Research Article

Cationic Emulsion as Artificial Tears: An *In vivo* Evaluation in Dry Eye Rabbit Models.

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ABSTRACT

Environmental factors like pollution, exposure to Video display terminals, ageing, drugs etc lead to ocular fatigue finally terminating into dry eye (keratoconjunctivitis sicca). Constant tear evaporation leading to exacerbated drying of ocular surface is an archetype. Hence the potential of a cationic submicronic emulsion as artificial tears was investigated. Submicronic emulsion system was formulated by dispersing a medium chain triglyceride in the aqueous phase with the aid of stirring, high speed homogenisation and employing a synergistic combinations of three emulsifiers; Solutol HS 15, Cremaphor EL and HPMC. Oleylamine (a cationic lipid) was used to impart positive surface charge to the oil globules. The emulsion was evaluated for pH, specific gravity, viscosity, osmolarity, globule size, zeta potential and surface tension.To evaluate the system potential as artificial tears, rabbit models resembling evaporative dry eye condition were developed using 0.2% Benzalkonium chloride. Efficacy of the emulsion system was evaluated by observing for chemosis, conjunctival hyperemia and mucosal discharge by digital photography. Tear production was measured using Schirmer tear test I.The developed cationic emulsion system revealed submicronic globule size (133nm), positive zeta potential (+63mV), desirable osmolarity(266 mOsms) and low surface tension (38milliN/m). Treatment involving emulsion instillation twice a day for 5 days was found to be therapeutically effective in alleviating the induced dry eye symptoms. Furthermorethe system increased the tear production from lower values (7-9mm) in dry eyes to basal values (15-16mm) in healthy eyes.

Keywords: Artificial tear, cationic, dry eye, emulsion, ocular, submicronic.

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INTRODUCTION

Keratoconjuncitivitis sicca (dry eye) pertains to a temporary or chronic eye ailment which occurs due to disruption of a healthy tear film finally culminating into ocular fatigue. The major contributing factors for this include environmental pollution, constant exposure to video display terminals, ageing, drugs, menopause etc. A healthy tear film is responsible for maintenance of ocular surface integrity; the absence of which leads to evaporation of the inherent aqueous layer thus decreasing the tear volume [1].

Tear film stability plays an essential role in minimising the evaporative loss and maintaining coverage over the ocular surface between eye-blinks. Tear film rupture occurs in dry eye due to formation of a partially wettable surface during interblink periods [2, 3]. Irrespective of the initial aetiology, inflammation is the key mechanism of ocular surface injury and is initiated by hyperosmolarity and tear film instability [4-6]. These factors further lead to redundant ocular surface wettability [7]. Viscoelastic artificial tears are commonly used formulations which act as substitutes for aqueous layer of the tear film. These

formulations comprise of solutions, suspensions, ointments or gels [8].

Conventional artificial tears show temporary relief by soothing the dry eye surface. Their therapeutic effect is limited by their attenuated residence time due to the rapid tear-turnover and naso-lacrimal drainage of the eye [7,8]. Frequently used artificial tear compositions containing corticosteroids have been associated with increase in intraocular pressure, cataract formation, and the risk of infection. Furthermore newer gel-, cellulose, and ointment-based formulation show long term inadequacies like vision-blurring, caking on the eyelids, corneal surface damage etc [9].

Stable and safe submicronic cationic emulsions have been explored as artificial tears of high therapeutic efficacy to the ocular surface [10]. Their efficiency lies in their ability to electrostatically interact with the negatively charged corneal surface and form a lipidic film that is difficult to wash off. This film prevents further evaporation of the aqueous layer [11].

The present work was an attempt to enhance the precorneal residence time of the formulation on a dry eye surface. The work involved formulation of a non-irritant 'oil-in-water' emulsion by dispersing a medium chain triglyceride (Labrafac Lipophile WL 1349) as submicronic globules (100 to 300 nanometers) in an aqueous phase with the aid of non-toxic, non-irritant, non-ionic surfactants like Solutol HS 15, Cremaphor EL and HPMC (5-cps). Oleylamine (a cationic lipid) was used to impart a positive zeta potential to the oil globules.

MATERIALS AND METHODS

Labrafac Lipophile WL 1349 was procured from Gattefosse India Pvt Ltd as gift sample. Lecithin was provided by Vav Life sciences (Mumbai, India). Solutol HS 15, Lutrol F68 and Cremaphor EL were provided by BASF India (Mumbai, India) as gift samples. Stearylamine and Oleylamine samples were gifted by Shri Sai Industries (Mumbai, India). Hydroxy propyl methyl cellulose (HPMC- 5 cps) was provided by Dow Chemicals (Mumbai, India). Cetrimide was purchased from Westcoast laboratories. (Mumbai, India). Glycerine and Alpha-tocopherol were purchased from S D Fine Chemicals (Mumbai, India). All other materials used were of analytical grade.

Six female rabbits of New Zealand White strain each weighing 2.5 to 3 kg were purchased from Haffkine Institute (Mumbai, India). The animals were housed in separate cages. The environmental conditions were maintained ensuring suitability for rabbit housing. The temperature and humidity conditions were monitored to prevent any interference with the dry eye studies. All animal experimental studies were conducted in accordance with guidelines from Institutional Animal Ethics Committee (IAEC). Approval for conducting irritation studies and other experimental studies on rabbits was acquired from IAEC. The IAEC approved protocols numbered ICT/IAEC/0910/21 and ICT/IAEC/0910/22 were obtained before conducting the study.

Formulation of Cationic submicronic emulsion

Oleylamine and alpha-tocopherol were successively weighed and added to Labrafac lipophile WL 1349 (MCT). The obtained oil phase was then stirred with slight heating at 40° C. 10 percent of the total aqueous phase was used to solubilise Cremaphor EL, Solutol HS, Hydroxy propyl methyl cellulose (HPMC) and Glycerine. The system was stirred with slight heating at 40°C to 55°C. Both phases were heated to a constant temperature of 65°C. The aqueous phase was added to the oil phase and stirred for 45 minutes at 75°C. Cetrimide was added to the remaining aqueous phase and heated at 75°C. Cetrimide containing aqueous phase was added to the coarse emulsion and the resultant system was stirred at 75°C for 25 minutes to obtain the final emulsion. The globule size of the emulsion was further decreased by high speed homogenisation for 15 minutes and the emulsion was brought to 25°C [11-14]. Final optimised procedure and formula was obtained after sequential production of five batches of emulsion based on formula I to V. (**Table 1**) Step-by-step optimisation was conducted to give a clear homogenous emulsion.

Table 1: Formulas I to V For Emulsion Formulation.

Formulas no →	I	II	III	IV	V
Ingredients↓					
MCT	2	2	2	2	2
Lecithin	0.1	-	-	-	-
Solutol HS	0.1	0.1	0.1	0.1	0.1
Lutrol F68	0.5	0.5	-	-	-
Stearylamine	0.1	-	-	-	-
Oleylamine	-	0.1	0.1	0.25	0.25
Cremaphor EL	-	-	0.5	1	1
HPMC	-	-	-	-	0.2
Cetrimide	0.005	0.005	0.005	0.005	0.005
Alpha-tocopherol	0.01	0.01	0.01	0.01	0.01
Glycerine	2.25	2.25	2.25	2.25	2.25
Distilled water(%w/w)	q. s. 100	q. s. 100	q. s. 100	q. s. 100	q. s. 100

Evaluation of Prepared Cationic submicronic emulsion

pH:

An adequate volume of the formulation was taken in a 50 ml beaker and the pH was recorded at 25° C using a standardised Siena Digital pH meter model no-UPH1.

Specific Gravity:

Specific gravity of the formulation was measured using specific gravity bottle of 10 ml capacity using purified distilled water as a standard. The later was calculated using the following formula:

Specific gravity of the formulation = Weight of 10 ml of formulation/Weight of 10 ml of distilled water

Viscosity:

Viscosity of the formulation was measured using Ostwald's Capillary viscometer. The viscosity was measured at constant temperature of 25°C using purified distilled water as the standard. The later was measured using the following formula:

$\eta_1/\eta_2 = \rho_1 t_1/\rho_2 t_2$

where:

η_1= Viscosity of the formulation

η_2= Viscosity of distilled water at 25°C

ρ_1= Specific gravity of the formulation

ρ_2= Specific gravity of distilled water at 25°C

t_1 = Time taken by formulation in seconds

t_2 = Time taken by distilled water in seconds

Osmolarity:

Formulation osmolarity was measured using a digital osmometer model number 3250 from Advanced Instrument, Inc. Direct measurements were made in milliOsms considering 287 milliOsms as standard.

Globule Size:

A drop of emulsion was taken in a clean test tube and diluted with 0.22 µ Millipore water(Milli-Q,Millipore Corp., USA) till a transparent solution was formed. The particle size was measured at 90° angle on Beckman Coulter N4Plus Submicron Particle Size Analyzer.

Zeta Potential Analysis:

The zeta potential of the prepared emulsion formulation was measured using Zetasizer 2000 (Malvern instruments). A sample was extemporaneously diluted in Millipore water (Milli-Q, Millipore Corp., USA) and injected in the zeta cell. The measurements were carried out in the fully automatic mode. Each sample was analyzed thrice, each analysis comprised of 100 runs of measurement.

Surface Tension:

The surface tension of the continuous phase of the emulsion was measured using Wilhelmy Plate method taking distilled water as standard.

Stability Evaluation:

Stability testing:

The emulsion formulation was subjected to stability studies at various temperatures; i.e. 25°C, 4 °C and in light. Emulsion stability after autoclaving was evaluated by placing it in autoclave at 15 psi at the temperature of 121 ° C for 15 minutes. The stability of the emulsion to centrifugation was

evaluated by centrifuging it for 15 minutes at 5000-7000 rpm. Similarly its stability to freeze-thawing was evaluated by exposing the formulation to 3 Freeze-thaw cycles. Each cycle comprised of placing the container containing formulation at 4 ° C for 2 days followed by placing the same at 25°C for 2 days. For stability evaluation, the formulation was observed for creaming or phase separation [15].

Pharmacological Evaluation of formulated Cationic submicronic emulsion

Ocular Irritation study of the emulsion:

Draize Eye Irritation Test as per OECD guideline No 405 (2) was performed on rabbits to evaluate the irritation potential of the cationic submicronic emulsions. The test was based on determining the presence of ocular pathophysiological changes like corneal opacity, congestion, swelling of iris, haemorrhage, destruction of iris, chemosis, reddening of conjunctiva (hyperemia), discharge of mucus leading to moistening of eyelids and hair etc. For the evaluation of the present formulation, the emulsion formulation was instilled in the rabbit eyes and the eyes were observed at durations of 0, 12, 24, 48 and 72 hours for any of the above pathophysiological changes. The observations were made using Canon Powershot Digital camera [16].

Dry eye rabbit model development and Tear production measurement:

The rabbits were induced with dry eye by administration of 2 drops of 0.2 % Benzalkonium chloride (BAC) solution twice a day for two days. Two days of topical instillation was found to be suitable for inducing the inflammatory symptoms and decreasing the tear production to produce moderate dry eye without causing permanent damage to the rabbit eye. The dry eye animal model produced by above procedure resembled the evaporative dry eye state in humans [17].

Schirmer I tear test was used to measure tear production from the rabbit eyes. (both healthy and dry eye state). The test comprised of placing a small strip of Whatmann 41 filter paper cut to dimensions; 35x5 mm after sterilisation inside the lower conjunctival sac after making a notch of 5 mm from one end of the strip. The eyes were closed for 5 minutes. The 30 mm segment was left to hang over the lower lid. After 5 minutes, the strip was removed and the wetted length was measured [18]. No anaesthetic was used as topical anaesthesia tends to decrease the test values and affects the reproducibility of the Schirmer tear test.

The dry eye induction was confirmed by decrease in tear production values and by the presence of the dry eye symptoms as observed by digital camera. The inflammatory symptoms produced were photographed.

Treatment with Cationic submicronic emulsion in Dry eye rabbit models:

Subsequent to two days of topical instillation of 0.2 % BAC in rabbits, the treatment of the rabbits was started. The formulation was instilled twice a day. The formulation was compared with saline. Post-instillation of the formulation, the gradual changes in the various components of inflammatory symptoms like reddening of the conjunctiva (bulbar and tarsal) and iris, conjunctival hyperemia, mucosal discharge from the eye, wetting of eyelids, hairs and surrounding regions of the eye, tearing and the ease of opening of eyes of rabbits were photographed and the increase in tear production values was measured. The treatment was continued till the treated eye was normalised to a healthy state with complete absence of inflammatory symptoms and the tear readings normalised to basal values (i.e. pertaining to healthy eye).The rise in tear production from day 1 of instillation to fifth day was compared to that of normal saline (control). A Two-way ANOVA test was applied to compare the efficacy of the emulsion formulation against saline.

RESULTS & DISCUSSION

Formulation studies

Opaque milky-white submicronic cationic emulsions were obtained by formulating systems as given in (**Table 1**). (**Fig. 1a-1e**) Formulas I to IV produced non-homogenous emulsions with creamy layer of undispersed emulsifier complex visible on the surface (**Fig: 1a-1d**). Emulsion produced by Formula V was found to be most uniform and homogenous with no traces of emulsifier on the surface or

throughout the emulsion system. Hence emulsion produced by Formula V was considered as the most optimised system (**Fig: 1e**). The interaction of negatively charged Lecithin with positively charged Stearylamine along with the absence of desirable quantity of emulsifier system may be a major contributing factor for the intense creaming seen in case of emulsion produced by Formula I. The substitution of Stearylamine with Oleylamine, the removal of Lecithin and the gradual increase in concentration of emulsifiers like Cremaphor EL was found to aid in arriving at an emulsion system with most desirable parameters devoid of creaming.

Fig: 1 a	Fig: 1 b	Fig: 1 c	Fig: 1 d	Fig: 1 e

Fig (1a-1e): 1 a) Emulsion by formula I, 1 b) Emulsion by formula II, 1 c) Emulsion by formula III, 1 d) Emulsion by formula IV, 1 e) Emulsion by formula V.

Pharmaceutical evaluation

pH: The pH of the optimised emulsion system was found to be 7.52 which was close to pH of normal tear fluid (7-7.4). Fomulation pH value close to pH of tear fluid is desirable and ensures the minimal physiological buffering action and hence minimal irritation after instillation of the system.

Specific gravity: Specific gravity of the formulation was found to be 1.005.

Viscosity: The formulation was found to show a viscosity of 1.32 mPa.s. The desirable viscosity for an ocular formulation to be compatible with tear fluid is required to be in the range of 1.3 to 5.9. Hence the formulation was found to be compatible with the corneal milieu in regard to viscosity [19].

Globule size: The submicronic size range of cationic emulsion enhances the kinetic stability profile of the emulsion. This ensures the emulsion to remain therapeutically effective during its shelflife. A desirable globule size of 133 nm was observed for the emulsion formulation.

Zeta potential: A positive zeta potential of 63 mV was observed for the formulation indicating the cationic nature of the dispersed oil globules. Zeta values higher than 30 mV are found to impart higher stability to the formulation. The zeta potential values depend on the cationic lipid. Hence the zeta potential of the emulsion formulation was found to be highly desirable thus ensuring its good physicochemical stability.

Osmolarity: The standard osmolarity of tears lies between 293 to 288 milliOsms. In dry eye syndrome, the osmolarity rises to a value of 308 milliOsms. Hyperosmolarity is a major cause of the inflammatory cascade initiated in dry eye. An artificial tear formulation; hypotonic or isotonic in nature is most desirable. The cationic emulsion was found to show an osmolarity of 266 milliOsms thus indicating its hypotonic nature [8, 19].

Surface Tension: The surface tension of tear fluid has been found to be between 44 to 50 milliN/m. The surface tension of distilled water was found to be 40 milliN/m. The use of emulsifiers in the present emulsion formulation contribute to decreased surface tension value of the aqueous continuous phase to 38 milliN/m thus aiding in enhanced wetting of the dry eye surface [19].

Stability testing: The emulsion showed no traces of creaming or phase separation when exposed to different temperature conditions of 25°C, 4°C and on exposure to light. The formulation was also found to be stable on autoclaving, centrifugation and freeze-thaw cycling.

Pharmacological evaluation

Draize test

No pathophysiological changes were observed after 0, 12, 24, 48 or 72 hours of topical instillation of the cationic

submicronic emulsion thus indicating the emulsion to be safe for ocular use.

Development of Dry eye rabbit models

Inflammation of bulbar and tarsal conjunctiva and swelling of membrane lining eyelids was seen. Opaque purulent mucous discharge causing wetting of hair and skin regions surrounding the rabbit eye was observed. Also the normal closure of the rabbit eye was affected. **(Fig. 2 a - 2d)**

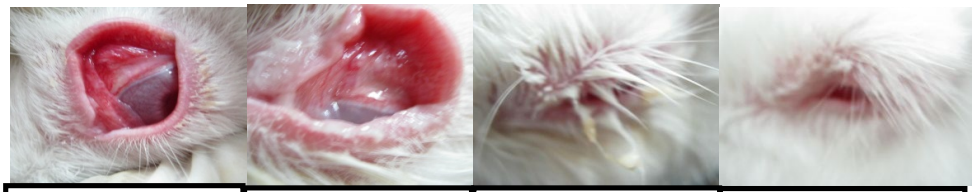

Fig: 2 a | Fig: 2 b | Fig: 2 c | Fig: 2d

Figure (2 a- 2 d): 2 a) Chemosis and irido-conjunctival inflammation, 2 b) Hyperemia and swelling of the eyelid membrane, 2 c) Mucosal discharge and wetting of regions surrounding the eyes, 2 d) Difficult eye closure.

Treatment of dry eyes of rabbit models with Cationic submicronic emulsion as artificial tears

A gradual decrease in the inflammation severity was observed after instillation initiation of the emulsion system in the rabbit models. A decrease in the irido-conjuncitval inflammation, mucosal discharge and chemosis was observed with complete recovery of the eye after five days of emulsion instillation. **(Fig. 3 a-3 e)** The rise in tear production from first day of instillation to fifth day was compared to that of control (saline) **(Fig. 4)**. Statistically significant results were observed for the rise in tear production with a P value of 0.0001. ($P<0.05$) The emulsion system as artificial tears was found to be more effective as compared to normal saline.

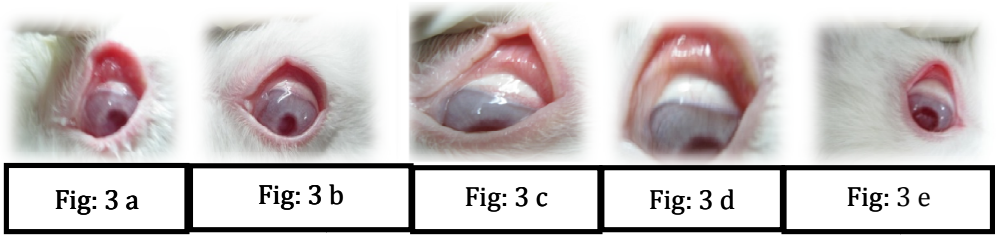

Fig: 3 a | Fig: 3 b | Fig: 3 c | Fig: 3 d | Fig: 3 e

Figure (3a-3e): 3 a) Eye one day post-instillation, 3 b) Eye two days post-instillation, 3 c) Eye three days post-instillation, 3 d) Eye four days post-instillation, 3 e) Eye fifth days post-instillation.

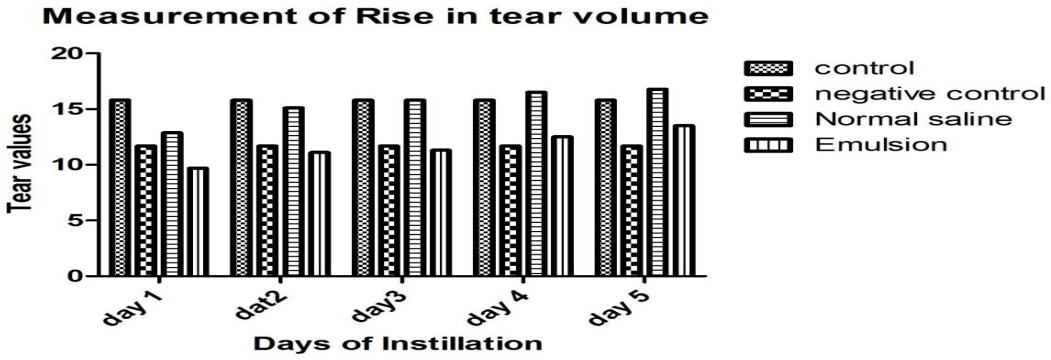

Figure 4: Measurement of Rise in Tear Production.

CONCLUSION

In the present research work, submicronic cationic emulsion is formulated using a medium chain triglyceride dispersed in the form of submicronic globules (<300 nm) in the aqueous phase. Non-toxic, non-irritant emulsifier systems like Cremaphor EL, Solutol HS 15 and HPMC (5-cps grade) are used as stabilisers for the emulsion. Step-by-step optimisation of the formulation is conducted to arrive at the final emulsion system which is homogenous and devoid of creaming or phase separation. Selection of emulsifier complex with compatible charge profile is a requisite to arrive at the stable formula. Since a cationic lipid (Oleylamine) is used to impart positive charge to the oil globules, the remnant emulsifier systems desirable to render stability are required to be non-ionic in nature (Cremaphor EL, Solutol HS 15 and HPMC) to prevent interaction and hampering of interfacial localisation of the emulsifiers. The interfacial localisation of the allocated emulsifiers is a prerequisite to obtain a stable submicronic emulsion. Considering the above-mentioned factors, emulsion produced by Formula V was found to be most desirable. Furthermore, to test the irritation potential of the system, *in vivo* Draize test is conducted. The Draize test helps to establish the ocular safety profile of the emulsion. The therapeutic efficacy is tested by developing dry eye rabbit models by use of 0.2 % benzalkonium chloride. Evaporative type rabbit models are used to study the formulation efficacy on dry eye with excess aqueous evaporation. Emulsion instillation twice-a-day for two days is found to alleviate all the induced dry eye symptoms with elevation of tear volume to basal values. The emulsion is found to show a higher therapeutic efficacy as compared to saline. Furthermore the emulsion can be studied as an ocular artificial tear formulation with a sustained release profile and can be used to delivery poorly soluble drugs by entrapment in oil globules for dry eye syndrome.

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