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Formulation, Development and Evaluation of Nanoparticulate Cyclosporine for the Management of Keratoconjunctivitis Sicca

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ABSTRACT:

Keratoconjunctivitis sicca is a disorder of the preocular tear film that results in damage to the ocular surface and is associated with symptoms of ocular discomfort. It is also called dry eye, keratitis sicca, sicca syndrome, xerophthalmia, dry eye disease (DED), ocular surface disease (OSD), or dysfunctional tear syndrome (DTS). Keratoconjunctivitis sicca is a Latin word and its literal translation is "dryness of the cornea and conjunctiva." It may be helpful to know that "Sicca" is part of the English word "desiccate." The disease in which the eyes do not produce enough tears is also known as "Sjogren's syndrome". Cyclosporine is currently the effective and safe therapeutic treatment of dry eye. The objective of this study was to evaluate the prospective effectiveness of cyclosporine nanoparticles for the treatment of keratoconjunctivitis sicca. Cyclosporine associated with the BCS Class II which belongs to immunosuppressive compounds having an anti-inflammatory activity. Arginine is having positive charge which act as a potent vasodilator. Hyaluronic acid is having a negative charge which act as a natural lubricating agent. Therefore, when these are mixed with cyclosporine by ionic-gelation method, it leads to formation of nanoparticles, consequently improve retention time and provide controlled release. Therefore, it will retard the evaporation of water and also improve water holding capacity of eyes. The aim of the proposed study was to develop a controlled release formulation of cyclosporine for the management of keratoconjunctivitis sicca. Therefore, objectives of the proposed study was to formulate cyclosporine nanoparticles for keratoconjunctivitis sicca treatment to achieve control release of cyclosporine from the formulation at the target site and to improve therapeutic outcome.

Keywords: Keratoconjunctivitis Sicca, Ocular Surface Disease, Lubricating Agent. Mucin Profile, Pro Inflammatory Effects, Prevalence, Immunosuppressive Compound, Cyclosporine, Arginine, Retention Time, Hyaluronic Acid.

1. Introduction

Keratoconjunctivitis Sicca is a disorder of the preocular tear film that results in damage to the ocular surface and is associated with symptoms of ocular discomfort. It is also called dry eye, keratitis sicca, sicca syndrome, xerophthalmia, dry eye disease (DED), ocular surface disease (OSD), or dysfunctional tear syndrome (DTS), or simply dry eyes (3).

Keratoconjunctivitis sicca is a Latin word and its literal translation is "dryness of the cornea and conjunctiva." It may be helpful to know that "Sicca" is part of the English word "desiccate." The dry eye syndrome in which the eyes do not produce enough tears is also known as "Sjogren's syndrome". (4-6).

Dry eye therefore can mainly be divided into two groups, namely, (7)

(1) Aqueous production deficient dry eye disease

(2) Evaporative dry eye disease

One of the most common complaints discussed in the offices of eye care specialists is dry eye disease (DED), or dysfunctional tear syndrome (DTS), which was formerly called dry eye syndrome (DES). Patients with DED often have ocular complaints of photophobia, a sandy or gritty feeling, burning and stinging, itching, dryness, eye fatigue, and pain. Patients may also develop blurry vision, contact lens intolerance, redness (hyperaemia), and possibly a mucous discharge. (8).

Patients with DED may present with complaints of excessive tearing that is considered a paradoxical reflex tearing because of reduced basal tear production. Dryness may often be worse later in the day. Some relief is often obtained from the use of topical artificial tears. However, the signs and symptoms may worsen; patients may require more tear supplements and/or seek advice from eye care professionals. (8-10).

Keratoconjunctivitis sicca is recognized as a consequence of disruption of lachrymal functional unit. The lachrymal functional unit consists of lachrymal glands, ocular surface including cornea, conjunctiva, eyelids, meibomian glands, ocular nerves, and goblet cells. The tear film is composed of three main layers. The innermost mucin or mucus layer is the thinnest, produced by cells of conjunctiva. The mucus helps the overlying watery layer to spread evenly over the eye. The middle or aqueous layer is the largest, thickest layer produced by the glands of upper lids and the accessory tear glands and contains essentially a very dilute saltwater solution. This layer keeps the eye moist and helps in the removal of any dust, debris, or foreign particles. Defects of this layer cause DES in most cases. The uppermost layer of tear film is a very thin layer of lipids. These lipids are produced by the meibomian glands and the glands of Zeis (oil glands in the eyelids). This layer helps to decrease evaporation of the watery layer beneath it. The mucous also reduces the surface tension between the lipid layer of the tear film and the water layer, thus contributing to the stability of the tear film. (9)

The tear fluid also consists of complex mixture of proteins, immunoglobulins, mucins, electrolytes, cytokines, lysozymes, lactoferrin, and growth factors. Lysozyme may act synergistically with IgA in lysis of bacteria. Tears also contain lactoferrin, which has some antibacterial effect. Average glucose concentration of the tears is 2.5 mg/dL and average tear urea level is 0.04mg/dL. Electrolytes such as K, Na, & Cl occur in higher concentration in the tears than in the blood. Osmolarity of tears is 309mOsm/litre. Average pH of the tears is 7.25 and refractive index of the tear film is 1.336 (10).

In addition to the natural occurrence of DED, iatrogenic causes induce a similar form of OSD in a much larger population subjected to anterior segment ocular surgery or using long-term topical medications. Specifically, cataract surgery is known to disturb the tear film and induce surface inflammation up to 2 months following surgery (11-13).

Nanoparticles systems accelerate the drug penetration, increase corneal uptake, and avoid systemic absorption. It is able to deliver more intact drug at site of action as compared to free drug (24). After administration, colloidal drug carriers can remain at the application site (cul-de-sac) and the prolonged release of the active ingredient starts by particle degradation or erosion, drug diffusion, or a combination of both, depending on the biodegradable or inert nature of the polymer (14).

Based on these considerations, various strategies have been employed to modify NPs properties, including the use of cationic or mucoadhesive polymers. We have evaluated the particle formation process of the NPs prepared from HA polymers with the benefit of positively charged in different ratios, which could interact with the anionic mucins present in the mucus layer at the surface of the eye (23). The current study aimed at developing and optimizing a bioadhesive nanoparticles of Cyclosporine for ocular delivery to improve corneal uptake efficiency to treat dry eye (11).

Prior studies with cyclosporine were only directed at patients with moderate to severe disease; we wished to include a cohort of patients with mild disease. The purpose of this study was to evaluate the effect of cyclosporine on treating mild, moderate, and severe dry eye disease that was unresponsive to artificial tears therapy (12-15).

2. Materials and Methods

2.1 Materials

Cyclosporine (Sigma Aldrich Pvt. Ltd.), Hyaluronic acid (CDH), Arginine (CDH), Cetrimide (CDH) etc.

2.2 Preparation of Cyclosporine Loaded Arginine-Hyaluronic Acid Nanoparticles

Hyaluronic acid-Arginine (HA-Arginine) nanoparticles was prepared by ionic-gelation method as reported by Oyarzun Ampuero *et al*. (2011). Nanoparticles were obtained using 0.1 molar of HA and 0.2 molar of Arginine in order to provide a molar equivalency. An arginine solution was dropped into a HA solution using a pipette under magnetic stirring (1,000 rpm). After this step drug which is dissolved in methanol, mixed drop by drop to the above solution. Final colloidal solution was then subjected for homogenization at 10000 rpm (14).

2.2.1 Optimization of ratio of Arginine and Hyaluronic acid

Different conc. Of arginine and hyaluronic solution were prepared in molar concentration such as 0.1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M and nanoparticles are prepared by ionic-gelation method to get the desired size range of nanoparticles at different speed and time by homogenization.

2.2.2 Optimization in the concentration of Cetrimide

Selected ratio of Hyaluronic acid and Arginine was kept constant and the concentration of Cetrimide was changed and the concentration with least particle size was selected.

2.2.3 Optimization in the Concentration of Water

Ratio of Hyaluronic acid and Arginine and concentration of Cetrimide with least particle size was kept constant and the percentage of stabilizers was changed to achieve the desired size of nanoparticles.

2.2.4 Optimization of Process Parameters

Homogenization speed (RPM), Time are the important processing parameters, which are used in the optimization of nanoparticles formulations. These are described in the following section (15-19).

A. Speed (rpm): The nanoparticles were prepared on different RPM to get the desired size.
B. Time: Time is also play a vital role in preparation of nanoparticles at different speed of homogenizer.

2.3 Physicochemical Characterization

2.3.1 Particle Size and Polydispersity Index (PDI)

Particle size and size distribution of prepared Hyaluronic acid and Arginine Nanoparticles were determined in the low volume quartz cuvette by using Delsa-Nano Zeta-Sizer. A minimum of three measurements per sample were made and the mean values was reported (18).

2.3.2 Scanning Electron Microscopy

The surface morphology and size of prepared Cyclosporine loaded Nanoparticles were analyzed using a Scanning Electron Microscope (SU8020, Hitachi). Scanning Electron Microscope uses a focused beam of high-energy over the nanoparticles, producing various signals that help to obtain information about the surface morphology and composition (17).

2.3.3 X-Ray Diffraction

X-Ray Diffraction screening was performed to detect the physical state of Cyclosporine loaded Nanoparticles. The sample was exposed to monochromatic nickel-filtered copper radiation (45kV, 40mA) in a wide angle X-ray diffractometer (D8 Advance, BRUKER, Germany) with 2Ɵ scan ranges from 4-450 with a step size of 0.1º (18).

2.3.4 Differential Scanning Calorimetry

DSC is a thermo-analytical technique, which is used to study thermal behavior and the decomposition of the prepared Cyclosporine loaded Nanoparticles. The phase transitions of Cyclosporine NPs was analyzed by Differential Scanning Calorimetry (SIIO 6300, SIIO, Japan) in a perforated aluminium pans at a heating rate of 50C/min from 30 to 4000ºC using nitrogen as blanket gas (50ml/sec) (19).

2.3.5 IR Spectroscopy

IR study was performed for identification and structural analysis of sample using FT-IR spectrometry. A small quantity of the sample was triturated with KBR preferably in the ratio of 1:100. This mixture was then compressed into a pallets which was then placed into the FT-IR assembly and the IR-spectrum was obtained out at temperature from 400 – 4000 (16).

2.3.6 Solubility

It is the amount of the solute which can be dissolved in a given amount of solvent under standard conditions of temperature, pressure and pH to form homogeneous solution. Solubility of drug was investigated in water, ethanol, chloroform and methanol (23).

2.3.7 pH

The pH values of optimized formulations were determined using digital pH meter (ME 963-P). The pH was repeated for formulations after 1day, 3days, 7days, 14days and 28 days (18).

2.3.8 In-Vitro Release Study

The in-vitro release study was performed using Franz diffusion cell to evaluate the drug release profile of the optimized formulations. The pre-treated dialysis membrane was used and mounted on the Franz diffusion cells. The receptor compartment contained PBS (100 ml) of pH 7.4. The temperature of diffusion media was thermostatically controlled at 37±0.5ºc by surrounding water in the outer jacket and the medium was stirred by magnetic stirrer at 100 rpm. Drug loaded nanoparticles were placed on the dialysis membrane, which was fixed in between donor and receptor compartment. The donor compartment was then capped to prevent evaporation. Samples of 1 ml were taken after 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,12, 13, 14, 15,and 16 hours and replaced by an equal volumes of fresh PBS to maintain the sink conditions. After suitable dilutions with PBS, samples were analysed by UV Spectrophotometer at 210 nm against the blank and atomic absorption spectrophotometer for nanoparticles. The release experiments of each sample were performed in triplicate and average value are reported (17).

2.3.9 Sterility Test

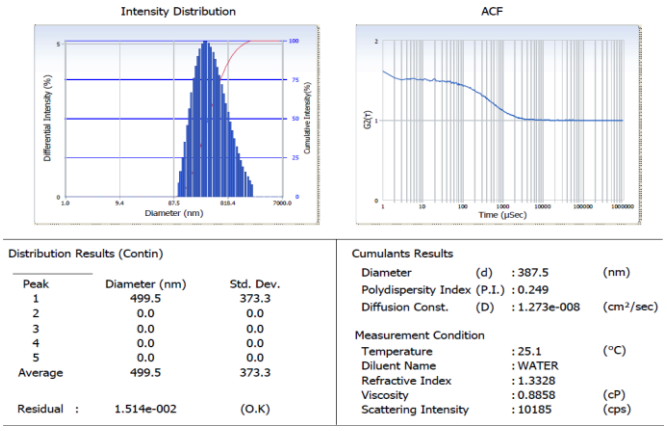
The sterility test of nanoparticles was performed by comparing turbidity of the test inoculation with test of McFarland standards. The culture media was prepared by using nutrient broth and it was autoclaved to sterilize the culture media. The nanoparticles were transferred into the culture media and incubated for 48 hrs. After the stipulate time period the samples were taken and analysed under UV-Spectroscopy. Solution consisting of 1% $Bacl_2$ and 1% H_2SO_4 was used to produce a series of McFarland standards with respect to their composition and equivalence. The absorbance of prepared dispersion was observed at 210 nm using UV-Spectrophotometer (19).

2.3.10 Isotonicity

Isotonicity of the developed formulation was observed for swelling, bursting and shrinking of RBC, the RBC were taken and diluted with the Hayem's solution, which was observed under the Motic microscope and the another part of test RBC was incubated with formulation. After incubation the diameter of red blood cells was measured. The mean diameter of RBC was determined by manual image analysis of approximately 200 to 500 RBC. Change in diameter of RBC was compared with control group to interpret toxicity (15).

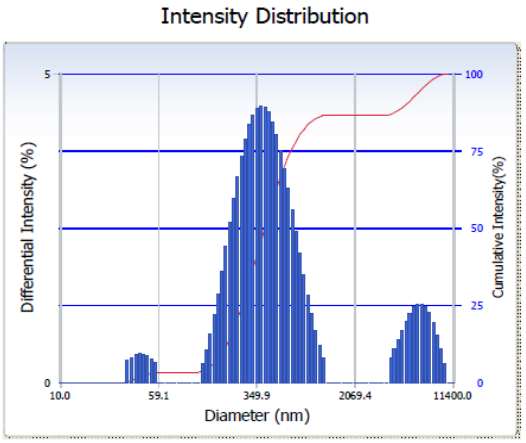
3. Result and Discussion:

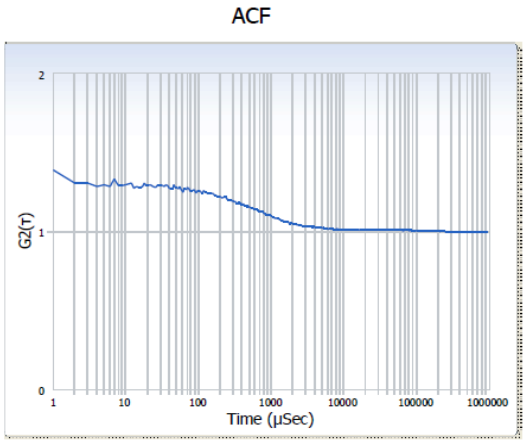
3.1 Particle Size and PDI of Nanoparticles without Drug



Polydispersity Index (PDI): 0.219
Diameter: 387.5 nm

3.2 Particle Size and PDI of Drug Loaded Nanoparticles





Distribution Results (Contin)

Peak	Diameter (nm)	Std. Dev.
1	43.3	6.7
2	433.7	211.2
3	6,702.6	1,692.7
4	0.0	0.0
5	0.0	0.0
Average	1,260.8	2,238.1
Residual :	1.790e-002	(O.K)

Cumulants Results

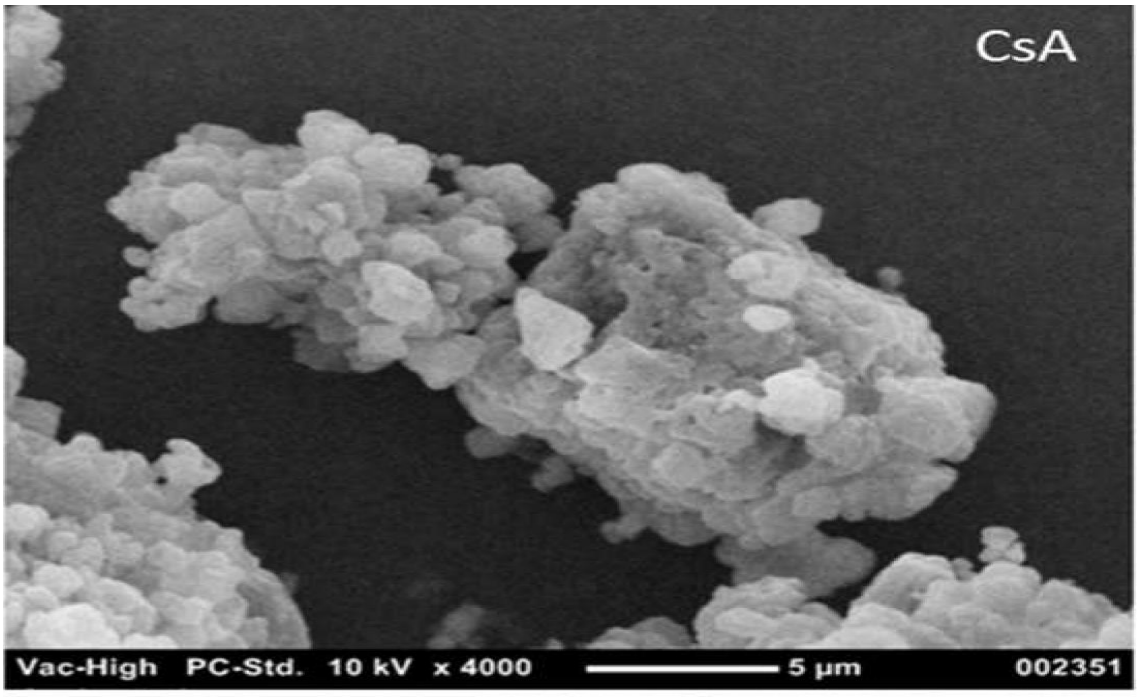
Diameter	(d)	: 620.0	(nm)
Polydispersity Index	(P.I.)	: 0.261	
Diffusion Const.	(D)	: 7.934e-009	(cm^2/sec)

Measurement Condition

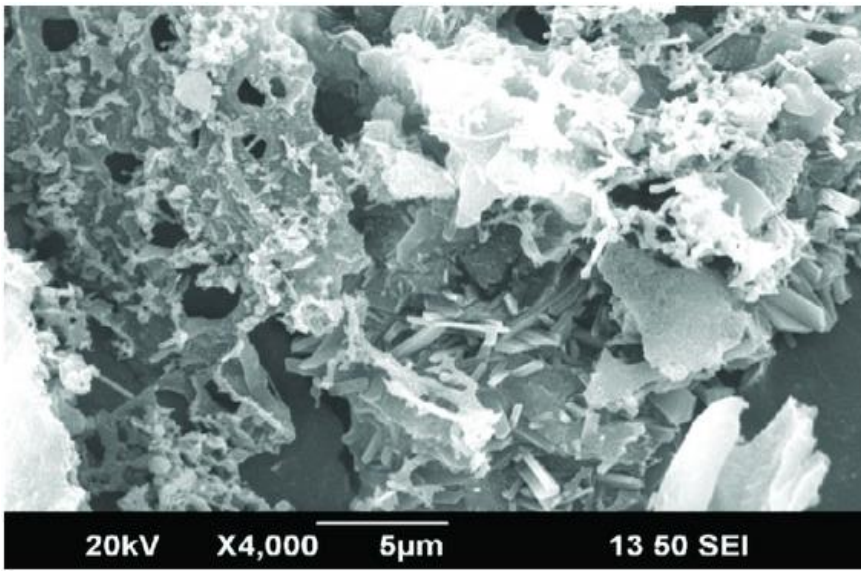
Temperature	: 25.0	(°C)
Diluent Name	: WATER	
Refractive Index	: 1.3328	
Viscosity	: 0.8878	(cP)
Scattering Intensity	: 8518	(cps)

Polydispersity Index (PDI): 0.261
Diameter: 620 nm

3.3 SEM of Cyclosporine and Formulation

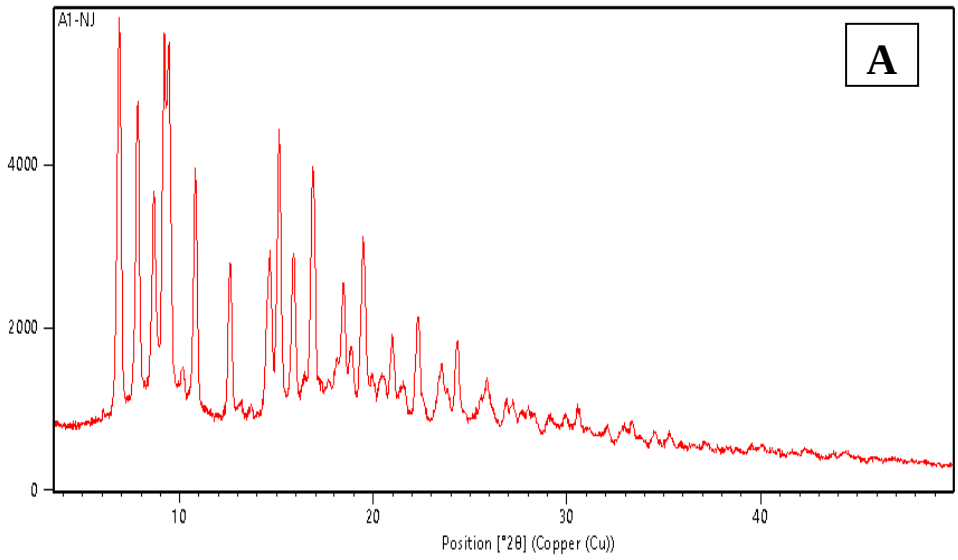


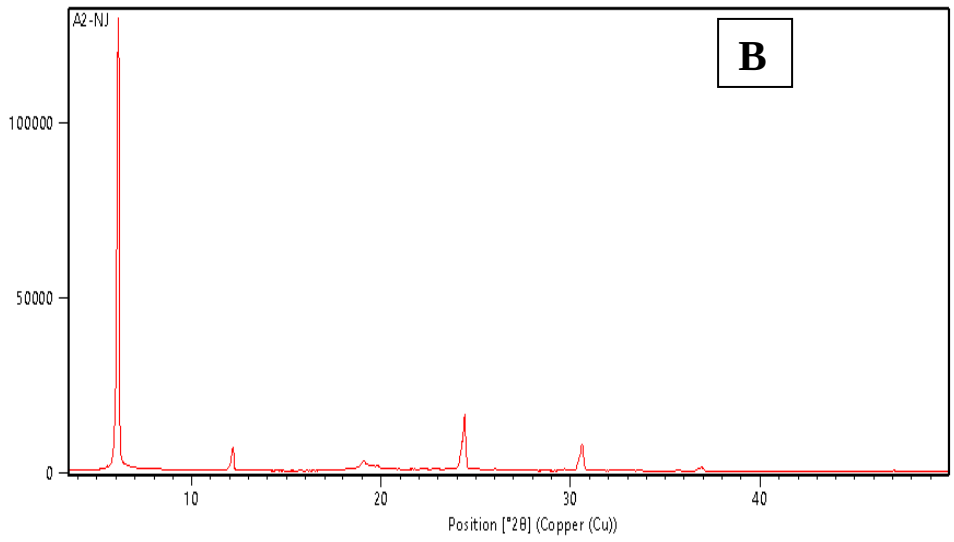
(SEM of Cyclosporine)



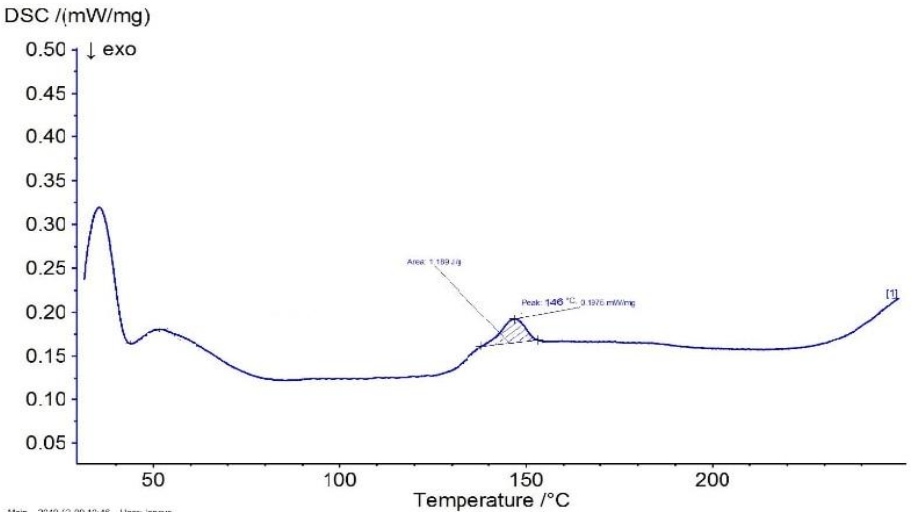
(SEM of Formulation)

3.4 X-ray diffraction of Plain Drug (A) & Formulation (B)

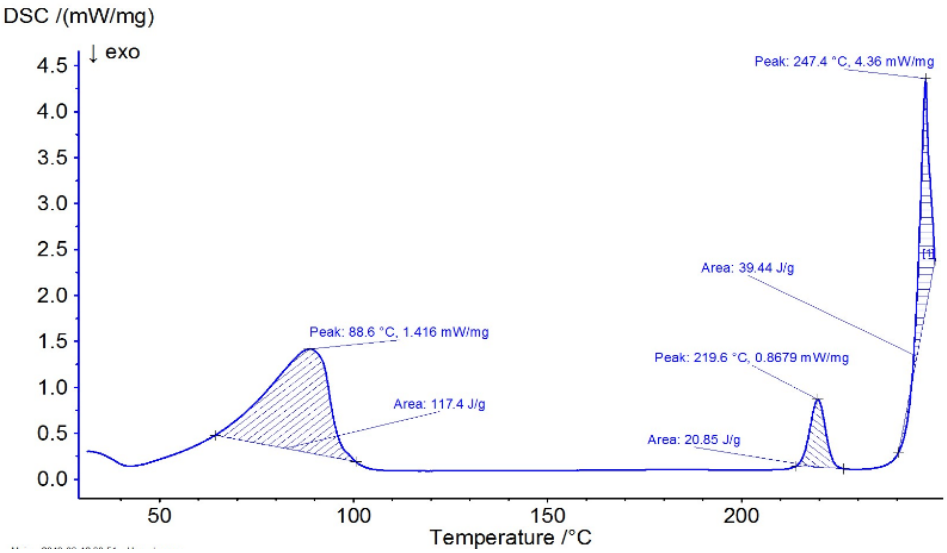




3.5 DSC of Cyclosporine and Physical Mixture

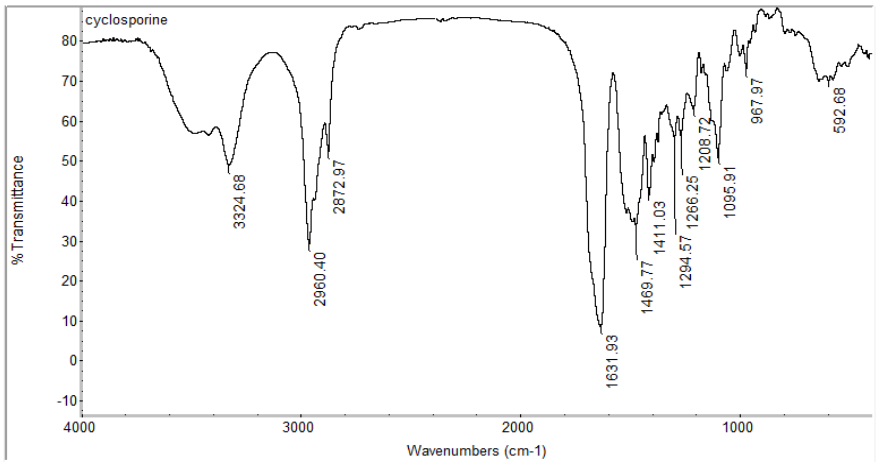


(DSC Graph of Cyclosporine)

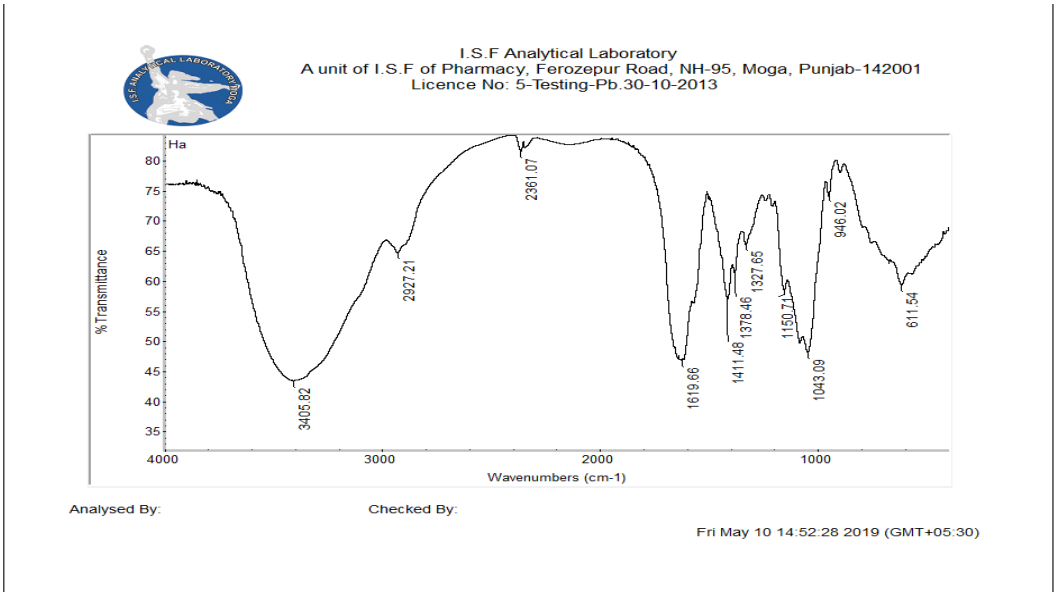


(DSC Graph of Physical Mixture: Cyclosporine+HA+Arginine)

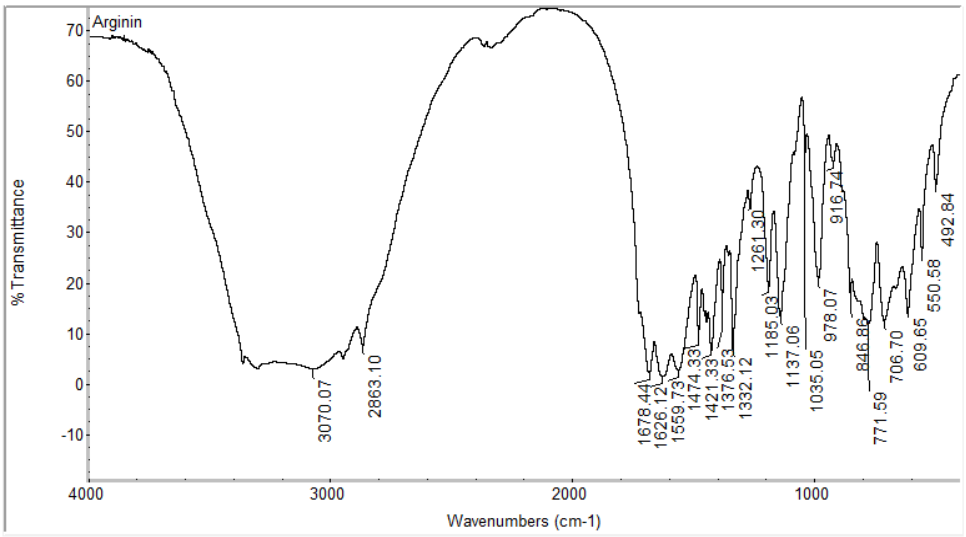
3.6 Infra-red (IR) Spectroscopy



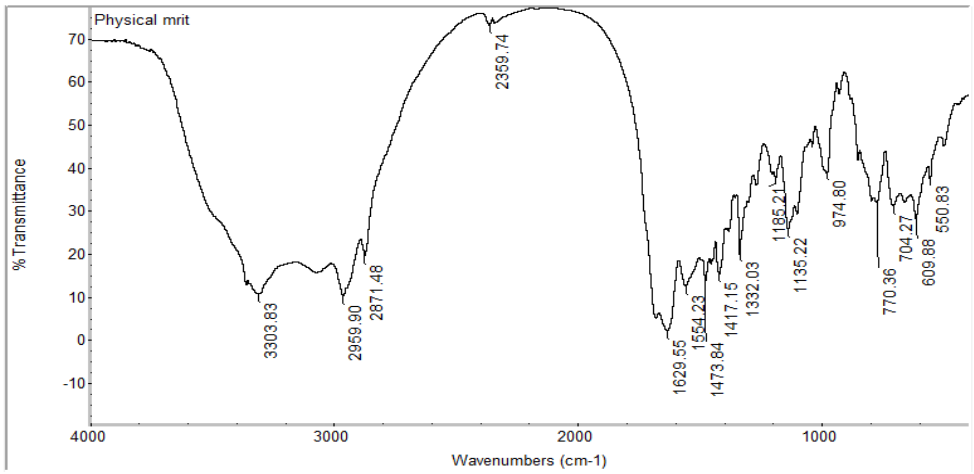
(IR Spectra of Cyclosporine)



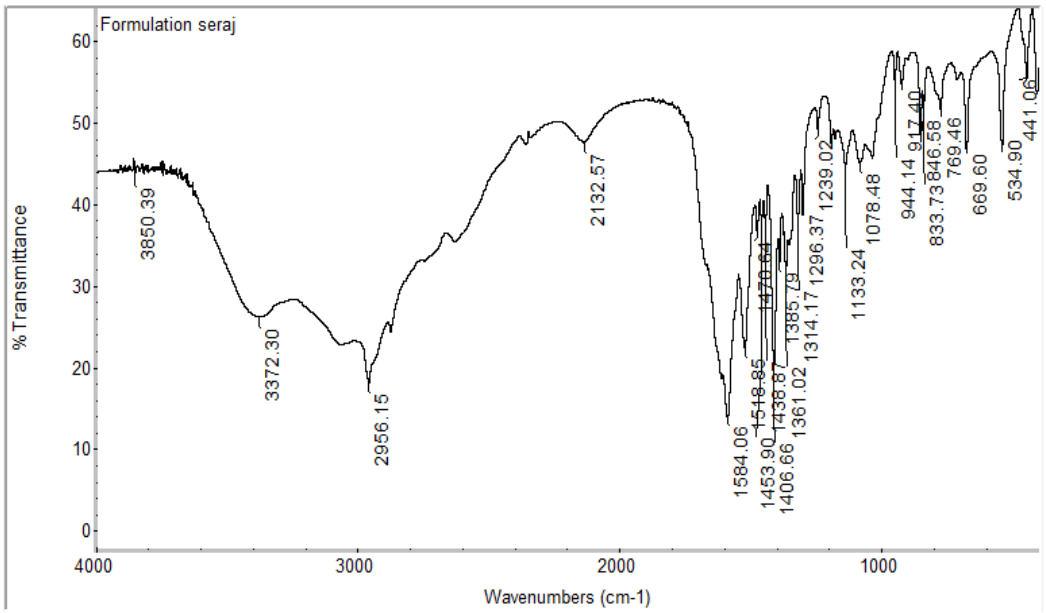
(IR Spectra of HA-Hyaluronic Acid)



(IR Spectra of Arginine)



(IR Spectra of Physical Mixture)



(IR Spectra of Final Formulation)

3.7 Solubility

The solubility trend of drug in different solvents has been given in the table. Results of solubility studies indicate that drug exhibited good solubility in methanol, acetone, ethanol and DMSO. It is slightly soluble in water.

Table: Solubility Profile of Cyclosporine

Solvent	Saturation solubility (mg/ml)	Parts of solvent required for one part of solute	Standard	Interpretation
Methanol	35	28	10-30 parts	Soluble
Acetone	36	27	10-30 parts	Soluble
Diethyl ether	32	26	10-30 parts	Soluble
Water	4	786	100-1000 parts	Slightly Soluble
Ethanol	32	25	10-30 parts	Soluble
DMSO	33	28	10-30 parts	Soluble

3.8 pH

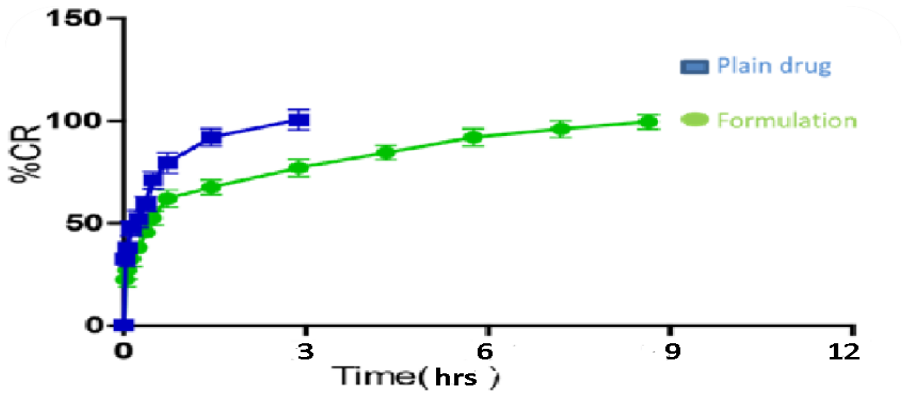
pH of Formulation at Different Time Intervals

Days	pH
Days 1	7.4
Days 3	7.4
Days 7	7.3
Days 14	7.3

Days 28	7.3

3.9 In-Vitro Drug Release

The % cumulative drug releases from optimized formulation with time are shown in figure which is given following. The release pattern shows that there was a sustained release followed by burst release from the nanoparticles which may be due to surface adherence. Initial burst release of approximate 40% of applied to surface drug, following burst release, an extended drug release was observed which is attributed to diffusion mechanism. Similar findings were observed in earlier studies by our group, where sustained release behaviour of cyclosporine was observed from HA nanoparticles up to 12 hours. Extended drug release mechanism also a function of poor drug solubility and slow degradation of polymeric constituents. Further the comparative study indicated that HA significantly reduced the drug release compared to plain drug solution.

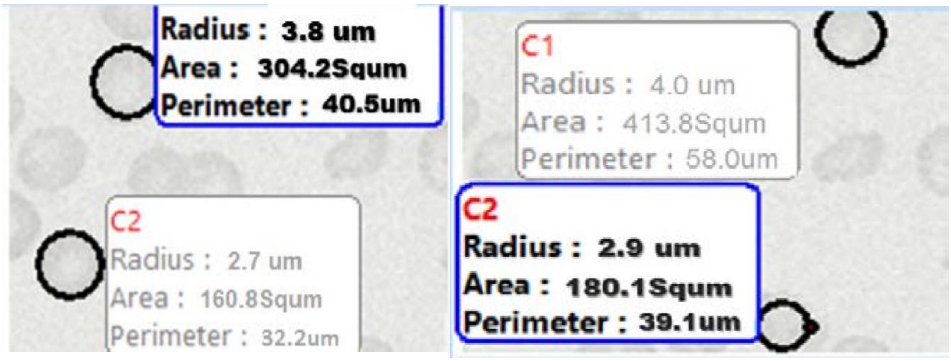


3.10 Sterility Test

The sterility test of nanoparticles was performed by comparing the turbidity of the test formulation with that of McFarland standard. It was found that the absorbance of both the standard and the test samples was found to be in the almost same range, which shows that there was no microbial growth. The observed absorbance of standard and the test samples are in range from 0.78- 0.79 which shows that there is no sign of microbial contamination in drug loaded nanoparticles.

3.11 Isotonicity

Isotonicity of the formulation was examined using RBC dilution method. 500 RBC were counted and their mean were reported. Results indicated that there is no change in mean diameter was observed in the treated group compare to untreated group, confirmed the isotonicity of the developed formulation. High molecular weight of hydrophobic polymer and insoluble nature of drug rendering fewer ionized particles to influence tonicity. Tonicity results also confirm that the developed product avoid discomfort and irritation, thus contribute useful platform for ophthalmic drug delivery. The mean value of 500 RBC were examined and reported in figure that is given below.



Standard (RBC with Hayems Sol.) Test (RBC with Hayems Sol.)

(Isotonicity of Standard and Test)

4. Conclusion

The present study reports the formulation and development of cyclosporine packed nanoparticles which combine the negatively charged hyaluronic acid with the positively charged properties of arginine.

Cyclosporine packed nanoparticles successfully prepared using ionic-gelation method. Polymer concentration, applied homogenizer speed, homogenization time seems to be the key determinant that accounts for the morphology of nanoparticles. In vitro characterization demonstrated that the prepared nanoparticles show high entrapment efficiency, controlled drug release behaviour, adequate mechanism, suitable for ophthalmic application. Above findings accounts for the suspected transition of drug in nanoparticles. In vivo observations clearly indicates the therapeutic potential of nanoparticles with higher drug availability at the target site. All the findings support the application of preservative free nanoparticles as a suitable carriers for drug delivery into the posterior eye segment. In a nut shell, we have demonstrated that nanoparticles with different properties could modulate the drug release in the cul-de-sac or in the ocular tissues after uptake by epithelial cells. The system interacts with eye surface, ensuring optimal contact between the formulation and the mucosa. It provides sustained drug release in the cul-de-sac for an extended period of time. These results indicate that cyclosporine packed nanoparticles have great potential as drug delivery systems and are promising formulations in the management of external inflammatory/autoimmune ocular diseases.

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