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Norfloxacin Loaded Cubosomal Nanodispersion as Ocular Drug Delivery for Allergic Conjunctivitis – Formulation, *In-Vitro* Permeation and Antibiotic susceptibility Studies

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ABSTRACT
As result of an abnormal immune response to harmless antigens (allergens), allergic conjunctivitis causes itching, weeping, burning, a foreign body sensation, and ocular dryness. The fluoroquinolone (FQ) antibiotic is one of the most regularly used to treat conjunctivitis. Norfloxacin (NOR) is a highly studied antibacterial drug that works against both gram-positive and gram-negative aerobic bacteria in-vitro. Hydrophobic character is reflected in its low oral bioavailability, despite its bactericidal effect this means that NOR needs to be improved for allergic conjunctivitis. Hydrophobic drugs' efficacy can often be improved through nanotechnology. This is why in our study, a nano dispersion particle size ranges from 10 to 500 nanometers was chosen as a drug delivery system to overcome the issues associated with NOR. Emulsification method was used to prepare NOR-loaded cubosomes. They were tested for their compatibility, particle size and polydispersity index, encapsulation efficiency, in-vitro drug permeation studies, and antibiotic susceptibility. A high entrapment efficiency and good compatibility were found in all of the formulations. According to in-vitro permeability and antibiotic susceptibility study result shows that F3 produced the highest drug permeation (97.21%) at 24 hours and more effective than other formulations. Based on these findings, NOR-loaded cubosomal nanodispersion could be treated with a new medication delivery system to improve transcorneal permeability, extend corneal retention, and increase its solubility and permeability characteristics and could help patients stick to their anti-allergy conjunctivitis treatment. Patient compliance is also improved as the frequency of dosing is reduced.
Keywords: *Norfloxacin, Allergic Conjunctivitis, Cubosomal dispersion, Nanotechnology, Emulsification*

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INTRODUCTION

Ocular allergy, also known as allergic conjunctivitis, is a series of hypersensitivity disorders in which the eyes create an aberrant immunological response to ordinarily harmless antigens (allergens), resulting in itching, weeping, burning, foreign-body sensation, and ocular dryness. Allergic disorders have increased exponentially over the previous four decades, with roughly 15–20 percent of the world's population suffering from ocular allergies alone. According to reports, up to 40% of individuals of all ages around the world are affected by allergy illnesses in one form or another. It is impossible to pinpoint a single cause for the rise in allergy disorders. As a result, experts are considering the role of a variety of factors, including genetics, air pollution, pets, and early childhood exposure [1]. Antibiotics are the medicine of choice for allergic conjunctivitis treatment. Fluoroquinolones (FQs), an antimicrobial agent, have been reported as current non-steroidal antibiotics/antibacterials and efficiently employed in the treatment of conjunctivitis, as well as being investigated for a range of ocular drug delivery methods [2]. Norfloxacin (NOR), a member of the FQs family, is the treatment of choice for E. coli, vibriocholerae, shigella, and campylobacter infections. It's used to treat gonorrhoea, eye infections, and urinary tract infections all throughout the world. It shows efficacy against a wide range of gram-positive and gram-negative aerobic bacteria in vitro. Literature reveals that the fluorine atom at position 6 confers greater efficacy against gram-negative bacteria, while the piperazine moiety at position 7 confers anti-pseudomonal activity. Norfloxacin is bactericidal and inhibits bacterial deoxyribonucleic acid production. It has a 35–45 percent

oral bioavailability and a 7-hour half-life. Its hydrophobic character is reflected in its low oral bioavailability. Because of its hydrophobic character, pharmaceutical experts have classified it as Class-IV of the Biopharmaceutical Classification System (BCS-IV), which denotes limited solubility and permeability. Though various technologies including solid Dispersion and cyclodextrin inclusion complexeshave been developed to improve Norfloxacin's solubility and bioavailability, there have been some reports of problems related to the aforementioned [3]. In recent years, Cubosomes have attracted increasing interest as ophthalmic nanocarriers because of their biocompatibilities and bioadhesive characteristics. Cubosomes can also hold a large variety of medications with varying degrees of hydrophilicity due to their great loading capacity. Drug delivery can be targeted and managed, enhancing drug stability, improving transcorneal permeability, extending corneal retention, and they can be manufactured using simple processes at low costs. Amphiphilic lipid molecules are dispersed in aqueous environments as a liquid crystal phase with cubic crystallographic symmetry to produce cubosomes, a unique structure. Its huge surface area is owing to the presence of two water channels separated by a constrained lipid bilayer, which gives it a large surface area. They feature a honeycomb-like (cavernous) structure and range in size from 10 to 500 nm [4].

In the current study meant to form Norfloxacin loaded cubosomes to deliver the drug as prompt with improving transcorneal permeability, extending corneal retention. Plan of such sort of measurements structure may improve patient's adherence to hostile to allergic conjunctivitis treatment. The fruitful result of this undertaking will give degree to new potential medication conveyance framework for the treatment of allergic conjunctivitis.

MATERIAL AND METHODS

Norfloxacin, Glyceryl Monooleate, Poloxamer 407 (Yarrow Chem Products Ltd., Mumbai), Tween80, Hydrochloric acid, Sodium acetate, Sodium chloride, Sodium bi carbonate, Calcium chloride, Potassium chloride, (LobaChemie. Industries, Gujarat). All the chemicals used were of analytical grade.

Formulation of Norfloxacin loaded cubosomes:

Norfloxacin loaded Cubosome dispersions were prepared by emulsification technique. The lipids phase (3.85–6.25%w/w of total dispersion), consist of glycerylmonooleate (GMO) and Poloxamer 407 (P 407) at different ratios [4:1,6: 1,and 8:1]were melted at 60 °C (heating magnetic stirrer, Eltek magnetic stirrer). Then, Norfloxacin was added to the molten oily phase and solubilized under stirring. Afterward, Tween 80 (1%w/w) were dissolved in water and heated to the same temperature and then was added to the molten mixture with stirring at 500 rpm. The formed dispersions were kept under stirring for 2 h at room temperature to allow solidification of the lipid droplets. Dispersions were then homogenized at 15,000 rpm and 60 °C for 1 min (Ultrasonic Homogenizers, Athena technology). After cooling, the dispersions were maintained at room temperature in glass vials. The composition of various Norfloxacin cubosmes dispersions were shown in Table 1

Table.1 Composition of various Norfloxacin cubosomes dispersion formulations.

S.NO.	FORMULATION CODE	GMO: P407	TWEEN80: H2O	DRUG(Mg)
1.	F1	4:1	1:10	200
2.	F2	6:1	1:10	200
3.	F3	8:1	1:10	200

Characterization of Norfloxacin loaded cubosomes:

Determination of particle size, polydispersity index (PDI) Norfloxacin-loaded cubosomes

The average particle size of the cubosomes and their size distribution (polydispersity index; PDI) were measured by a NanoLaser Particle Sizer (Globolytics instrument Pvt, Lmt. It is based on the principles of laser diffraction by measuring the angular variation in intensity of light scattered as a laser beam passes through a dispersedparticulatesample.Allmeasurementswereperformedintriplicatesat25°C [4].

Determination of % entrapment efficiency(EE)

The prepared Norfloxacin-loaded cubosomes dispersions were centrifuged at 13500 rpm and 4°C for 60 min (Research centrifuge, REMI instrument Mumbai, India, 3–30 K, Sigma, Germany). Then, the supernatant was collected to determine the amount of free (non-entrapped) NOR by spectrophotometric analysis (UV spectrophotometer; Shimadzu,USA) at 278 nm. Blanks were prepared by centrifuging plain cubosomes dispersions contain the same concentrations of all ingredients except NOR. The entrapment percentage of NOR in the cubosomes was calculated by subtracting the amount of free NOR from the total

amount of norfloxacin used to prepare the cubosomes. All measurements were performed in triplicates [4].The entrapment efficiency (EE) was estimated according to the following equation:

$\%EE$=[$(Totaldrug–Freedrug)/Totaldrug$] ×100

Determination of pH

The pH values of the prepared Norfloxacin-loaded cubosomes nano-dispersions were determined by digital pH meters (Vision plus,alpha01) [4]

***In-vitro* Drug permeation study**

The in vitro release studies of NOR from the formulation were studied using the dialysis membrane of molecular weight cut off 12000–14000 kDa. The diffusion medium used was freshly prepared simulated tear fluid (pH7.4). Composition of simulated tear fluid (NaCl-0.68g, $NaHCO_3$-0.22g, $CaCl_2$-0.008g, KCl-0.14g, Distilled water-100ml) A dialysis membrane previously soaked overnight in the diffusion medium was tied from both ends and 2ml volume of the formulation was accurately pipette into this assembly. The dialysis membrane was suspended in a beaker containing 100ml of diffusion medium at (37°C±0.5). Here, 100 ml was selected keeping the sink condition in mind and the formulation remained in the eye for the complete duration of study. Also, in meaning for in vitro release for ocular drug delivery was keeping simulated condition. This assembly was kept on magnetic stirrer at 50 rpm. The aliquots were diluted with dissolution medium and analyzed by UV Spectrophotometer at 278nm [5].

Antibiotic susceptibility study:

Preparation of Muller Hington Agar (MHA)

38 gm of agar medium was suspended in one liter of distilled water. Heated it with frequent agitation and boiled for one minute to completely dissolve the medium.Autoclavedat121°Cfor15minutes and cooled at room temperature. The cooled Mueller Hinton Agar was poured into four sterile petridish on a level, horizontal surface to give uniform depth and allowed to cool at room temperature. pH was analyzed and stored at 2-8 ºC for further process [6].

Dilution of sample

The cubosomal dispersions of the three formulations was diluted using water in the ratio of 10^{-1} from the stock solutions. From the F1 formulation dilutions of 2, 4 and 6 are selected. Similarly, from the F2 formulation dilutions from 1, 3 and 5are selected and from the F3 formulation dilutions of 1 to 6 are selected to check the antibacterial susceptibility of E. Coli (*Escherichia Coli*) by Agar well diffusion method [7].

Agar well diffusion method:

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. Similarly, to the procedure used in disk-diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculums over the entire agar surface.Then, a hole with a diameter of 6 to 8mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested [8].

Susceptibility test:

TheNorfloxacincubosomeswereevaluatedformicrobiologicalstudy*in-vitro*.Muller Hington agar was seeded with the test organism E. Coli was allowed to solidify in the petridish. Norfloxacin cubosomes was placed over the agar layer. The plates were then incubated at 37 ± 0.5°C for 24h. After incubation the zone of inhibition around the ocular cubosomes was measured with the help of ruler [9].

RESULTS AND DISCUSSION

Preparation of Norfloxacin loaded cubosomes

Blank and Norfloxacin loaded cubosomal dispersions were prepared by emulsification method using glyceryl mono oleate and poloxamer 407 in oil / water emulsion. The dispersions emerged as uniform dense white mixtures with no visible signs of aggregate. It was observed that cubosomes of this composition have reasonable physicochemical properties with minimum particle size and maximum encapsulation efficiency.

Characterization of Norfloxacin loaded cubosmes dispersions:

Determination of Particle Size, Polydispersity Index and Zeta Potential

The mean particle size, polydispersity index, and zeta potential of the prepared cubosomes are listed in Table2.Particle size greatly influences the physical stability of cubosomes and their penetration through the corneal membrane and accordingly the ocular bioavailability of the loaded drug [10].In addition, by comparing F1, F2, and F3 which were prepared using ascending values of GMO: P407 ratio, it was clear

that as that ratio increases from 4:1 to 8:1, least particle size is obtained, as shown in Figure1a-c. This observation could be explained by the importance of P407 in the reduction of interfacial tension between the oil droplets and the aqueous phase, thus allowing the formation of homogenous and uniform emulsions with smaller droplet sizes [11].

It is clear that all of the formulations have negatively charged zeta potential which may be attributed to the ionization of the free oleic acid that present in the monooleate (anionic) that makes the dispersion anionic in nature [12]. In addition to the presence of P 407 that interact by hydroxyl ions with the aqueous medium. It was previously reported that the particles with negative charge could permeate through the skin via channels created by the repulsive forces between negatively charged skin lipids and particles [4]. Evaluation of the zeta potential of the prepared cubosomal dispersion is important to ensure the long-term stability of the colloidal dispersion. Although the values of zeta potential of all formulations were not high enough (between10.1 and 13.7 mv) to avoid the aggregation of particles by electric repulsive effect, the presence of P 407, would stabilize the cubosomes by the adsorption of its polypropyleneoxide copolymer (hydrophobic moieties) on the outer surface of the cubosomes that lead to shielding of the inverted-type self-assembled lipid nanoparticles from the surrounding aqueous medium, whereas the polyethylene oxide copolymer(the hydrophilic moieties) will suspend in water.

Table 2. The mean particle size, polydispersity index, and zeta potential of the prepared cubosomes

Formulation Code	Particle size(nm)	Polydispersity index	Zetapotential(mV)
F1	158.56±0.030	0.23±0.306	-26.21±1.544
F2	73.83±0.0306	0.35±0.030	-33.331±0.583
F3	23.65±0.031	0.44±0.026	-42.37±1.015

Mean ±SD, n=3

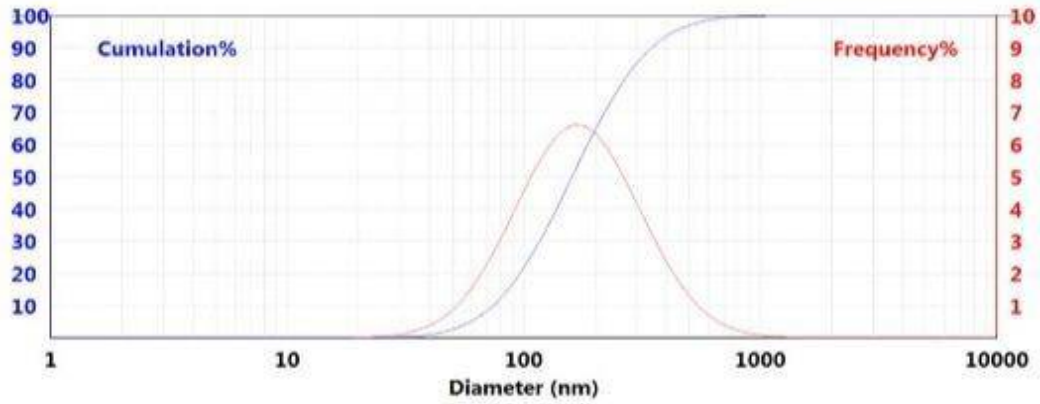


Fig.1a Particle size distribution of F1

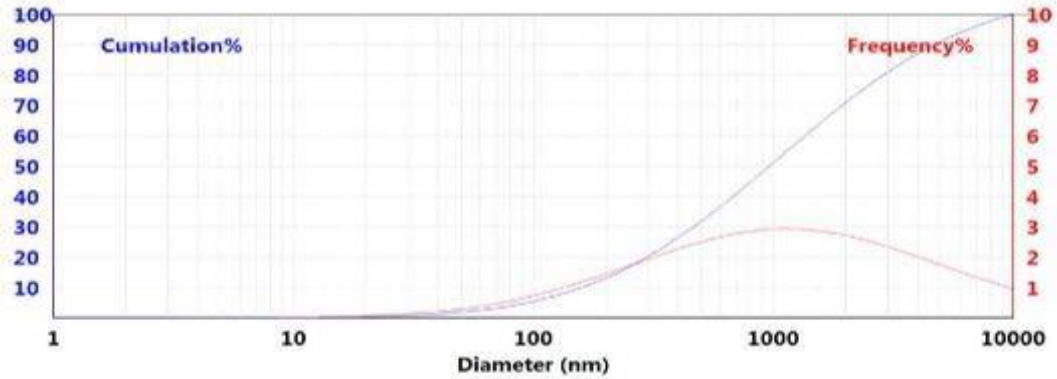
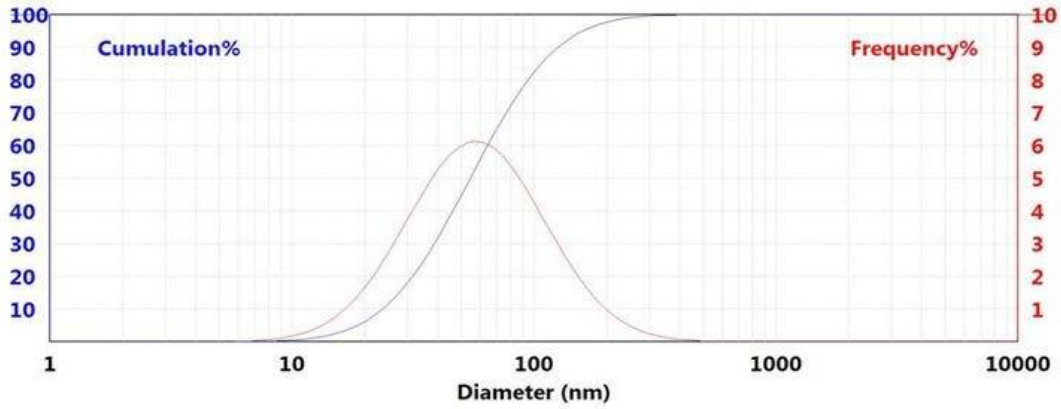


Fig.1b Particle size distribution of F2

Fig.1c Particle size distribution of F3



Determination of % Entrapment Efficiency and pH values

The E.E % of NOR in the prepared cubosomes is presented in Table 3. All of the formulations showed E.E % ranged from 70.5±0.20to 90.5±0.20. Less E. E % of NOR was observed in F1 & F2 compared to F3. It could be noticed that formulation factors that increase NOR solubility in the aqueous phase had a negative effect on drug E.E%, namely; type and concentration of aqueous stabilizer and oil: water ratio.In general, drug solubility is higher in liquid lipid than in solid lipid, which increases the entrapment efficiency. Incorporation of liquid lipids to solid lipids leads to reduced crystallinity and increased imperfections in the crystal lattice which leaves enough space to accommodate drug molecules, thus, leading to improved drug loading capacity and drug entrapment efficiency. Therefore, decreased particle size shows maximum entrapment efficiency [13].

However, the eye can tolerate a pH range between 4.5 and 11.5 due to the buffering action of the tears. the pH in the range of 7.2 ± 0.2 is considered ideal for maximum comfort [14]. All of the prepared formulations of cubosomes showed pH values between 7.02 and 7.41and it is given in Table 3 and the formulations were in the normal pH range of the physiological pH of the eye. Therefore, no ocular irritation may occur due to pH

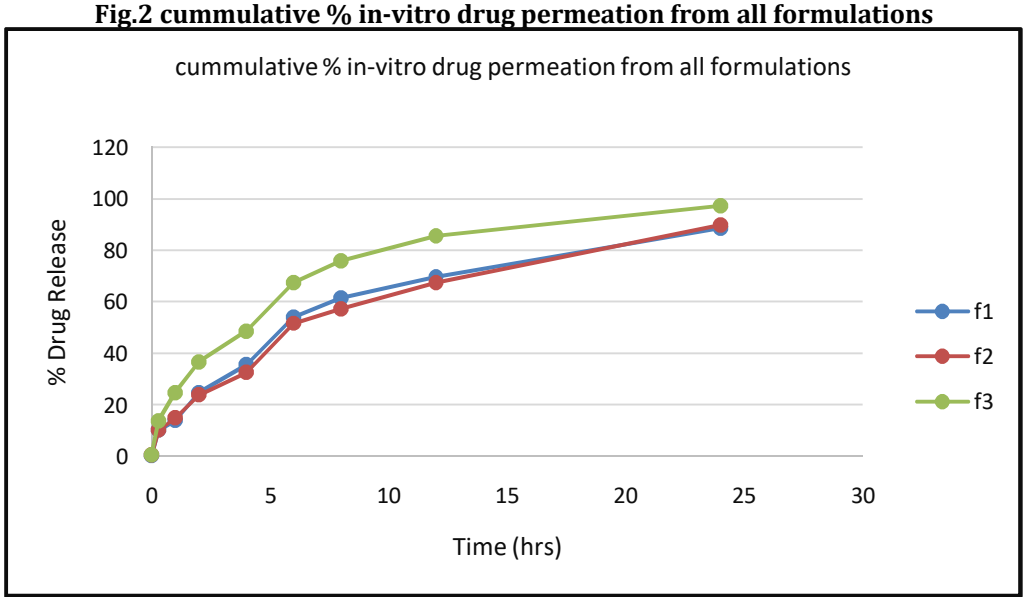
Table 3. Entrapment Efficiency and pH value of all formulations. *In-vitro* % drug permeation from prepared Cubosomes

Formulation Code	%Entrapment Efficiency	pH value
F1	70.5±0.20	7.02
F2	78.4±0.30	7.23
F3	90.5±0.20	7.41

Mean ±SD, n=3

The poor solubility and permeability of NOR in both lipid and water limits its transfer through the corneal epithelium and endothelium, and also the transfer through the hydrophilic stroma, respectively [15]. The amounts of NOR permeated per unit area through the semipermeable membrane at different times from different formulations are represented graphically in Figure 2. The results of the permeation study showed that there was no lag time in NOR permeation across semipermeable membrane from the tested formulations. This may be due to the permeation enhancing effect of both GMO and P407. The permeation results revealed that F3 shown higher and sustained effect due to the permeation enhancing effect of both GMO and P407 in higher concentration and structural similarities between the bicontinuous lipid bilayer of cubosomes and cell membranes of corneal epithelium. This similarity neither facilitates membrane fusion and therefore allowed the direct passage of NOR into corneal cells may result from the increase in corneal permeation and ocular residence time by the effect of cubosomes [16].

Fig.2 cummulative % in-vitro drug permeation from all formulations



Antibiotic susceptibility study

The Agar well diffusion susceptibility method is simple and practical and has been well-standardized. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted. The results were given in Fig.2a-c and it revealed that all formulations produced antibiotic effect with various levels. Among these formulations F3 showed more effect. This may be due to presence of higher concentration of drug loaded in the formulation.

CONCLUSION

Norfloxacin loaded cubosomal nanodispersions were successfully prepared and characterized. The all formulation showed an optimum particle size of 23.65±0.031, polydispersity index 0.44±0.026,and zeta potential-42.37±1.015mV. *In-vitro* permeation study showed 4-folds enhancement in permeability coefficient of NOR when compared with that stated in the literature. Antibiotic susceptibility result showed effective inhibition of growth against gram –ve bacillus (E. Coli). Therefore, NOR-loaded cubosome provide a novel topical drug delivery system for conjunctivitis management. It extended the release, increased permeation and contact time by a tremendous decrease in the used dose. Further study such as ex-vivo corneal permeation, corneal irritancy will be needed to predict the *in-vitro-in-vivo-correlation* in the enhancement of bioavailability and irritant effect of NOR.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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