitro drug release	Zero-order kinetics	First order kinetics	Higuchi model
Coefficient of regression (R^2)	0.976	0.982	0.976

For drug content determination, Propane-2-ol and the gel corresponding to 1-mg of CsA was transferred into a volumetric flask, and the mixture (100 mL) was then sonicated at room temperature for 30 minutes while shaking it occasionally, in a water bath. The solution was filtered through a PVDF filter before being injected into an HPLC column. For optimal formulations, drug content ranged from 98.5 to 101%. A Franz diffusion cell with a pre-saturated dialysis membrane (12,400 Da, Himedia, Mumbai) was used for diffusion studies *in-vitro*. The diffusion medium (22 mL) *viz*. Phosphate buffer pH 7.4: propane 2 ol (50:50) was maintained at 32° temperature and 200 rpm speed. The %drug diffused (membrane diffusion area of 2.26 cm^2) from an emulgel equivalent to 100 μg CsA was calculated at hourly intervals with reference to the *in-vitro* drug release linearity plot. The cumulative drug release (CRD) percentage was plotted against time (hr) as shown in Figure 3. The regression coefficients (R^2) and release constants were calculated and are shown in Table 2.

RESULTS AND DISCUSSION

CsA being a slightly soluble drug[19] the nonaqueous vehicle *viz* oleic acid was selected as the maximum solubility of CsA

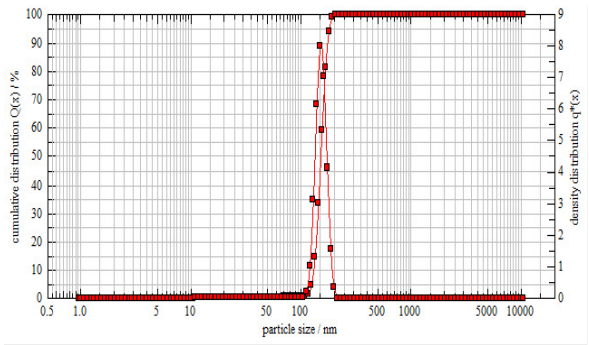

Figure 1: Unimodal particle size distribution of CsA nanoemulgel formulation by DLS technique

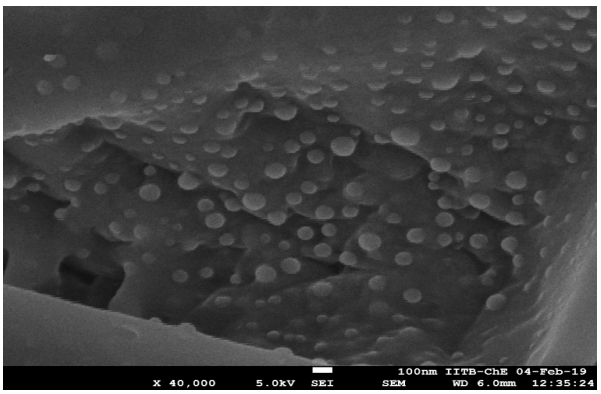

Figure 2: Morphology of CsA nanoemulgel by cryo scanning electron microscopy.

viz. 22.50 ± 0.76 mg/mL was observed, as compared to other lipids (Table 1). All test batches employed 1% oleic acid as an oil solvent and a 1:1 ratio of oil to surfactants mix (Smix). The advantages of the various oils and surfactants used in ocular formulations for treating eye conditions.[20] have been compiled. Although 10 to 30% oil can be used to prepare typical emulsions, the percentage of oil was restricted to no more than 5% for ocular formulation to reduce the risk of ocular toxicity and irritation.[21-23]

Table 2 of ANOVA for particle size response showed that the model F value of 494.8 indicates that this model was found significant and resulted in a p-value of less than 0.05. The study of the influence of formulation variables (surfactant type and concentrations) on particle size (nm) showed that the particle size of nanoparticles decreased as the concentration or ratio of HS: LS was raised. The effects illustrated in the form of a normal plot, and Pareto plot showed that both types of surfactants exhibit significant effects and there was a greater influence of hydrophilic surfactant on particle size of emulsion. Fit statistics indicated a good fit for the selected model. The model also showed that the formulation variables studied were independent of each other. The data in Table 3 demonstrated a high coefficient of regression (R^2) value for the response, with the difference between the anticipated and adjusted R^2 being less than 0.2. This model was seen as suitable because the measurement of the signal-to-noise ratio with a value of 53.444 (value > 4) suggested an adequate signal.

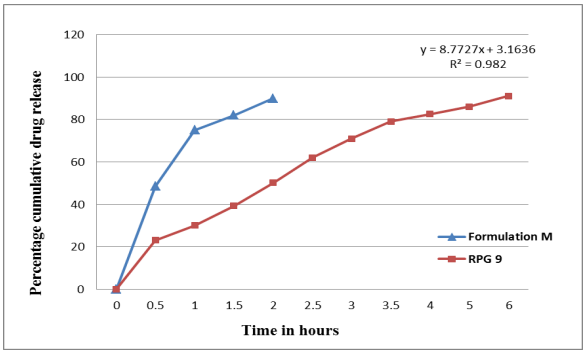

Figure 3: Percentage cumulative drug release of prepared nanoemulgel RPG 9A. All values are expressed in ± SD (n = 3)

Following is polynomial equation that fits the statistics produced.

$Y= 527.95- 284.72 X_1- 41.07 X_2$ where Y is the dependent variable and the regression coefficient of term X- surfactant concentration was found as 527.95.

There was no evidence of an interaction between the two factors under study; instead, the major effects (X_1-HS and X_2=LS) characterized the overall result of both factors as they changed from their low (-1) to high value (+1).

During drug excipient compatibility investigations, the DSC thermogram of CsA demonstrated a strong peak at its M.P. of 116.1° and corresponded with available cyclosporine literature.[18] The absence of the peak at 116° for the chosen nanoemulgel 9A showed that the CsA drug particles have been thoroughly dispersed in the internal lipid phase of the O/W nanoemulsion and that molecular level dispersion has occurred. Lipid emulsion physically resembled a simple aqueous eye drop as more than 90% of the continuous phase in a lipid emulsion was aqueous, despite the presence of additional components. For emulgel preparation, mucoadhesive and biocompatible gelling agents were selected. A mixture of HPMC K4M and carbopol at 2 and 1% weight ratios were chosen, resulting in a translucent attractive liquid gel. Additionally, gelling agents provided good colloidal stability and simple incorporation into lipid emulsion without altering the particle size.

With a mean size of less than 200 nm and a polydispersity index (PDI) of 0.198, the PCS method demonstrated the unimodal particle size distribution as shown in Figure 1. A very minimal PDI score (0.198) suggested that the gel formulation had a narrow globule size distribution in gel formulation. The O/W emulgel system's morphology was investigated using cryo-scanning electron microscopy and the monodisperse nanocarrier system could be seen in the microscopic picture. At a magnification of 40,000, spherical oil globules with a smooth surface and uniform size of less than 200 nm were found embedded regularly in a gel-based system, as illustrated in Figure 2. The results of the cryo-SEM study complemented the size dispersion data obtained from the PCS method.

Characterization of different parameters as shown in Table 4 illustrated that the selected nanoemulgel complies with all requirements including drug content, globule size, pH, viscosity, etc.

Liquigel showed a viscosity of 60-70 cps and CsA drug content was 99.5% as determined by the validated HPLC method of analysis. The particle size analysis (PSA) of the O/W emulgel system was performed using Nano ZS90 and the unimodal globule size distribution was obtained in the range of 190 to 200 nm. The suitability of liquigel for ocular administration, physical stability, and therapeutic efficacy are all influenced by the particle size distribution, mean size, and PDI, among other critical physical variables.[24]

In-vitro permeation studies (Figure 3), showed that 90% of the drug was released in 2 to 3 hours for marketed formulation M, however, *in-vitro* study for nanoemulgel RPG 9A showed that the drug was released for 6 hours, approximately 75% of the drug was released within 3.5 hours indicating a prolonged drug release by the nano-carrier based gel system. The diffusion mechanism was shown to suit linear regression by first-order kinetics (Table 4), with a coefficient of regression (R^2) value of 0. 982.

The RPG 9A CsA nanoemulgel were stored for three months in glass bottles at 40 and 4°C. Samples were taken out and tested every 15 days, tested for properties *viz*, physical appearance, osmolarity, pH, drug release, and assay. Following evaluation of the physicochemical properties, it was discovered that all emulgel formulations stored for up to three months were stable.

CONCLUSION

The lipid carrier-based stable topical cyclosporine ophthalmic nanoemulgel was formulated containing uniform, smooth surfaced oil globules of mean size < 200 nm, and showed prolonged drug release up to 6 hours, indicating increased corneal-residence and improved bioavailability by nanocarrier based gel system. The resultant nanoemulgel would give patient compliance and prolonged drug retention.

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