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Mucoadhesive Nanoparticles as Carrier Systems for Prolonged Ocular Delivery of Gatifloxacin/Prednisolone Bitherapy

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Abstract: A fluoroquinolone/glucocorticoid combination for the treatment of bacterial keratitis in the form of mucoadhesive nanoparticle suspensions was developed to prolong the release and improve patient compliance. Gatifloxacin/prednisolone loaded nanoparticles were prepared using Eudragit RS 100 and RL 100 and coated with the bioadhesive polymer, hyaluronic acid. FT-IR and DSC studies revealed no interaction between gatifloxacin and prednisolone. The effects of the drug:polymer ratio (D:P) and the RS/RL ratio were studied. The obtained nanoparticles were distinct and spherical with a solid dense structure. They have average particle size range of 315.2 to 973.65 nm. Increasing the D:P ratio significantly lowered the entrapment efficiency for both drugs ($p < 0.05$). The nanoparticle suspensions revealed significantly prolonged drug release comparing to the free drugs ($p < 0.05$) with no burst effect. Increasing the polymer concentration and the Eudragit RS ratio significantly decreased the release efficiency values. Gatifloxacin showed anomalous release ($n = 0.4943$) from 1:1 D:P ratio nanoparticle suspension and Fickian diffusion mechanism ($n < 0.45$) from formulas prepared at higher D:P ratios. Gatifloxacin showed better bioavailability and sustained action in aqueous humor and corneal tissue from the nanoparticles compared to the commercial eye drops. The resulting nanoparticle suspension is promising in reducing dose frequency and improving patient compliance.

Keywords: Mucoadhesive; nanoparticle suspensions; Eudragit; bacterial keratitis; gatifloxacin; prednisolone

Introduction

Bacterial keratitis is an ocular emergency that requires immediate and appropriate treatment to limit corneal morbidity and vision loss. It is characterized by corneal ulceration, stromal abscess formation, surrounding corneal edema, and anterior segment inflammation. *Streptococcus, Pseudomonas, Enterobacteriaceae* and *Staphylococcus* species are the most common groups of bacteria responsible for bacterial keratitis.[1] Treatment of bacterial keratitis starts with rapid and intense antibiotic therapy (one drop every 5 min for the first 30 min followed by treatment every 15−30 min for 48−72 h). This intensive regimen of treatment is disruptive to patient and family and often necessitates hospitalization for compliance with frequent therapy.[2]

Topical fluoroquinolones are recommended as antibiotics for bacterial keratitis based on their widespread availability,

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long shelf life, broad spectrum coverage and lack of toxicity.[3] Gatifloxacin is a fourth-generation fluoroquinolone with good aqueous solubility, better penetration and an improved antibacterial spectrum, particularly against resistant *Staphylococcus* and *Streptococcus* pathogens, compared with older fluoroquinolones.[4,5] Gatifloxacin is commercially available as 0.3 and 0.5% eye drops. The literature included formulating it as in situ gelling systems[6,7] and chitosan−sodium alginate nanoparticles.[8]

In conjunction with antibiotics, a cycloplegic agent should be used for treatment of bacterial keratitis to minimize inflammation and increase patient comfort. Additional therapy can include nonsteroidal anti-inflammatory drugs (NSAIDs), tissue adhesive and subconjunctival injections once or twice daily for 1 to 2 days.[9] Prednisolone is one of the most effective drugs of the glucocorticoid group expressing anti-inflammatory, desensibilic and antiallergic activity that is available as ophthalmic drops and suspension. It is used to inhibit the inflammatory response to a variety of inciting agents. It inhibits the edema, fibrin disposition, capillary dilation, leukocyte migration, capillary proliferation, fibroblast proliferation, disposition of collagen and scar formation associated with inflammation.[10]

Controlled drug delivery to the eye is one of the most challenging fields of pharmaceutical research due to the low drug contact time and the precorneal loss of conventional eye drops. Nanosized carriers and mucoadhesive ocular drug delivery systems are promising to treat external and intraocular eye infections and diseases that require frequent eye drop instillation in order to maintain therapeutic drug levels.[11]

Eudragit RS and RL are copolymers of poly(ethylacrylate, methyl methacrylate and chlorotrimethyl-ammonioethyl methacrylate). They are insoluble in water at physiological pH values and capable of swelling, so they are good for the dispersion of active compounds.[12]

Hyaluronic acid is a natural, nonirritating bioadhesive polymer of polysaccharide that shows pseudoplastic behavior. Several studies demonstrated its possible use as a mucoadhesive adjuvant in ocular delivery systems.[13]

Based on the above considerations, this study aimed to evaluate the potential of hyaluronic acid coated Eudragit nanoparticle suspensions containing gatifloxacin and prednisolone bitherapy as a long-term extraocular drug delivery without comprising intraocular structures and/or systemic drug exposure. The effect of formulation variables was studied and the formulations were characterized both *in vitro* and *in vivo*.

Experimental Section

Materials. Gatifloxacin and prednisolone were kindly provided as free samples by Cangzhou Goldlion Chemicals Co., Ltd., China, and Memphis Company, Cairo, Egypt, respectively. Hyaluronic acid sodium salt (HA), from *Streptococcus equi* sp. was purchased from Fluka, USA. and Eudragit RS 100 and Eudragit RL 100 from Röhm Pharma (GMBH, Darmstadt, Germany). HPLC grade solvents (acetone and acetonitrile) were from Sigma-Aldrich Chemical Co. (USA). Tymer eye drops (gatifloxacin 0.3%), batch no. HE140, Exp. 5/2010, was provided by Jamjoom Pharmaceuticals, (Jeddah, Saudi Arabia) and Benox eye drops (Oxybuprocaine HCl 0.4%), batch no. 0803-4, Exp. 11/2010, was provided by the Egyptian International Pharmaceutical Industries Co., Cairo, Egypt. Sodium chloride, sodium bicarbonate, calcium chloride, benzalkonium chloride and disodium edetate were of analytical grades and obtained from El-Nasr Pharmaceutical Chemical CO., Cairo, Egypt.

Compatibility Study between Gatifloxacin and Prednisolone. Fourier transform infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC) experiments were performed on gatifloxacin, prednisolone and their 1:1 physical mixture.

Samples for DSC (±5 mg) were weighed into aluminum pans (TA Instruments, Brussels, Belgium) and hermetically sealed. Runs were performed over a temperature range 0−250 °C, at 5 °C/min. Octadecane and indium standards were used to calibrate the DSC-7 calorimeter (Perkin-Elmer, Norwalk, CT). IR spectra were obtained with a Perkin-Elmer

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1600 spectrophotometer, using the KBr disk technique (about 10 mg sample for 100 mg of dry KBr).

Method of Determination of Drugs in Mixture. Direct, first and second derivative UV spectroscopy were investigated as cheap and fast methods for the *in vitro* determination of gatifloxacin and prednisolone in mixture. Calibration curves were constructed for both drugs in artificial tear fluid prepared according to Van Ooteghem.[14]

Preparation of Nanoparticle Suspension. The nanoparticles were prepared adapting the spontaneous emulsification technique previously described by Bodmeier et al.[15] Specified amounts of Eudragit polymers and both drugs were dissolved in 25 mL of ethanol. Gatifloxacin and prednisolone weight ratio was fixed at 3:10 according to their ocular therapeutic doses. The alcoholic solution mixture was added dropwise (3 mL/min) to 50 mL of distilled water under continuous magnetic stirring at 800 rpm. The formed nanoparticles were further stirred for 48 h at ambient temperature and pressure. Nanoparticles were collected by centrifugation (REMI high speed, cooling centrifuge, REMI Corporation, India) and then stored in glass bottles with buffer solution for subsequent evaluation.

All the prepared formulas contained 0.01% w/v benzalkonium chloride and 0.01% w/v disodium edetate as preservatives and permeation enhancers for gatifloxacin.[16]

Coating of Nanoparticles. Coating the prepared nanoparticles with HA was done applying two strategies:[17] (1) coating of the performed nanoparticles by HA adsorption onto the nanoparticle surface by mixing a given volume of the resulting suspension with an equivalent volume of 0.025% w/v HA aqueous solution; (2) coating during particle formation by replacing the distilled water in the aforementioned procedure with 0.025% w/v aqueous HA solution.

Nanoparticle Morphology, Particle Size Analysis and Zeta-Potential Determination. Morphological analysis of the nanoparticles was performed using transmission electron microscopy (TEM, Philips CM-10, USA). Samples of the nanoparticle suspension (5−10 μL) were dropped onto Formvar-coated copper grids. After complete drying, the samples were stained using 2% w/v phosphotungstic acid. Digital Micrograph and Soft Imaging Viewer software were used to perform the image capture and particle sizing.

The mean particle sizes of the formulations were also determined by observing 10 slide mounts of each formulation, each containing at least 20 nanoparticles, under a computerized microscope (processing and analyzer system: Leica DMLB, U.K). The polydispersibility index (PI) was calculated according to the method described by Vora et al.[18]

The zeta potential of nanoparticles was measured by a laser zetameter (Zeta Sizer 2000, Malvern Instruments, U.K.) using a sample of 0.01 g in 50 mL of double distilled water at ionic strength of 2×10^{-2} M NaCl. The pH was adjusted to 7.4 during determination. The used samples were first separated from their aqueous suspension by ultracentrifugation and redispersed in artificial tear fluid.

Drug Loading Determination. The encapsulation efficiency of nanoparticles was determined according to Motwani et al.,[8] where drug-loaded nanoparticles were separated from the aqueous medium containing nonassociated gatifloxacin and prednisolone by ultracentrifugation (REMI high speed, cooling centrifuge, REMI Corporation, India) at 18,000 rpm and 4 °C for 30 min. The amount of each drug loaded into the nanoparticles was calculated as the difference between the total amount used to prepare the nanoparticles and the amount that was found in the supernatant.

Release Study. The nanoparticles were separated from their aqueous suspension by ultracentrifugation, redispersed in 1 mL of artificial tear fluid, placed in a dialysis membrane bag (Spectrapor membrane tubing No. 2 Spectrum Medical Industries, USA), and suspended in 200 mL of artificial tear fluid. The entire system was kept at 37 ± 0.5 °C at 50 rpm. At appropriate time intervals, 2 mL of the release medium was removed and replaced with fresh medium. The amounts of gatifloxacin or prednisolone were measured after appropriate dilution. All measurements were performed in triplicate and the SD was calculated.

The release extent was determined by calculating the release efficiency after 24 h (RE_{24h}) for all formulas according to Khan.[19]

The kinetics of gatifloxacin release from the prepared formulas were determined by finding the best fit of the release data to the zero order, first order, Higuchi[20] and Hixson-Crowell cube root[21] models. To evaluate the mechanism of release, data for the first 60% of the drug release were plotted in the Korsmeyer−Peppas equation[22] as log cumulative percentage of drug released versus log time, and the exponent n was calculated through the slope of the straight line. Where the drug transport mechanism from spherical matrices is by

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Fickian diffusion (case I transport) when $n = 0.43$, if $0.43 < n < 0.85$, it indicated anomalous (non-Fickian) transport and, for values of $n = 0.85$, case II or zero-order release kinetics was indicated. Occasionally, values of $n > 0.85$ for release from spheres have been observed, which has been regarded as super case II kinetics.

Effect of Coating with HA. The nanoparticles were re-evaluated for their morphology, diameter and zeta potential after coating with HA. The release profile of gatifloxacin and prednisolone was tested after coating with HA. The similarity factor f_2 was calculated from the mean release data before and after coating.[23]

Determination of Gatifloxacin Levels in Cornea and Aqueous Humor. Gatifloxacin was measured in the aqueous humor and in the corneal tissue applying the HPLC method previously described by Al-Dgither et al.,[24] and its behavior was used as a marker for the bioavailability of the developed nanoparticle suspension. Separation was achieved on an X Terra MS C_{18} (3 mm × 50 mm, 5 μm) column. The mobile phase, 0.025 M disodium hydrogen phosphate (pH 3.0) and acetonitrile (80:20 v/v), was delivered at a flow rate of 1.0 mL/min. The eluent was monitored using spectrophotometric detection at 293 nm.

Eighteen New Zealand albino rabbits were used where each rabbit received the selected nanoparticle formula in one eye and the commercial Tymer eye drops as a control in the other eye (two drops). At time intervals of 0.5, 1, 2, 3, 4, and 6 h, samples of aqueous humor (three rabbits each) were removed from the anterior chamber of each eye using a 26-gauge needle attached to 1 mL tuberculin syringe immediately after local anesthetic instillation (Benox eye drops). Then, the rabbits were sacrificed. The ocular surface was irrigated with isotonic phosphate buffered saline and dried with soft tissue. The cornea from each eye was separated and collected in preweighed tubes. For each cornea, 0.5 mL of phosphate buffered saline was added, then vortexed (Jank Kunkel, IKA, VF_2, Germany) for 5 min and the homogenate was kept at 4 °C for 6 h to ensure maximum diffusion of the drug. The animal experiments adhere to the recommendations of the European Community guidelines for the use of experimental animals.

The degree of drug penetration is expressed as the maximum ocular tissue concentration measured in μg/mL aqueous humor or μg/g corneal tissues. However, it should be noted that the volume of the aqueous humor is less than 1 mL (about 0.2 mL) and the weight of cornea is less than 1 g (ranging from 75−115 mg) so that values expressed in the units given represent data extrapolated from small quantities.[25]

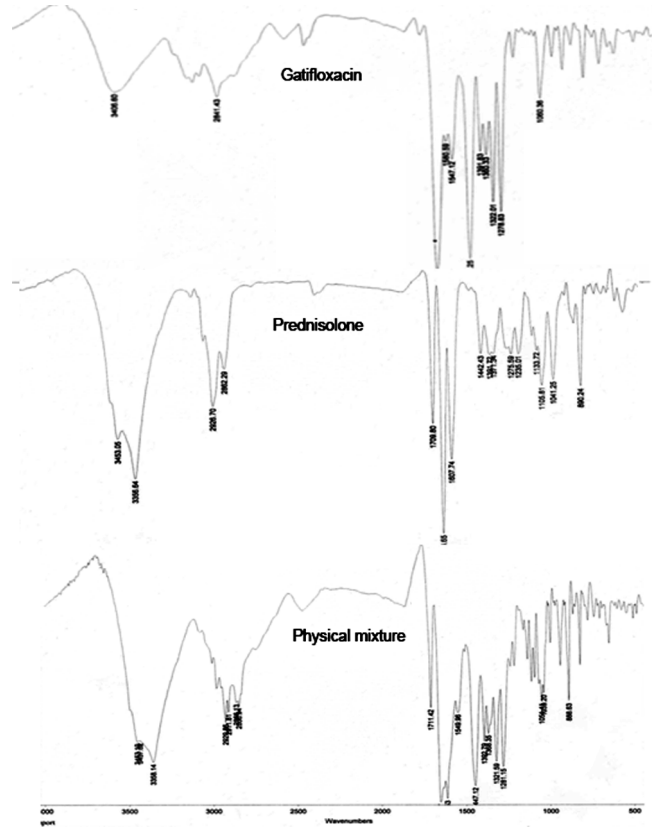

Figure 1. FT-IR spectra of gatifloxacin, prednisolone and their physical mixture.

The pharmacokinetic parameters including the maximum drug concentration (C_{max}) and the time of maximum drug concentration (t_{max}) were estimated from concentration versus time profiles. The area under the concentration−time curve (AUC_{0-6}) was estimated by the trapezoidal method.

Paired t test (at $p < 0.01$) was used to evaluate the statistical difference between the two treatments for the extent of drug absorption in both ocular tissues (AUC_{0-6}).

Results

Compatibility Study. FT-IR was used to evaluate the strength of interaction between gatifloxacin and prednisolone (Figure 1). The FTIR spectrum of gatifloxacin is characterized by absorption bands of −OH and −NH group stretching vibrations at 3401.3 cm^{-1} and C═O group stretching vibrations at1723 and 1637.1 cm^{-1}. The FT-IR spectrum of prednisolone showed absorption bands of the two O−H group stretching vibrations at 3453.05 and 3356.64 cm^{-1} and the C═O group stretching vibrations at 1709.80, 1653.65, and 1603.34 cm^{-1}. These bands were also observed for the physical mixture of the two drugs with the same absorbance. These results confirm that there is no interaction between gatifloxacin and prednisolone in the physical mixture.

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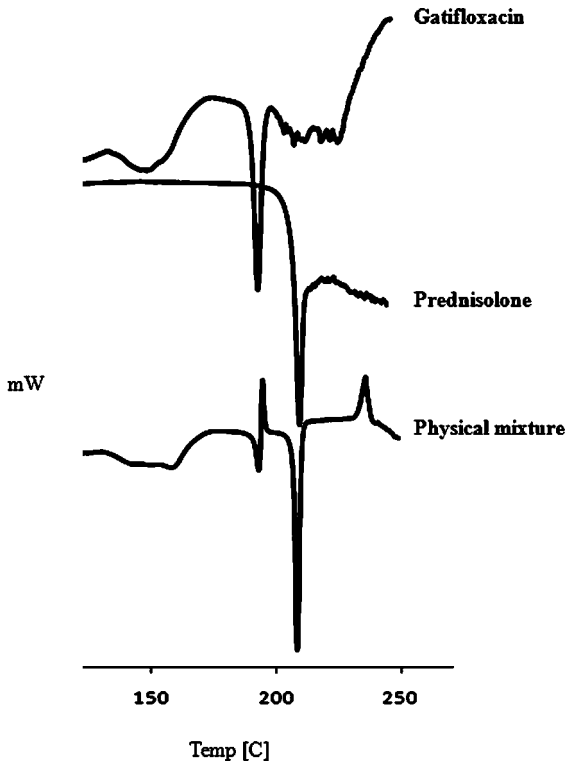

Figure 2. DSC thermograms of gatifloxacin, prednisolone and their physical mixture.

Thermal behavior of the pure drugs and their physical mixture is depicted in Figure 2. The DSC thermograms of gatifloxacin and prednisolone showed sharp endothermic peaks at 192.66 and 219.40 °C, respectively corresponding to their melting and indicating their crystalline nature. The physical mixture also showed these two persistent melting peaks indicating the absence of physical interactions between the two drugs and their existing in a crystalline form.

Method of Determination of Drugs in Mixture. Applying of direct UV spectral analysis for gatifloxacin and prednisolone in artificial tear fluid (Figure 3a) showed overlap between the two curves that prevented direct measurement of the two drugs in the mixture. However, the second derivative spectroscopy showed two separate peaks at 246.8 and 231.6 nm for prednisolone and gatifloxacin, respectively (Figure 3b). Thus, second derivative spectroscopy was adopted for determination of the combined drugs in the concentration range of 2−20 ug/mL. The coefficient of determination (R^2) values for the calibration curves were 0.996 and 0.994 for gatifloxacin and prednisolone, respectively.

Preparation and Evaluation of Nanoparticle Suspension. The TEM showed the nanoparticles as distinct spherical particles with solid dense structure, Figure 4a.

The mean sizes of the prepared formulas ranged from 315.2 to 973.65 nm (Tables 1 and 2). Increasing the polymer concentration and thus the viscosity of the inner drug/polymer alcoholic solution during preparation led to a shift toward higher particle sizes, Table 1. The nanoparticle diameter values were 404, 554, 714 and, 973 nm at drug:polymer (D:P) ratios of 1:1, 1:5, 1:10 and 1:20, respectively. An increase in viscosity of the organic phase decreases the distribution efficiency of the polymer−solvent phase into the external phase leading to formation of larger nanoparticles.[26]

Higher Eudragit RL ratios produced nanoparticles of smaller particle sizes, Table 2.

The polydispersibility index of all the prepared formulas was low (Tables 1 and 2), showing that this method of preparation results in nanoparticles highly uniform in size.

Table 2 shows the effect of Eudragit RS/RL ratio on the surface charge of the nanoparticles. All formulas were positively charged with zeta potential values in the range 30−45 mV. Nanoparticles prepared with higher amounts of Eudragit RL acquired significantly higher zeta potential values due to the availability of more quaternary ammonium groups.

Drug Loading Study. Tables 1 and 2 show high encapsulation efficiency percentages for both drugs (70.18−86.36% for gatifloxacin and 61.11−79.66% for prednisolone). This could be attributed to the low water solubility of both drugs; they were preferentially partitioned into the polymer phase rather than escaping to the aqueous phase.[27] Increasing the D:P ratio significantly lowered the encapsulation efficiency for gatifloxacin and prednisolone ($p < 0.05$), Table 1. The polymer composition showed no obvious effect on the % encapsulation efficiency of the two drugs (Table 2).

Release Study. Figure 5 shows that formulating gatifloxacin and prednisolone as Eudragit nanoparticle suspensions significantly prolonged their release in relation to the free drugs ($p < 0.05$). No burst effect has been observed, indicating that the drugs were homogeneously dispersed in the Eudragit matrix and that no significant amount of drug was adsorbed onto the nanoparticle surface.

Increasing the D:P ratio from 1:1 to 1:5 significantly retarded gatifloxacin release from the nanoparticle suspension and it was clearly reflected on the release efficiency (RE_{24h}) values (Table 1). However, no remarkable retarding effect was noticed on further increasing of the D:P ratio to 1:10 and 1:20. Such behavior may be explained by the interaction of the entrapped gatifloxacin with Eudragit by means of electrostatic bindings between the carboxyl moiety of the first and the quaternary ammonium groups of the later.[12] No more gatifloxacin was available to react with higher percentages of Eudragit at D:P ratio of 1:10 and 1:20. Analysis of variance and further discrimination between the RE_{24h} mean values by the 5% allowance test showed that, for gatifloxacin, the 1:1 drug:polymer ratio was significantly different from the three higher ratios; the critical value above which means are considered significantly different was 3.830.

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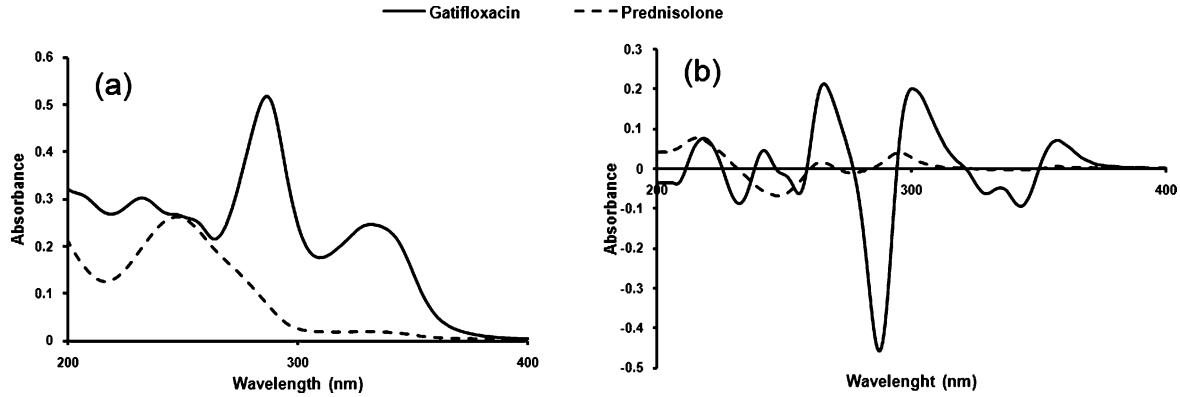

Figure 3. Direct (a) and second derivative (b) UV spectra of gatifloxacin/prednisolone mixture in artificial tear fluid.

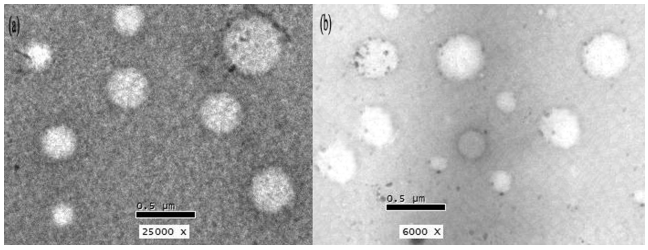

Figure 4. TEM photographs of (a) uncoated nanoparticles at magnification power $x = 25000$ and HA coated nanoparticles at magnification power $x = 6000$ (b).

On the other hand, the release profiles of prednisolone showed a gradual decrease in the release efficiency (RE_{24h}) values on increasing the D:P ratio (43, 30, 29 and 26% for 1:1, 1:5, 1:10 and 1:20, respectively). This proportional retarding effect could be explained by the gradual increase in organic phase viscosity.[28]

Statistical analysis showed that the D:P ratio of 1:1 was significantly different from 1:5 and 1:10 ratios, which were similar but significantly different from 1:20. The critical value was 2.812.

It is evidenced in Figure 6 that increasing Eudragit RL percentage enhanced the release of gatifloxacin from the nanoparticles. This is because of the greater water permeability of the Eudragit RL due to its higher quaternary ammonium group content. Eudragit RL contains an amount of quaternary ammonium groups ranging between 8.8% and 12% in relation to 4.5− 6.8% for Eudragit RS.[29] Statistical analysis of the gatifloxacin release data showed that its release profile from the formulas containing high Eudragit RL amounts (RS/RL ratios of 10/90 and 30/70) showed statistically similar RE_{24h} mean values. Both values were significantly different from that of 50/50 RS/RL ratio, which was significantly different from those of the formulas prepared applying high RS/RL ratios (70/30 and 90/10). On the other hand, changing polymer composition (RS/RL ratio) did not significantly affect the release of prednisolone from Eudragit nanoparticles, Figure 6. Such behavior could be attributed to the low solubility properties of the drug which predominate over the polymer matrix permeability as a limiting factor.

Release Kinetics and Mechanism. Gatifloxacin release from nanoparticle suspension prepared at a D:P ratio of 1:1 showed better fit with zero order ($R^2 = 0.9962$) and Hixon−Crowel ($R^2 = 0.9827$) plots than with Higuchi diffusion plot ($R^2 = 0.9375$). Fitting the release data up to 60% release to the Krosmeyer−Peppas equation showed anomalous release ($n = 0.4943$), indicating both diffusion and polymer erosion mechanisms. The erosion probably resulted from the low proportion of the polymer in this formula. Contrarily, formulas prepared at higher D:P ratios (1:5, 1:10 and 1:20), exhibited a Fickian or diffusion mechanism of drug release ($n < 0.45$) and the release data showed higher R^2 values on fitting them into Higuchi diffusion plot. These results may be due to the diffusion of the release medium into the nanoparticles, thereby solubilizing the drug and releasing it slowly. Changing the polymer composition (RS/RL ratio) did not alter the release kinetics or mechanism ($0.5136 > n > 0.7328$).

Effect of Coating with HA. The coated nanoparticles appeared spherical and exhibited a broader size distribution, Figure 4b. The particle size of the coated formulas was larger for both coating strategies, Table 3. The higher increase in particle size on coating by adding HA during preparation is probably due to its effect on the viscosity of the external aqueous phase. Similar results were reported by Barbault-Foucher et al.[17] for poly-ε-caprolactone nanospheres coated with HA.

Simultaneously, the PI for the coated formulas reached higher values than the uncoated one indicating that aggregation took place. Probably, these aggregates resulted from interactions between free HA and free Eudragit in the suspension medium.

Zeta potential drastically changed after HA coating: initially positive due to the cationic Eudragits, then became

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Table 1. Evaluation Parameters for Gatifloxacin/Prednisolone Nanoparticle Suspensions Prepared at a 50/50 Eudragit RS/RL Ratio and Different D:P Ratios

D:P ratio	particle size (nm) (mean ± SD), $n = 200$	PI[a]	encapsulation efficiency (%) (mean ± SD), $n = 3$		RE_{24h}[b] (mean ± SD), $n = 3$	
			gatifloxacin	prednisolone	gatifloxacin	prednisolone
free drug					92.041 ± 0.3	56.323 ± 0.1
1:1	404.15 ± 20.3	0.050	82.689 ± 0.3	74.208 ± 0.2	77.131 ± 0.3	43.104 ± 0.2
1:5	554.15 ± 24.4	0.044	82.822 ± 0.2	73.731 ± 0.5	42.275 ± 0.2	30.285 ± 0.3
1:10	714.45 ± 28.9	0.040	81.085 ± 0.4	67.859 ± 0.4	43.257 ± 0.5	29.493 ± 0.2
1:20	973.65 ± 26.2	0.026	70.179 ± 0.2	61.114 ± 0.3	39.836 ± 0.3	26.416 ± 0.1

[a] PI = the polydispersibility index obtained as PI = standard deviation/mean particle size in nm. [b] RE_{24h} = release efficiency after 24 h calculated as $RE = \int_0^t (y\,dt)/(y_{100}t) \times 100$.

Table 2. Evaluation Parameters for Gatifloxacin/Prednisolone Nanoparticle Suspensions Prepared at a D:P Ratio of 1:1 and Different Polymer Compositions

Eudragit RS/RL ratio	particle size (nm) (mean ± SD), $n = 200$	PI[a]	mean zeta potential (mV), $n = 3$	encapsulation efficiency (%) (mean ± SD), $n = 3$		RE_{24h}[b] (mean ± SD), $n = 3$	
				gatifloxacin	prednisolone	gatifloxacin	prednisolone
10/90	315.2 ± 27.9	0.088	45.1 ± 0.2	85.101 ± 0.5	77.680 ± 0.4	85.623 ± 0.6	42.467 ± 0.4
30/70	383.45 ± 17.3	0.045	42.0 ± 0.4	84.812 ± 0.6	77.184 ± 0.6	83.859 ± 0.4	44.152 ± 0.3
50/50	404.15 ± 20.3	0.050	35.5 ± 0.5	82.689 ± 0.3	74.208 ± 0.2	77.131 ± 0.3	43.104 ± 0.2
70/30	453.5 ± 27.0	0.059	32.4 ± 0.4	84.039 ± 0.2	75.994 ± 0.3	74.458 ± 0.4	39.890 ± 0.6
90/10	495.4 ± 29.3	0.059	30.4 ± 0.5	86.356 ± 0.5	79.664 ± 0.5	72.563 ± 0.5	41.511 ± 0.4

[a] PI = the polydispersibility index obtained as PI = standard deviation/mean particle size in nm. [b] RE_{24h} = release efficiency after 24 h.

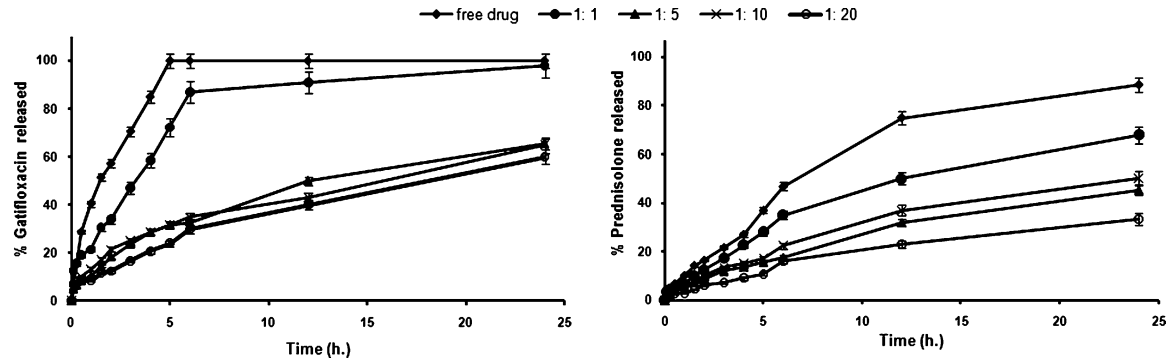

Figure 5. Effect of D:P ratio on the *in vitro* release of gatifloxacin and prednisolone from nanoparticle suspensions prepared at 50/50 Eudragit RS/RL ratio in relation to the free drugs, error bars indicate SD, $n = 3$.

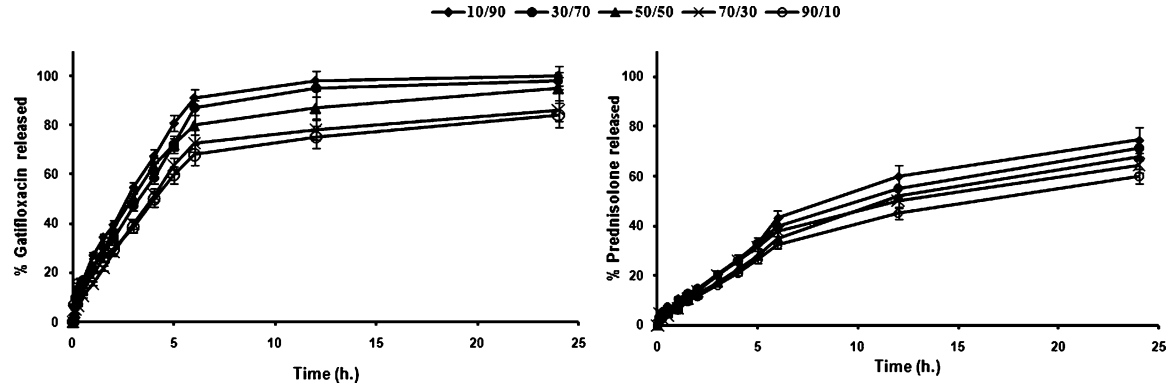

Figure 6. Effect of polymer compositions (RS/RL ratio) on the *in vitro* release of gatifloxacin and prednisolone from nanoparticle suspensions prepared at D:P ratio of 1:1, error bars indicate SD, $n = 3$.

negative proving the adsorption of the negatively charged HA on the nanoparticle surface by electrostatic interaction, Table 3.

The high values of the similarity factor (f_2) prove that coating with HA does not significantly affect the release profile of gatifloxacin and prednisolone from the nanoparticle suspension.

Table 3. Evaluation Parameters for HA Coated Eudragit Nanoparticles Prepared at a D:P ratio of 1:1 and a 10/90 Eudragit RS/RL Ratio in Relation to Uncoated Formula

nanoparticle formula	particle size (nm) (mean ± SD), $n = 200$	PI[a]	mean zeta potential (mv) $n = 3$	f_2[b]	
				gatifloxacin	prednisolone
uncoated	404.15 ± 20.3	0.050	+45.1 ± 0.2		
coated during particle formation	678.00 ± 38.4	0.198	−47.0 ± 0.3	91.2	90.3
coated by adsorption on performed particles	460.30 ± 46.2	0.137	−45.2 ± 0.2	93.3	94.1

[a] PI = the polydispersibility index obtained as PI = standard deviation/mean particle size in nm. [b] f_2 = similarity factor calculated as $f_2 = 50 \log\{[1 + (1/n)\sum_{n=1}^{n} W_t (R_t - T_t)^2]^{-0.5} \times 100\}$.

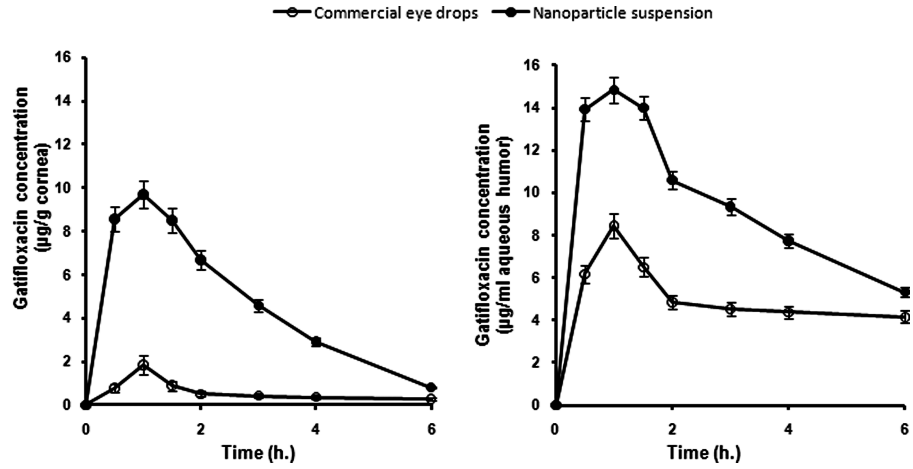

Figure 7. Mean ocular concentration−time profiles of gatifloxacin, error bars indicate SD, $n = 3$.

Table 4. Pharmacokinetic Parameters for Gatifloxacin (mean ± SD, $n = 3$) after Topical Instillation of Nanoparticle Suspension Prepared at a D:P Ratio of 1:1 and a Eudragit RS/RL Ratio of 10/90 and Coated by 0.025% HA Using Adsorption Technique and Commercial Eye Drops in Healthy Rabbits

treatment	corneal tissue			aqueous humor		
	C_{max} (µg/g)	T_{max} (h)	AUC_{0-6} (µg·h/g)	C_{max} (µg/mL)	T_{max} (h)	AUC_{0-6} (µg·h/mL)
commercial eye drops[a]	1.85 ± 0.35	1	3.55 ± 0.75	8.45 ± 0.54	1	30.88 ± 1.51
nanoparticle suspension	9.69 ± 0.87	1	28.02 ± 1.23	14.85 ± 0.93	1	54.75 ± 1.78
statistical test (p)			0.12			0.05

[a] Tymer eye drops (gatifloxacin 0.3%).

Determination of Gatifloxacin Levels in Cornea and Aqueous Humor. Figure 7 shows gatifloxacin levels in the corneal tissue and aqueous humor after the topical instillation of the selected nanoparticles and the commercial gatifloxacin eye drops. It is clear that both treatments reached their maximum corneal concentration (C_{max}) after 1 h, but this concentration was 5.23-fold higher in case of the nanoparticle suspension, Table 4. In addition, the effect of the commercial eye drops almost ended after 2 h, while that of the HA coated nanoparticles lasted for more than 6 h. Similarly, the nanoparticles showed a 1.76-fold increase in the C_{max} of gatifloxacin in the aqueous humor in comparison to the eye drops, Figure 7.

Statistical analysis proved the presence of significant difference between the mean (AUC_{0-6}) values for both treatments at $p < 0.01$.

Discussion

The objective of combining gatifloxacin and prednisolone was to gain the benefits of both antibiotics and anti-inflammatory drugs to treat a serious ocular disease. The nanorange obtained is preferable for ocular application to prevent patient discomfort and provide a good drug diffusional release.[30,31] Eudragits were selected due to their reported advantages such as good stability, reproducible release rates of the active drug and ocular tolerability.[12] The main advantage of the used spontaneous emulsification technique is the avoidance of toxic organic solvents, commonly used in micro- and nanoparticle solvent evaporation techniques, which increases the potential ophthalmic application of the system. This method also produced nanoparticles highly uniform in size. Benzalkonium chloride and disodium edetate of 0.01% w/v concentration each were added as preservatives to the prepared formulas. Rathore and Majumdar[16] proved that a 1:1 benzalkonium chloride to

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disodium edetate mixture showed the maximum permeation enhancing effect on *in vitro* transcorneal permeation of gatifloxacin from aqueous drops.

Two variables were studied each at a time. First, four D:P ratios (1:1, 1:5, 1:10 and 1:20) were tested at a fixed polymer composition of 50/50 Eudragit RS/Eudragit RL. Changing D:P significantly affected the particle size and encapsulation efficiency, as well as the *in vitro* release profile and mechanism. Higher polymer amounts resulted in higher particle sizes as previously stated for gliclazide[32] and terbutaline[33] loaded Eudragit nanoparticles. Increasing the D:P ratio lowered encapsulation efficiencies for gatifloxacin and prednisolone. This is in contrast to Dillen et al.[34] and Pignatello et al.,[35] who reported that the D:P ratio did not affect the drug loading encapsulation efficiencies.

Increasing the D:P significantly retarded the release of gatifloxacin and prednisolone. In accordance with our results, it was reported that increasing the D:P ratio reduced the ibuprofen release rate from Eudragit RS 100 nanoparticles.[36] On the other hand, our results were in contrast to Pignatello et al.,[35] who stated that changing the D:P ratio from 1:1 to 1:2 did not significantly affect the dissolution rate of cloricromene from Eudragit nanoparticles.

The release data of gatifloxacin was presented as a model for the release kinetics analysis from the prepared nanoparticles. Increasing the D:P ratio from 1:1 to 1:5 changed the mechanism of gatifloxacin release from anomalous release to Fickian diffusion, while no further change was recorded for the higher D:P ratios (1:10 and 1:20). Contrarily to that, no significant effect was reported for the D:P ratio on the release mechanism of flurbiprofen[37] and indomethacin[38] from Eudragit particles within the conditions used in each study.

Eudragit RS/RL ratio has no significant effect on gatifloxacin release mechanism of the prepared nanoparticles. Similarly, Dillen et al.[34] and Kristmundsdottir et al.[39] proved that changing the Eudragit composition (RS:RL ratio) and even using each Eudragit type as a single polymer did not change the release kinetics and mechanism.

The literature stated different release mechanisms for Eudragit micro- and nanoparticles. The Higuchi's square root equation showed the best fit for piroxicam[40] and acetazolamide[41] release from Eudragit nanoparticles. On the other hand, the flurbiprofen release resulted from coexisting dissolutive and diffusion phenomena.[36] Malamataris and Avgerinos[37] stated that the indomethacin release from Eudragit RS/RL microspheres was described on the basis of two biexponential, first-order models. Zero-order release kinetics provided the best correlation for acetazolamide Eudragit microspheres prepared using solvent evaporation technique.[28]

Based on the above evaluations, the nanoparticle suspension prepared at 1:1 drug to polymer ratio showed the lowest mean particle size, the highest drug content and acceptable release profile with zero order kinetics. This ratio was selected for further optimization by applying five RS/RL ratios (10/90, 30/70, 50/50, 70/30 and 90/10). The obtained formulas were evaluated as above in addition to their Zeta potential determination.

Evaluating the prepared nanoparticles showed that the smallest particles were obtained with the highest Eudragit RL ratio. Similar results were reported by Pignatello et al.[12] On the other hand, it was reported that the mean particle sizes of ciprofloxacin-loaded Eudragit nanoparticles[34] and acetazolamide loaded Eudragit microspheres[41] were not affected by the Eudragit type. The positive charge increased on increasing the Eudragit RL ratio.

Neither drug encapsulation efficiencies nor release kinetics was affected by changing the polymer composition. High Eudragit RS 100 ratios significantly retarded gatifloxacin release from the nanoparticles, while no significant effect for this factor was recorded for prednisolone release. Similarly, different results were reported according to the drug nature. The most retardant effect was obtained by using Eudragit RS for furosemide loaded microspheres prepared by spherical crystallization,[42] ibuprofen microspheres[43] and flurbiprofen nanosuspensions.[37] No significant difference

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between ciprofloxacin release rate constants of Eudragit RS or RL containing nanoparticles was observed.[34]

Gatifloxacin/prednisolone loaded nanoparticles prepared at Eudragit RS/RL ratio of 10/90 produced the smallest particle size (315 nm) and the highest zeta potential (+45 mv). This high positive charge developed on the nanoparticles was important to bind to the bioadhesive negatively charged HA and thus this formula was selected to be coated with HA and was re-evaluated.

The coated particles remained in the nanorange and acquired negative charge due to the HA adsorption. Similarly, a drastic change in surface charge was reported for PLGA nanoparticles coated with the chitosan.[44] Results showed that no significant changes were recorded in gatifloxacin encapsulation efficiency or release profile after coating with both strategies.

From the above results, the nanoparticles prepared at a D:P ratio of 1:1 and Eudragit RS/RL ratio of 10/90 and coated by 0.025% HA using adsorption technique were selected to be tested *in vivo*. They showed the smallest particle size and PI. This nanoparticle formula showed significant increase and prolongation in gatifloxacin concentration in the cornea and aqueous humor in comparison with commercial eye drops. The adhesion of nanoparticles to the eye surface prolonged the residence time of the drug in the conjunctival sac and improved its penetration across the cornea. Moreover, the small particle size of the prepared nanoparticles resulted in a good binding capacity.

Conclusion

Gatifloxacin and prednisolone were successfully formulated as a single medication for the treatment of bacterial keratitis. The prepared nanoparticle suspension could improve patient compliance due to its easy instillation in the eye and its prolonged action. Eudragit nanoparticles were easily coated with HA resulting in improved ocular bioavailability in comparison to the commercial eye drops. The particle size, the surface charge and the *in vitro* release profile can be tailored by changing the drug:polymer ratio and the polymer composition.

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