Indian Journal of Novel Drug Delivery

IJNDD

An Official Publication of Karnataka Education and Scientific Society

Research Article

Moxifloxacin Hydrochloride Loaded Polymeric Nanoparticles for Ocular Drug Delivery: *In-Vitro* and *Ex-Vivo* Studies

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ARTICLE DETAILS

Article history:
Received on 27 August 2019
Modified on 5 October 2019
Accepted on 16 October 2019

Keywords:
Moxifloxacin Hydrochloride,
Poly D,
L-lactide,
Nanoparticle,
Ocular Drug Delivery.

ABSTRACT

Abstract

Ocular bioavailability of drugs from conventional eye drops is very poor due to the physiologic barriers of the eye. In general, ocular efficacy is closely related to ocular drug bioavailability, which may be enhanced by increasing corneal drug penetration and prolonging precorneal drug residence time. Therefore, the present study involves the development, characterization and evaluation of biodegradable moxifloxacin hydrochloride nanoparticles intended for ocular use. Nanoparticles were prepared by nanoprecipitation techniques using Poly D, L-lactide. To optimize the drug formulation, 3^2 factorial designs was applied. The effect of independent variables such as drug-to-polymer ratio and speed of homogenizers on entrapment efficiency (EE %) and particle size were investigated. Further studies such as differential scanning calorimetry (DSC), X-ray diffraction (XRD) and scanning electron microscopy were carried out on the optimized formula. In vitro release study showed extended drug release. DSC and XRD indicated the dispersion of the drug within the nanoparticles. Ocular tolerance was evaluated using hen's egg chorioallantoic membrane (HET-CAM) test which showed nonirritant efficacy of developed formulation. These results demonstrate the feasibility of encapsulating moxifloxacin hydrochloride biodegradable polymeric nanoparticles for ocular delivery.

INTRODUCTION

Ocular drug delivery route is the most preferable route of drug delivery. Ocular drug delivery systems are developed to treat eye locally, whereas past formulations are targeted to reach systemic circulation and these are designed to overcome all the disadvantages of conventional dosage forms such as ophthalmic solutions [1]. The main problem with conventional dosage forms is eye irritation (due to drug particle size and shape) which induces lacrimation i.e. overflow on to lids, tear turn over, and due to pharmacokinetic responses like metabolism, non-specific binding and different mechanisms like diffusion, dissolution and erosion the conventional dosage forms are less advantageous [2].

The eye drop dosage form is easy to instill but suffers from the inherent draw back that the majority of the medication it contains is immediately diluted in the tear film as soon as the eye drop solution is instilled into the cul-de-sac and is rapidly drained away from the precorneal cavity by constant tear flow, a process that proceeds more intensively in inflamed than in the normal eyes, and lacrimal-nasal drainage [3].In general, ocular efficacy is closely related to ocular drug bioavailability, which may be enhanced by increasing corneal drug penetration and prolonging precorneal drug residence time. A variety of ocular drug delivery systems such as inserts and collagen shields and colloidal systems such as liposomes, nanoparticles, and nanocapsules have been designed and investigated for improved ocular bioavailability. The use of nanotechnology-based drug delivery systems such as microemulsions, nanosuspensions, nanoparticles, solid lipid nanoparticles, niosomes, dendrimers, and liposomes has led to the solution of various solubility-related problems of poorly soluble drugs [4]. Polymeric nanoparticle formulation is one of the strategies currently used to improve drug absorption across biological membranes.

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Considering the fact that there is short residence of dosage form in ocular cavity, it was proposed that the use of mucoadhesive polymers, that increase the concentration and residence time of the associated drug [5]. Based on literature data, the three most commonly used polymers in ophthalmic drug formulations are poly (alkyl cyanoacrylates), polycaprolactone, and Poly D,L-lactide. The literature data also reveals that in the case of ophthalmic drug delivery, an appropriate particle size and a narrow size range, ensuring low irritation, adequate bioavailability, and compatibility with ocular tissues, should be sought for every suspended drug. Among the wide variety of mucoadhesive polymers reported in the literature, the Poly D, L-lactide has been selected as a polymer of choice because of its unique properties including acceptable biodegradability, biocompatibility as well as the ability to increase membrane permeability [6, 7].

Moxifloxacin is an 8-methoxy fluoroquinolone having half-life nearly 12 h. It has a broad spectrum antibiotic activity, with efficacy against various Gram-positive and Gram-negative microorganisms through inhibition of DNA gyrase and topoisomerase IV and is indicated for treating bacterial conjunctivitis [8]. As compared to other fluoro-quinolone moxifloxacin has highest potency against Staphylococcus aureus and Staphylococcus epidermis.

Hence, the present work was aimed towards the development and characterization of biodegradable nanoparticles containing moxifloxacin hydrochloride to improve precorneal residence time and ocular bioavailability.

MATERIALS AND METHODS

Moxifloxacin hydrochloride was received from Cipla Ltd, Mumbai, India as a gift sample. Polyvinyl alcohol (PVA) M. Wt. 22000 Dialysis tubing cellulose membrane which is having molecular weight cut-off 12000-14000 g/mole was purchased from Sigma Aldrich Pvt Ltd. Mumbai, India. Poly (D, L-lactide) ester having viscosity of 0.34 dL/g was purchased from Sigma Aldrich Pvt. Ltd. Mumbai. All other reagents used were of analytical grade.

Preparation of Drug Loaded Nanoparticles Using Nanoprecipitation Technique

The nanoprecipitation technique was used for the preparation of Moxifloxacin Hydrochloride nanoparticles. Organic solution of biodegradable polymer (PLA) and exact amount of Moxifloxacin Hydrochloride (50 mg) in 10 mL of acetone were prepared. The organic phase was added drop wise into 20 mL of aqueous solution containing PVA (1%) and stirred magnetically. After 30 min of stirring the volume of nanoparticles dispersion was concentrated to 10 mL under reduced pressure using a Rota evaporator with vacuum (KNF, vaccum pumps & system). The aggregates were removed by filtration through a 0.45µm syringe filter. Separation of non-encapsulated drug was performed by ultracentrifugation (Beckman Coulter) at 50,000rpm at 4^0C for 30 min. The supernatant was discarded and separated nanoparticles were washed twice with distilled water to remove excess surfactant. The washed particles were resuspended in 5 mL of water solution containing 5 %(w/v) mannitol as cryoprotectant and freeze dried for 48 hrs. The whole experimental carried out in aseptic area. The nanoparticles were kept at 2 to 8^0C for further investigation.

Design of the Experiment

The effect of different parameters on the physicochemical properties of the prepared nanoparticles was studied using 3^2 full factorial design.

Table 1: Experimental plan of the 3^2 factorial designs

Factor	Level		
	++	+	-
Polymer Concentration (mg)	150	100	50
Speed of Homogenizer	1200	1000	800

Table 2: Composition of Moxifloxacin Hydrochloride loaded nanoparticles formula prepared according to 3^2 factorial designs.

Formula Number	Drug concentration	Polymer Concentration	Speed
F1	50	50	800
F2	50	100	800
F3	50	150	800
F4	50	50	1000
F5	50	100	1000
F6	50	150	1000
F7	50	50	1200
F8	50	100	1200
F9	50	150	1200

Concentration of polymer and different speed were selected as the independent variables. The particle sizes of colloid system and encapsulation efficiency of the drug were selected as the dependent variables.

Characterization of the Nanoparticles

Determination of the Particle Size

Size distribution, average particle size and PDI were determined by photon correlation spectroscopy using Zeta-particle size, Model Nano ZS. The separated nanoparticles were subjected to measurement followed by dilution with distilled water. The particle size and PDI measurements were carried out at a scattering angle of 90^0 and at a temperature 25°C. All experiment done in triplicate [9].

Encapsulation Efficiency (EE %) Measurements

Extraction and quantification of the encapsulated Moxifloxacin Hydrochloride was performed for the determination of the encapsulation efficiency of moxifloxacin hydrochloride in polymeric nanoparticles. The portion of the encapsulated moxifloxacin hydrochloride was obtained by ultra-centrifugation 1ml of the nanoparticle suspension at 18000 rpm for 1hour using a cooling centrifuge at 4°C. The supernatant was removed and the formed pellets were re-suspended in phosphate buffer saline pH 7.4 to ensure the complete removal of all free moxifloxacin hydrochloride. The supernatant (free moxifloxacin hydrochloride) was collected and measured using HPLC. A reverse phase C-18 column was equilibrated with the mobile phase Ammonium formate: Acetonitrile (70:30) and pH adjusted 4.0 with formic acid. Mobile phase flow rate was maintained at 1ml/min and eluents was monitored at 295 nm for moxifloxacin. The sample was injected using a 20 µl fixed loop. Mobile phase was prepared by mixing 700 ml of 20mM ammonium formate solution with 300 ml of HPLC grade acetonitrile to get the proportion of 70:30 v/v and finally the pH was adjusted to 4.0 with formic acid. The mobile phase was sonicated for 10 minutes and filtered through 0.45µ membrane filter.

EE of the drug= (amount of encapsulated drug) / (total amount of the drug) X 100

……Equation No.1

The entrapped moxifloxacin hydrochloride concentration was expressed as percentage entrapment efficiency which can be defined as the percent fraction of the total input drug encapsulated in the polymeric nanoparticles [10].

Redispersibility of Nanoparticles

The selected formulation was freeze-dried to obtain a dry powder for further investigation. In addition to that it was taken to study the effect of cryoprotectant on freeze-drying and dispersibility of the prepared nanosuspension. Mannitol at a concentration of 5 times the total solid contents in the formulation used as a cryoprotectant. Two samples of nanosuspension each were placed in a flask, the amount of mannitol required added to one and shaken to dissolve, the second sample left with out of cryoprotectants. These flasks were frozen in a deep freezer at −20°C for 12 h for primary freezing. Then the container was attached to the vacuum adapter of the lyophilizer. The solvent sublimed under a pressure of 80 mmHg for 48-72 h [11].

Swelling Index

The accurately weighed nanoparticles were placed in a glass vial containing pH 7.4 phosphate buffer 10 mL at 37 ± 0.5 °C in incubator and was stirred occasionally. The nanoparticles were periodically removed by blot using filter paper and the change in weight of particulates was measured till equilibration. The weight was recorded after a period of 3 h in triplicate and the swelling ratio (SR) was calculated using formula (Eq. 2) [12].

Swelling index (%) = W1-W2 X 100 / W1

…….. Equation No. 2

Where,

W1 = Weight of nanoparticles after swelling

W2 = Initial weight of nanoparticles

Differential Scanning Calorimetry

Differential scanning calorimetric measurements were carried out by using differential Scanning Calorimeter (DSC DA 60 Shimadzu, Japan) equipped with a liquid nitrogen subambient accessory. The DSC was performed for the pure moxifloxacin, the PLA and the drug loaded nanoparticle formulation. Sample 2 mg were loaded in a flat-bottomed aluminium pan and subjected to a heating cycle from 40 to 400^0C with a heating rate of 10^0 C/min. A stream of nitrogen gas was used to control the heating and cooling rate. The temperature and energy scales of the instrument were calibrated using purified indium as the reference material [13].

X-ray Diffraction

X-ray diffraction analysis was employed to detect the crystallinity of the drug in nanoparticle formulation, which was conducted using a Philips PW 3710 x-ray diffractometer (XRD) with a copper target and nickel filter. Powders were mounted on aluminum stages with glass bottoms and smoothed to a level surface. The XRD pattern of each sample was measured from 10 to 50 degrees 2-theta using a step increment of 0.1 2-theta degrees and a dwell time of 1 second at each step [14].

Scanning Electron Microscopy

The scanning electron microscopy (JEOL Model JSM - 6390LV) was used to characterize the surface morphology of nanoparticles. The nanoparticles were mounted directly on the SEM stub, using double sided, sticking tape and coated with platinum and scanned in a high vacuum chamber with a focused electron beam. Secondary electrons, emitted from the samples were detected and the image formed [15].

***In Vitro* Release Studies**

The release study of nanoparticle formulation for the release of moxifloxacin hydrochloride from polymeric nanoparticles was carried out using a membrane diffusion technique. In vitro diffusion cell was made using dialysis membrane as a semi permeable membrane. To retain the nanoparticles as well as to enable the free drug to diffuse freely into the release media a dialysis membrane of 12,000-14,000 Molecular weight cut-off was used. Formulated nanoparticles equivalent to 1 mg of the drug were dispersed in 1 mL of isotonic phosphate-buffered saline at pH 7.4. The nanoparticle dispersions were packed in dialysis membrane secured with two clamps at each end. To maintain sink condition, the dialysis bag was immersed in tightly-capped glass vials (7 × 2.8 cm) containing 10 mL of 0.5 % (w/v) of sodium lauryl sulphate solution in distilled water. The release test was performed by placing the glass vials in a thermostatically-controlled shaking water bath adjusted to 37 ± 0.5 °C with a constant shaking rate of 100 rpm. At predetermined time points, the whole release medium was withdrawn and replaced with fresh release medium. The concentration of the drug released was measured by HPLC. All experiments were carried out in triplicate [16].

Chorioallantoic Membrane (Het-Cam) Test and Irritation Score Calculation

A chorioallantoic membrane (CAM) testing as a mucous-membrane irritation test was performed for Draize eye irritation test. Commercially available fertilized white chicken eggs without micoplasms were used for the test. For CAM testing, the hen's eggs were put in incubator trays with the large ends up; the trays were placed in the incubator, which automatically rotates and was maintained at an optimum temperature of 37.5±0.5°C. The eggs were candled on day 5 of incubation and every day thereafter; nonviable embryos were removed. On day 10 of incubation the egg shell was scratched around the air cell by a dentist's rotary saw and then pared off. After careful removal of the inner egg membranes the vascular CAM was exposed. The test sample in volume of 0.2ml was applied on the CAM surface. A series of four eggs was used; two eggs, treated with vehicle only, serve as controls. After the application of the test substance, the CAM, the blood vessels, including the capillary system, and the albumen were examined and scored for irritant effects (hyperaemia, haemorrhages, coagulation) at 0.5, 2 and 5 minutes after treatment. The numerical time-dependent scores for hyperaemia, haemorrhages and coagulation (Table 3) were summed to give a single numerical value indicating the irritation potential of the test substance on a scale with a maximum value of 21. The mean value of four tests makes possible an assessment by a classification scheme analogous to the Draize categories (Table 4) [17].

Table 3: Scoring scheme for irritation testing with the hen's egg chorioallantoic membrane.

Effect	Score (Time in Second)		
	0.5	**2**	**5**
Hyperemia	5	3	1
Hemorrhage	7	5	3
Coagulation	9	7	5

Table 4: Classification of cumulative scores in the chorioallantoic membrane test

Cumulative Irritation	Score assessment
0-0.9	Practically none
1-4.9	Slight
5-8.9	Moderate
9-21	Strong

Stability Studies

Stability studies were carried out on optimized formulation at 30 ± 2°C in stability chamber (Thermolab) for 6 months. The optimized formulation stored in the sealed in glass bottle. After 6 months, drug content, particle size and redispersibility studies were carried out [18].

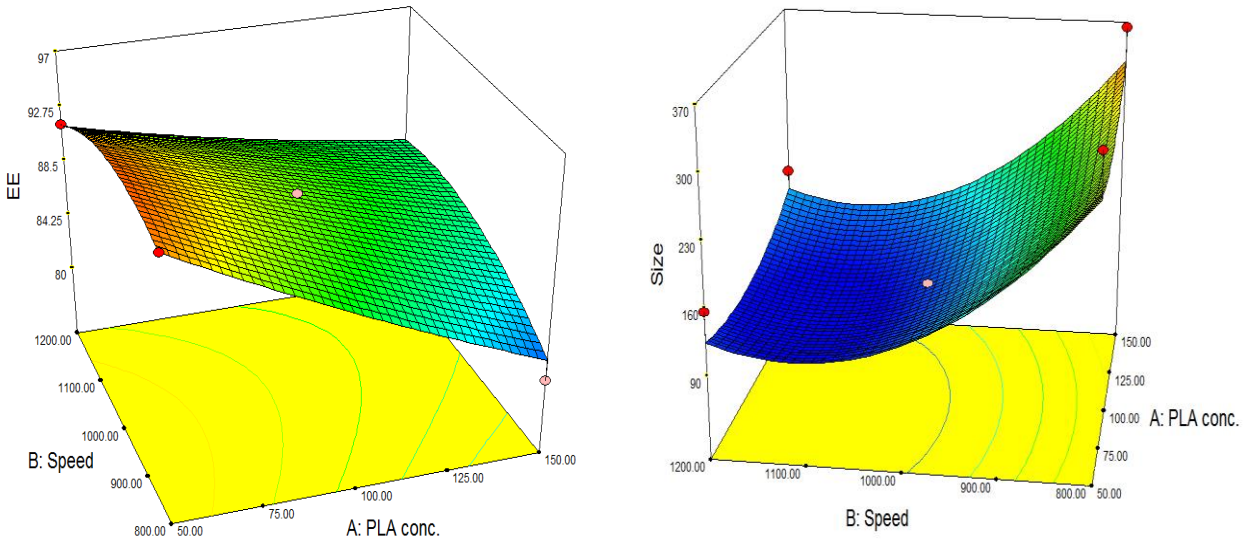
Sterility Testing

All parenteral preparations should be sterile. Sterility studies were carried out to ensure the sterility of finished product. Since it is administered by parenteral route, direct inoculation method was preferred to carry out sterility testing. In this method, the specified quantity of sample under test was drawn aseptically from the containers and transferred to fluid thioglycollate medium (20 mL) and Soybean-Casein digest medium (20 mL), separately. Mixture of nanoparticles with the medium was incubated for not less than 14 days at 30°C–35°C in case of fluid thioglycolate medium and 20°C–25°C in case of Soybean-Casein digest medium. The growth of any microorganisms in the medium was observed [19].

RESULTS AND DISCUSSION

Experimental Design

Based on the experimental and above studies two factors (Concentration of polymer and speed of the homogenizer) were determined crucial factor for particle size and entrapment efficiency of the prepared polymeric nanoparticles. The concentration of polymer was found to have a significant effect on entrapment efficiency as optimum concentration of polymer. The speed of the homogenizer is also effect on optimization formulation [20].



(A) **(B)**

Figure 1: Surface plots showing the effect of (A) Encapsulation Efficiency (B) Particle Size

Preparation of Drug Loaded Nanoparticles

Moxifloxacin hydrochloride loaded PLA Polymeric nanoparticles were successfully prepared by using nanoprecipitation technique as it is rapid and easy to perform. Nanoparticle formation is one step and instantaneous procedure. When the polymer solution is added to the non-solvent, rapid desolation occurs followed by nanoprecipitation. As soon as the polymer-containing solvent has diffused into the dispersing medium, the polymer precipitates, involving immediate drug entrapment. Moreover, this method always produces a carrier size in the nanometer range and uses ingredients with low toxic potential which is suitable for ocular route.

Characterization of Nanoparticles

Determination of Particle Size

When considering irritation and comfort, the particle size is an important factor in the development of an ocular drug delivery system. The mean particle size of prepared nanoparticle formulae is shown in Fig. 2. The particle size varies from 108.1 to 363.1 nm.

The effect of different formulation variables namely polymer concentration and speed had significant effect on particle size. Concerning the effect of polymer concentration Fig. 2 show that the particle sizes obtained by using lower polymer concentration were significantly smaller than those obtained by using higher polymer

concentration. It showed that as the polymer concentration increases, the particle size also increases. Different speed also affects the particle size as shown in Fig. 2. Particle sizes obtained by using low speed were significantly larger than the particle sizes obtained by using high speed. But it is to be noted that if speed is further increased it did not decrease the particle size to the significant extent. So the optimum speed which gave the smaller particle size was speed used for F4.

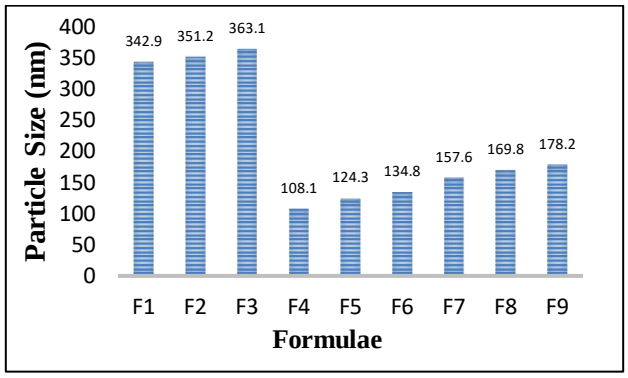


Figure 2: Particle size of the Moxifloxacin hydrochloride nanoparticles formulations

Particle size distribution represented by polydispersity index (PDI) was measured for all nanoparticles formulations. The mean PDI values ranges from 0.555 to 1.0. The narrow distribution represented by the small PDI values denotes particle size uniformity in the nanoparticle formulae.

Encapsulation Efficiency (EE)

Nanoparticle encapsulation efficiency % ranged between 80.2 to 96.8%. All tested variables have a significant effect on EE%. It was observed that the increase in polymer content resulted in decrease in EE% of the nanoparticles formulae (Fig. 3).

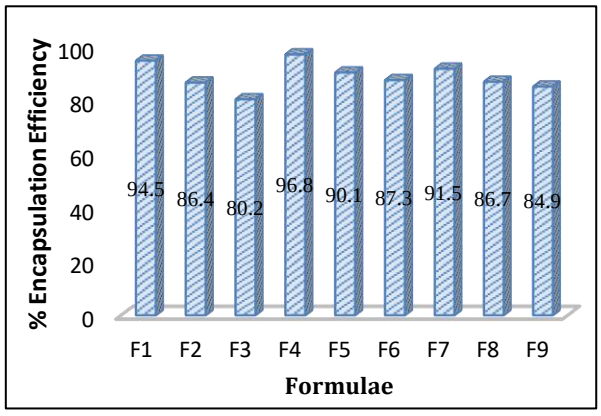


Figure 3: Encapsulation Efficiency of the Moxifloxacin hydrochloride in nanoparticles

This result may be attributed to the increasing viscosity of the organic phase upon increasing polymer content.

Redispersibility Test

It was found that the dispersibility was improved when using mannitol as cryoprotectants and products were spontaneously dispersed into primary nanosuspension within 1-3 min in both media (0.1 N HCl and phosphate buffer pH 6.8). It suggested that mannitol in the products would improve the wetting of the hydrophobic drug and accelerate the penetration of water into the products. On the other hand, the products without cryoprotectants could not be dispersed well and transformed into the original nanosuspension within 15 min as expected from their agglomerated structure.

Swelling Index

As polymer concentration is increase from 50 mg to 150 mg exploration increase in swelling index from 55% to 85% indicates that being PLA as hydrophilic polymer uptake excessive amount of water responsible for swelling for polymeric nanoparticles.

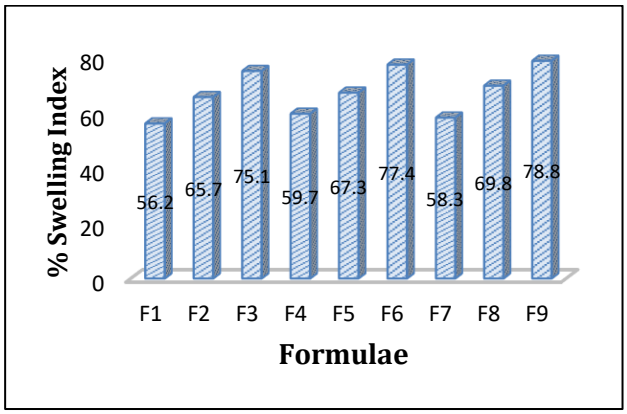


Figure 4: Percentage of Swelling Index of Nanoparticles Formulation.

Differential Scanning Calorimetry (DSC)

To verify the existence in the physical interaction between moxifloxacin hydrochloride and excipients, each sample was analyzed by differential scanning calorimetry (DSC). DSC thermogram of Moxifloxacin hydrochloride, PLA and lyophilized drug-loaded nanoparticles is shown in Fig. 5. In the drug loaded nanoparticles thermogram, the characteristic endothermic peaks at 254^{0}C of the drug disappeared. It could therefore be concluded that moxifloxacin hydrochloride was entrapped in an amorphous or molecular dispersion state in the polymer matrix [21, 22].

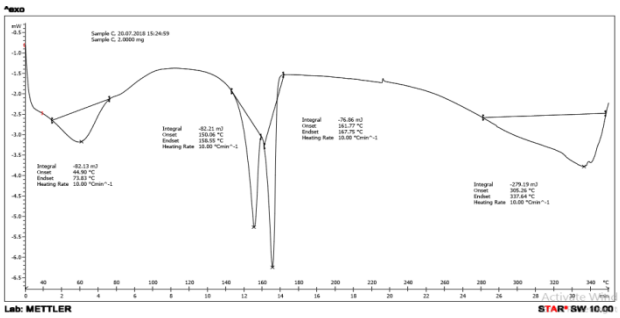


Figure 5: DSC thermogram of the optimized nanoparticles formulation (F4).

X-ray Diffraction (XRD)

The crystallinity of the moxifloxacin hydrochloride in the nanoparticles has been carried out by powder x ray diffraction. Fig. 6 shows the powder x ray diffraction patterns of optimized formulation (F4).It indicates that the drug was present in the nanoparticles in an amorphous state, confirming DSC results.

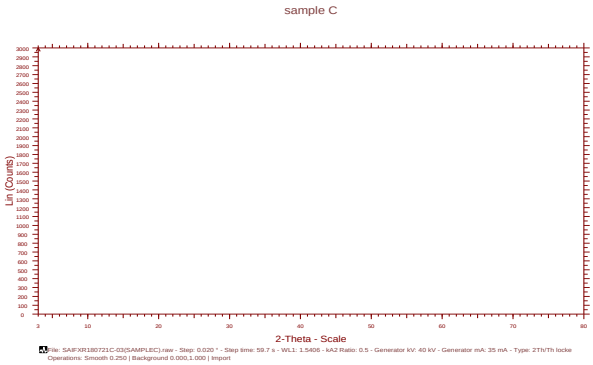


Figure 6: XRD of the optimized nanoparticles formulation (F4).

Scanning Electron Microscopy (SEM)

The morphology of drug loaded nanoparticles (F4) was accessed using SEM and is shown in Fig. 7. This figure indicates that the nanoparticles were cylindrical in shape and their size was in the nanometer range with smooth surface essential for ocular drug delivery.

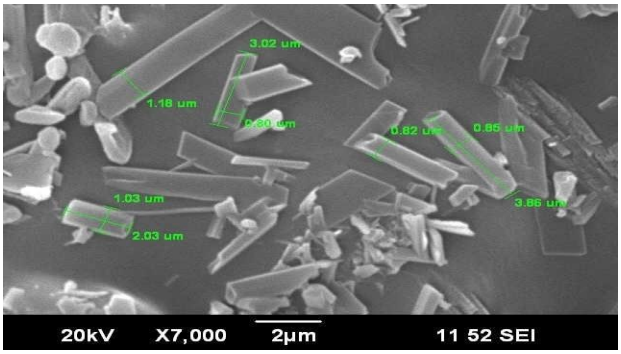


Figure 7: SEM of the optimized nanoparticles formulation (F4).

In Vitro Drug Release from Nanoparticles

The drug formulae prepared with nanoprecipitation technique were subjected to in vitro release study. The amount of Moxifloxacin Hydrochloride released from nanoparticles was evaluated using a dialysis technique. The release profiles of moxifloxacin hydrochloride are shown in Fig. 8.

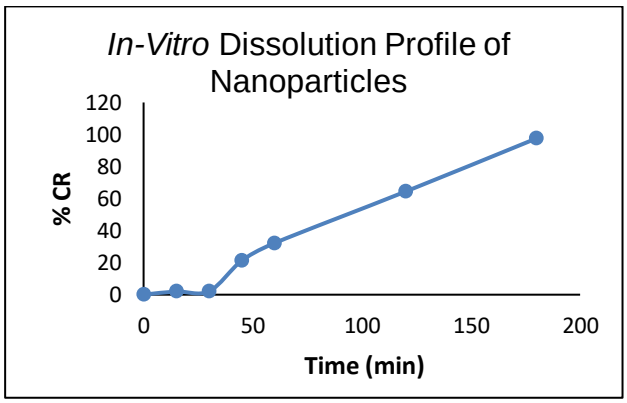


Figure 8: *In vitro* release study of the Moxifloxacin hydrochloride nanoparticles formulation.

Drug release from PLA based nanoparticles reveals sustained release up to 1 hrs due to wetting followed by immediate release as results of rapid diffusion of drug release from swollen polymeric nanoparticles.

CAM Test

It is found to that formulation is non irritant as score value for Hyperemia, Hemorrhage and coagulation is zero, as compared to phosphate buffer solution pH 7.4 should coagulation after five minutes (Score value 1.4) which is slightly irritant.

Figure 9: CAM Test of the optimized nanoparticles formulation (F4).

Stability Studies

Stability studies were carried out on optimized formulation at 30 ± 2°C in stability chamber (Thermolab) for 6 month. The optimized formulation stored in the sealed in aluminum

foil. After 6 months, drug content, particle size and redispersibility studies were carried out.

Table 5: Stability of Moxifloxacin Hydrochloride nanoparticles during storage (F4)

Parameter	0 Day	1 Month	3 Month	6 Month
Drug Content	96.8	96.0	95.4	94.9
Particle Size	108.1	110.6	112.3	114.3

Sterility Testing

During sterility testing, we found that there was no evidence of microbial growth when formulations were incubated for not less than 14 days at 30°C to 35°C in case of fluid thioglycolate medium and at 20°C to 25°C in case of Soybean-Casein digest medium demonstrating that formulation passes the test for sterility.

CONCLUSION

Moxifloxacin hydrochloride was successfully suitable within biodegradable nanoparticle using nanoprecipitation technique. The formulation study using 3^2 factorial designs is used for the optimum formulation to be obtained. The drug-polymer ratio and speed had a significant effect on the particle size and encapsulation efficiency of the nanoparticle. The formulated moxifloxacin nanoparticles was found to be a suitable and potential natural carrier in terms of their particle size, drug loading capacity, redispersibility *in vitro* release characteristics, sterility and better ocular tolerability. The stability study of moxifloxacin from nanoparticles has shown suitable results.

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