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Original article

Poly (D, L-lactide-co-glycolide) nanoparticles for sustained release of tacrolimus in rabbit eyes

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ABSTRACT

Tacrolimus (TAC)-loaded Ploy-lactide-co-glycolide nanoparticles (PLGA-NPs) was developed by emulsification-diffusion method for topical ocular delivery in certain ocular conditions where therapeutic level of immunomodulator into eyes is required for sufficient duration. So, we optimized TAC-loaded PLGA-NPs with higher TAC payload. The mean particle-size and its distribution, polydispersity, zeta-potentials, morphology, drug encapsulation and loading capacity of NPs were analyzed. Transcorneal permeation through excised rabbit cornea revealed instant and controlled permeation of TAC from TAC-aqueous suspension (TAC-AqS) and from PLGA-NPs, respectively. Stability study results indicated that there were no significant changes in above characteristics for 1-month storage at 25 °C. The safety of PLAG was established by modified Draize's test after its topical administration in rabbit eyes. The adopted liquid chromatography-electrospray ionization tandem mass spectrometry was successfully applied for TAC quantification in ocular tissues and aqueous-humor. PLGA-NPs improved corneal, conjunctival and aqueous humor bioavailability of TAC. A considerably higher TAC-concentration from F2 was found in ocular tissues even at 24 h and in aqueous humor till 24 h following its topical ocular administration as compared to TAC-AqS. The PLGA-NPs significantly enhanced ocular bioavailability of TAC than that of aqueous suspension.

1. Introduction

Topical applications of conventional formulations in the eyes are limited by low and poor ocular availability because of rapid tear-turnover and impermeability of drugs to the cornea due to its defensive mechanism [1]. Cornea is consisting of transparent connective tissues known as stroma, which is protected by epithelia on both the sides. The inner endothelium is a monolayer that outlines the anterior chamber while the outer layer of cornea is made of stratified non keratinized squamous epithelia, which protects the stroma from outer environment by luminal junctions and provides a strong physical barrier against any external materials. The physical barrier is also accompanied by a physicochemical barrier (mucin layer) that defends the entry of any drug or antigen. Moreover, special effects of mechanical washing by tear fluid and wiping of eye lids collectively enhance protective action of the proteins [2]. As a consequence, free drugs in solution form is rapidly eliminated from the ocular surface after instillation in to eyes, thus only 2–5% of the applied dose actually available for the intraocular tissues after corneal and conjunctival permeation and hardly offers high drug availability [3–5]. So, frequent administration is required to get the therapeutic effects [6], which may cause adverse drug reactions and affect therapeutic and patient compliance. Hence, there is a need of an ideal ocular drug delivery system that would offer a sustained and controlled release of drugs in the eyes. Ploy (D,L-lactide-co-glycolide, 50:50 D, L-lactide:glycolide) nanoparticles (PLGA-NPs) are encouraging ophthalmic delivery systems that are used as sustained and controlled delivery of numerous drugs for ocular disorders [7]. PLGA-NPs are of suitable size for ocular use [8]. Being a biodegradable, biocompatible polymer and its non-toxic degradation byproducts; PLGA has been approved by FDA for ocular use and have strong potential for ocular drug carriers as it resulted very low eye irritation [8,9]. Polyvinyl alcohol (PVA) and Pluronic-F68 were chosen as stabilizer for the PLGA-NPs development, because PVA [10,11] and Pluronic F-68 [12,13] are the surfactants those are free from cytotoxicity.

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The immunosuppressants as topical ocular application help in treating the ocular autoimmune diseases, to manage corneal graft rejection, uveitis, ocular pemphigoid, allergic conjunctivitis, dry-eye conditions, keratitis, atopic and vernal keratoconjunctivitis [14–20] and other ocular inflammatory conditions. PLGA-NPs would provide a sustained ocular delivery of tacrolimus.

Tacrolimus (TAC) is a potent macrolide lactone immunosuppressive agent [21] which was first derived from *Streptomyces tsukubaensis*. TAC and cyclosporine-A (CsA) are the most common topical immunomodulators having similar mechanism of actions, but TAC is around 10–100 times more potent than CsA [22]. TAC also subdues the immune responses by preventing the release of many other inflammatory cytokines (like, IL-3, IL-4, IL-5, IL-8, Gamma-interferon and tumor necrosis factor-α) [23]. TAC has effectively been used as therapeutic agent in various immune mediated conditions or diseases. TAC was also found to be effective in treating eye conditions such as, delay the incidence of corneal allograft rejection and prolong the allograft survival period [24], ocular inflammation, ocular pemphigoid [25] and uveitis [15].

Topical use of 0.03% TAC eye drops was found effective in severe allergic conjunctivitis and successfully improved ocular surface status and tear stability in dry eye conditions [14]. Similarly, topical dexamethasone and TAC treatments were found effective in mouse allergic conjunctivitis model to suppress the infiltration of eosinophil and lymphocyte into subconjunctival tissues in mouse [26]. Due to complications associated with corticosteroids, other alternatives are sought for similar therapeutic applications. Latest clinical trials revealed that TAC has equivalent effect as corticosteroids in ocular allergic crisis control and maintenance therapy with very low complications and adverse effects [27–29].

TAC was found to inhibit the histamine release and its action in addition to the inhibition of CD4-lymphocytes activation. It also inhibited prostaglandin synthesis in mast cells and basophils [15,30]. Due to its potential to reduce activated T-cells, the TAC efficacy has been investigated for topical ocular use to prevent the corneal graft rejection and hence topical ointment of 0.03% TAC has been evaluated as a second-line treatment in high-risk corneal grafts patients [31].

In the present investigation TAC-loaded PLGA-NPs was formulated by emulsification-diffusion method to determine the ocular bioavailability of TAC after topical administration in rabbit eyes. For comparison 0.03% TAC aqueous suspension (TAC-AqS) was also incorporated in the investigation.

2. Materials and methods

2.1. Materials

"Tacrolimus, cyclosporine-A, ploy (D,L-lactide-co-glycolide; lactide: glycolide (50:50)) with molecular weight 30,000–60,000 and Pluronic-F68 (Poloxamer-188) were purchased from Sigma Aldrich Co. (St. Louis MO, USA)". Polyvinyl alcohol (PVA, Mw 17,200), ethyl acetate and acetone were purchased from "AVONCHEM Ltd. (Wellington House, Waterloo St. West, Macclesfield, Cheshire, UK)". Chloroform and dichloromethane (DCM) were purchased from MERK (Darmstadt, F.R. Germany) and PANREAC QUIMICA SA, (Barcelona, Spain), respectively. Tween-80, acetic acid glacial and HPLC grade methanol were obtained from "BDH Ltd. (Poole, England)". Phosphate buffer saline was obtained from GIBCO, Life Technologies Ltd. (Paisley, Scotland). Ammonium acetate was purchased from Fluka Chemika, (Switzerland). "Acetonitrile (HiPerSolv Chromanorm, HPLC-grade) were purchased from BDH, PROLABO®, LEUVEN, EC. Milli-Q® water purifier (Millipore, France) was used for purified water and all other used chemicals were analytical grade and solvents were HPLC grade".

2.2. Methods

2.2.1. Development of TAC- loaded PLGA-NPs

PLGA-NPs were formulated by emulsification-diffusion method [32]. Briefly, 100 mg PLGA and 15 mg TAC was solubilized in 2.5 mL DCM or chloroform, the obtained organic phase was added drop by to 7.5 mL aqueous phase containing PVA in deionized water as stabilizer with and without Pluronic F-68 and Tween-80, then emulsified using homogenizer (21,500 rpm, 10 min) to get primary emulsion. Further addition of large volume of aqueous phase (3-times of primary emulsion) to the emulsion under continuous magnetic stirring (1000 rpm) allowed the organic phase to leave the droplets. Thereafter, the excess organic solvent was removed through evaporation during magnetic stirring for 3 h to give NPs-suspension. The suspended NPs were then isolated and purified by washing with Milli-Q® water using ultracentrifugation (30,000 rpm, 30 min). The obtained NPs were then lyophilized for further studies. To prepare the TAC-AqS, TAC was suspended in sterile water (particle size, 600 nm). The sterile water was containing PVA (1%, w/v), phenyl mercuric nitrate (0.003%, w/v) as preservative and NaCl (0.9%, w/v) [33].

2.2.2. Particle size, polydispersity, zeta-potentials and morphology of PLGA-NPs

The mean particle size, size distribution and polydispersity index of the purified PLGA-NPs were performed by the dynamic light scattering (DLS) using Zetasizer Nano-Series (Nano- ZS90, Malvern Instruments, England) at 25 °C at 90° scattering angle for optimum detection. The zeta-potential of the NPs was determined using the same instrument using software DTS-Version 4.1 (Malvern, England). The surface morphology of PLGA-NPs was observed by scanning electron microscope (SEM). The NP samples were coated with gold using an Ion Sputter at 20 mA for 6 min. Observation was done at 10–20 kV accelerating voltage, 8.5 mm working distance and at 2.48 KX magnification power. For drug encapsulation and loading around 10.5 mg of TAC loaded NPs was dissolved in chloroform and acetone (1:1, v/v), the solvents were evaporated. The residue so obtained was dissolved in methanol with sonication and magnetic stirring. The solution was centrifuged (15 min at 13,500 rpm), supernatant was obtained and free TAC (indirect method) was analyzed by HPLC. The HPLC system (Waters® 1500 series controller, USA) was equipped with UV-detector (Waters® 2489, dual absorbance detector, USA), pump (Waters® 1525, Binary pump, USA), an automated sampling system (Waters® 2707 plus autosampler, USA) and the system was monitored by "Breeze (Waters®)" software. TAC was analyzed by injecting 30 μL of the supernatant to a C_{18} column (Macherey-Nagel, 4.6 × 150 mm, 10 μm particle size). The mobile phase was consisted of 75:25 (v/v) of acetonitrile and MilliQ water and the pH of water was adjusted to 3 by orthophosphoric acid. The detection wavelength was set 215 nm, the flow rate of the mobile phase was 1 mLmin^{-1} and column temperature was set to 60 °C [34,35]. The drug concentration was calculated by using straight line equation: $y = 16.784\,x + 13529$; $R^2 = 0.9993$. The encapsulation (%EE) and drug loading (%DL) was calculated with the following equations (Eqs. (1) and (2)):

$$\%\,E.E. = \left(\frac{Initial\,drug\,amount\,(mg) - Freedrug\,(mg)}{Initialamountofdrug\,(mg)}\right) \times 100 \quad (1)$$

$$\%D.L. = \left(\frac{Initial\,drug\,amount\,(mg) - Freedrug\,(mg)}{Amountofnanoparticles\,(mg)}\right) \times 100 \quad (2)$$

2.2.3. Freeze-drying and stability of TAC-loaded PLGA-NPs

Around 1 mL of the developed PLGA-TAC-NPs suspensions were filtered through Millipore® syringe filters (0.45 μ) and freeze-dried by using a FreeZone-4.5 Freeze Dry System (Labconco Corporation, Kansas City, MO, USA). The process was performed at −40 °C for 24 h and at 0.05 mm Hg of pressure. The stability study of TAC loaded PLGA-NPs was conducted as reported elsewhere [36]. 10 mg of freeze-dried sample was put in tightly capped glass vials and stored for 30 days at 25 ± 1 °C. The variations in the particle-size, zeta-potential, encapsulation and release of drug were periodically monitored to assess the PLGA-NPs stability and the concentration of TAC was analyzed by HPLC [34,35].

2.3. In vivo *animal study*

Male Albino rabbits weighing 2.5–3.5 kg were obtained from "College of Pharmacy, Animal care and use center, King Saud University, Riyadh, Saudi Arabia, for the *in vivo* studies". All the animals were kept and housed as per the recommendations of the "Guide for the Care and Use of Laboratory Animals" permitted by the center.

2.3.1. Transcorneal permeation study

Rabbit cornea was excised and fitted between donor and receptor compartments of "double jacketed automated transdermal diffusion cells (sampling system-SFDC 6, LOGAN, New Jersey, USA)" where the corneal epithelial surface was towards the donor compartment. Simulated tear fluid (pH 7.4) was filled in to receptor compartment and water (at 37 ± 1 °C) was allowed to flow in outer jacket of the diffusion cell with 95% air + 5% CO_2 ventilation. The diffusion cells were placed on different stations of the instrument and the air bubbles from receptor compartment was expelled out through continuous magnetic stirring. 600 μL of each TAC-AqS and F2-nanosuspension having a predetermined TAC dose (0.03%, w/v) was put in the donor compartments of three diffusion cells ($n = 3$) and the set-up was started. Sampling was done from receptor compartment at different time points till 4 h and TAC content was analyzed by HPLC [34,35] Permeation parameters for TAC from AqS and NPs (F2) were deliberated by plotting the TAC permeated (μg cm^{-2}) across the cornea on y-axis *versus* time (h) on x-axis, then slope of the linear portion of the plot was assessed. The flux (J) and permeability coefficients/apparent permeability (P_{app}) in each case were estimated by means of following equations (Eq. (3) and (4)):

$$J\ (\mu g\, cm^{-2}\, s^{-1}) = dQ/dt \quad (3)$$

$$Papp\ (cm\, s^{-1}) = J/C_0 \quad (4)$$

"where 'Q' denotes the amount of TAC crossing cornea or else (dQ/dt) is the linear portion of the slope, 'A' is the area of contact of cornea, 't' is contact time and C_0 is the initial TAC concentration (μg mL^{-1}) inside donor compartment [5].

2.3.2. Ocular-irritation study

Based on physicochemical characteristics and transcorneal permeation parameters, formulation F2 was chosen for ocular irritation and pharmacokinetic studies. Eye-irritation study was done as per the modified Draize's test [5,37] in rabbit eyes, for acute eye-irritation and corrosion examination. About 50 μL of F2-nanosuspension was applied in the lower conjunctival sac of right eyes of rabbits ($n = 6$) while left eyes were served as control and treated with saline (0.9% NaCl solution) [38]. The eyelids were gently held for 5 s to avoid the loss of instilled formulations because of tear dilution. Suspension of PLGA-NPs was administered three times a day for 7 days, and treated eyes were categorized by visual examination of iris, cornea, and conjunctiva according to the arithmetical scoring systems as our previous experiment [5]. Irritation magnitude was assessed by discomfort to the animals, signs, symptoms in conjunctiva, cornea and eyelids [5,37,39]

2.3.3. Tacrolimus biodistribution in rabbit's aqueous humor and ocular tissues

The drug concentration in cornea, conjunctiva and aqueous humor was detected for evaluating the TAC ocular bioavailability from PLGA-NPs (F2, 0.03%, w/v equivalent to 15 μg) and compared with TAC-AqS (0.03%, w/v) [40]. Rabbits were separated in two groups; each group was consisting of three animals. The right eyes of first group of animals were subjected to a single topical instillation of F2 (50 μL) containing 0.03%, w/v (15 μg) TAC and right eyes of second group of animals were treated with 50 μL of TAC-AqS containing same amount of TAC (15 μg). Thirty minutes after dosing, TEKAM (HIKMA Pharmaceuticals, Amman, Jordan) anesthesia comprising Ketamine. HCl was intravenously injected in the marginal ear vein of each rabbit thereafter a 29-gauge insulin syringe-needle system was used to take out 50–60 μL aqueous humor samples at different time points (1, 2, 4, 6, 12 and 24 h). The aspirated samples in 0.5 mL Eppendorf tubes were wrapped and sealed in aluminum foil to avoid any photo degradation of samples and kept at −80 °C till the analysis [5,40–42]. Quantitative analysis of TAC in corneal and conjunctival tissues was done only at 24th h of the experiment because of the constraints in number of animal use, otherwise for each time point we had to sacrifice the animal which was not favorable. At 24th h post instillation rabbits were sacrificed with excess intravenous injection of Ketamine.HCl, eyes were proptosed and rinsed with saline. Cornea and conjunctiva were dissected, washed with saline, dried with blotting paper to remove any adhering TAC and kept in pre-weighed vials. The vials were re-weighed; mass of the collected tissues was calculated (Average weight of corneal and conjunctival tissues was 0.089 and 0.415 g, respectively) and TAC was extracted from the collected tissues.

The ocular tissues were cut, grinded and homogenized for 2 min twice with extraction buffer (10 mM sodium-molybdate, 2 mM DL-dithiothreitol and 0.1 M Tris-hydrochloride, all were in equal volume) [43]. For cornea (0.089 g) 1.75 mL and for conjunctiva (0.415 g) 8.25 mL of extraction buffer was used separately. The mixture was centrifuged (13,000 rpm; 10 min) and the supernatant was collected. The supernatants were aliquoted to 10 mL capacity tubes and 20 μL (50 ng mL^{-1}) of working IS was added. The mixtures were vortexed for 2 min and 2.5 mL of dichloromethane was added in each tube. The samples were again vortexed and centrifuged to separate the organic and aqueous layers. The organic layer was transferred in to glass vials. For TAC analysis, the organic layer was evaporated to dryness at 50 °C under nitrogen stream. The obtained residue was then resuspended in mobile phase (200 μL) and vortexed at the time of analysis. The stored aqueous humor and tissue extracted samples were then analyzed by injecting 10 μL of each through the adopted UPLC–MS method [40].

3. Data analysis

The TAC concentration was estimated from the drug recovery in the collected samples. By using non-compartmental approach, the pharmacokinetic parameters ($t_{1/2}$, t_{max}, C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$) were computed by a software (PK-Solver, Nanjing, China through MS-Excel-2013) [44]. One-way ANOVA was applied to compare the obtained pharmacokinetic parameters, where $p < 0.05$ was assumed as statistically significant.

3.1. UPLC–MS conditions for TAC analysis

The UPLC system (Waters Acquity H—Class) coupled with triple quadruple mass spectrometer (Waters, Milford, USA) was used for TAC aqueous humor sample examination. The elution of drug was done on C_{18} Acquity UPLC BEH^{TM} (Waters, USA) column (1.7 μm, 2.1 × 50 mm). The mobile-phase (20 mM ammonium acetate aqueous solution and methanol at 15:85, v/v ratios) was pumped at 0.20 mL min^{-1} flow rate and the injection volume was 10 μL. Mass spectrometric detection was performed through electrospray ionization (ESI) probe operated in the positive ionization mode and single ion recoding (SIR) for TAC and CsA as internal standard as per the reported methods [40,45,46].

3.2. Calibration and sample preparation

TAC and CsA (IS) standard stock solutions were prepared in acetonitrile (ACN) to get 1000 μg mL^{-1} concentrations. Working standards of TAC was prepared by diluting the stock solution with ACN. Working solution of TAC was prepared in ACN; 20 μL of working solution was mixed with 50 μL blank aqueous humor and 930 μL ACN to get 0.25–1000 ng mL^{-1} spiked calibration standards. Working solution of IS (2500 ng mL^{-1}) was obtained by diluting CsA stock solution with ACN. Aqueous humor samples were thawed and vortexed, and in 50 μL of aqueous humor 20 μL (50 ng mL^{-1}) of IS was added and mixed. Afterwards, 930 μL ACN was further added and vortexed (2 min) for protein precipitation then centrifuged at 13,000 rpm for 10 min 500 μL supernatant was taken out from the centrifuged samples and transferred to HPLC vials, finally 10 μL of each sample was injected for analysis [5,40].

4. Results and discussion

4.1. Formulation of TAC-NPs

The TAC-NPs were formulated by emulsification-solvent diffusion method where, PLGA concentration in the internal organic phase was a significant factor in increasing the NPs-size, the size increases with increasing the PLGA concentration. Thus, 1%, w/v, PLGA concentrations was chosen to formulate the small sized NPs based on the reported study [47]. The PVA as stabilizer at 0.5–1.0%, w/v concentrations with or without Pluronic F-68 (0.5–1.0%, w/v) and Tween-80 (0.1%, w/v) were applied to assess the effect of stabilizer/surfactant concentrations on size of PLGA-NPs and drug encapsulation [48]. The nanoparticles in the size range of 164 to 375 nm were obtained (Table 1). The results of physicochemical characteristics indicated that 1.0% w/v, was the optimal concentrations for PVA: Pluronic F-68 (1:1) whereas PVA: Tween-80 (1: 0.1). Our results indicated that to get the smaller sized NPs with higher encapsulation 1.0%, w/v of PVA and Pluronic F-68 were the optimal concentrations [32]. At this PVA and Pluronic F-68 concentrations, the average sizes of PLGA-NPs was 164.53 ± 12.52 nm and the drug encapsulation was 83.35 ± 4.52% with 15.92 ± 2.75% loading. Moreover, these two surfactants are considered as free from cytotoxic effect. Pluronic F-68 has shown more than 90% cell viability up to 5.56 mg mL^{-1} strength, while PVA has shown about 90% cell survival at 1.85 mgmL^{-1} strength. Thus, the high cell viability and cell survival potential of Pluronic F-68 and PVA makes them the most cytocompatible polyglycol surfactants [49]. Therefore, PVA and Pluronic F-68 were selected to formulate the TAC-loaded PLGA-NPs for ocular use. In this method, the droplets stabilization and preliminary NPs development after solvent diffusion is critical step to avoid coalescence and aggregation. When the oil-water interface is formed, drive to lower the system's energy and to obstruct the coalescence of NPs is the adsorption of stabilizers at the interface [47,48].

4.2. Characterization of PLGA-NPs

At the selected and optimized concentrations of polymer and stabilizers, the mean particle sizes were in the range 164 nm (F2) to 239 nm (F5). It is noteworthy to mention here that the particles for ocular use should never be higher than 10 μm, as larger particles cause scratching, discomfort and severe irritation to eyes [50], whereas smaller particles improve the patient comfort. The physicochemical results indicated the acceptable particle size which could be appropriate for ocular delivery of TAC, sufficient magnitude of polydispersity and zeta-potential with highest drug encapsulation was found at 1.0% w/v, PVA and 1.0% w/v, Plurionic-68. Specifically, at these concentrations, the average size, polydispersity index (PDI) and zeta-potential were 164.53 ± 12.52 nm, 0.108 ± 0.004 and −15.56 ± 2.41 mV, respectively, with 83.35 ± 4.52% encapsulation and 15.92 ± 2.75% drug loading. The width of particle size distribution was indicated by polydispersity index and its smaller values indicating the stable PLGA-NPs dispersion. Our results indicated a smaller polydispersities (Table 1), suggesting the unimodal distribution of the NPs. The negative or positive zeta-potential predicts the physical stability of any dispersion or suspension. The absolute high magnitudes of zeta-potential have strong inclination towards the stabilization of any colloidal carrier systems by avoiding the NPs-aggregation. Based on the zeta-potential values obtained in the present study (Table 1), these absolute values of zeta-potentials indicated an average electrical charge on the surfaces of the PLGA-NPs which may repel NPs to prevent their aggregation [4]. Normally, zeta potentials more than ±30 mV are considered stable for colloidal dispersion [51]. The results in the present study indicated that PLGA-NPs (F1–F5) may not be stable in colloidal state, so the NPs particles would be stored in lyophilized state and they should be reconstituted just before instillation.

The morphological characterization of NPs was performed through SEM observation, which has shown spherical structured solid dense NPs with smooth and even surfaces. SEM image (Fig. 1) of NPs indicating, that they are distinguished from each other and representing themselves separately as evidenced by the polydispersity values (Table 1) of formulation F2. These results exhibited

Table 1
Physical characteristics of tacrolimus-loaded PLGA-NPs with varying concentration of stabilizers, prepared through emulsification-diffusion method, with 100 mg of PLGA and 15 mg of drug (mean ± SD, $n = 3$).

Formulations	Particle size (nm)	Polydispersity index	Zeta-potential (mV)	% Encapsulation efficiency	TAC loading (% DL)
With polyvinyl alcohol (PVA) and Pluronic-F68 as stabilizer (% w/w)					
F1 (0.5: 1.0)	277.59 ± 19.57	0.169 ± 0.008	−11.83 ± 1.82	82.38 ± 3.72	13.23 ± 2.89
F2 (1.0: 1.0)	164.53 ± 12.52	0.108 ± 0.004	−15.56 ± 2.41	83.35 ± 4.52	15.92 ± 2.75
F3 (1.0: 0.5)	375.98 ± 16.89	0.236 ± 0.017	−10.97 ± 2.05	79.72 ± 5.91	12.71 ± 3.63
With polyvinyl alcohol (PVA) and Tween-80 as stabilizer (% w/w)					
F4 (0.5: 0.1)	344.19 ± 8.83	0.213 ± 0.004	−12.15 ± 1.76	75.95 ± 6.61	13.85 ± 2.62
F5 (1.0: 0.1)	239.02 ± 6.58	0.291 ± 0.012	−10.63 ± 2.02	68.93 ± 5.26	11.79 ± 2.76

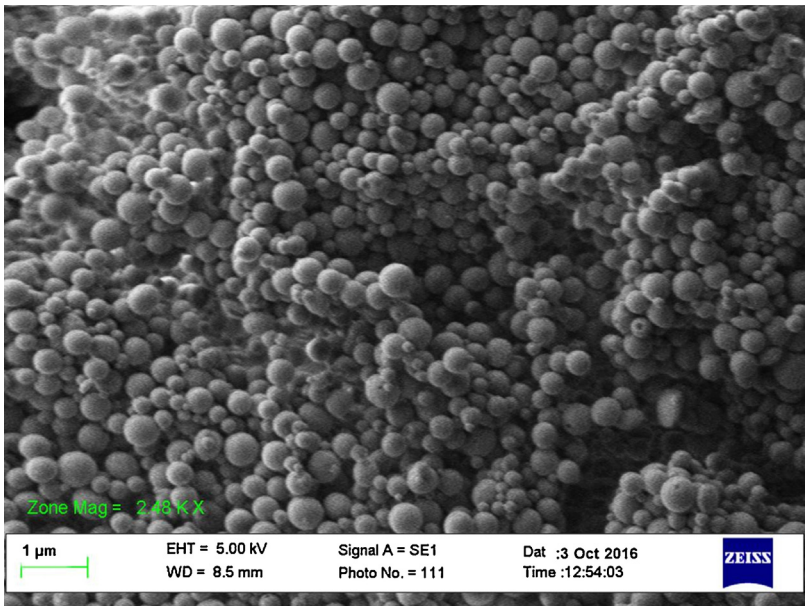

Fig. 1. Scanning electron microscope of TAC loaded PLGA-NPs (F2).

that TAC-loaded PLGA-NPs of approximately 164 nm size with perfect morphology were successfully developed by the selected emulsification-diffusion technique.

Encapsulation (%EE) of PLGA-NPs was found adequately high that was in the range of 68.93 ± 5.26% (F5) to 83.35 ± 4.52% (F2) (Table 1). The sufficiently high drug encapsulation in NPs might be endorsed due to the immediate trapping of TAC in PLGA-matrix because of the presence of PVA and Pluronic F-68 in the aqueous phase and drug encapsulation essentially followed the core-shell model with TAC-enhanced core surrounded by polymer [52,53].% DL of PLGA-NPs was satisfactory, that was in the range of 11.79 ± 2.76% (F5) to 15.92 ± 2.75% (F2). From the results of our study the highest encapsulation and drug loading was found in case of F2. This might be assumed because of the presence of little higher concentrations of PLGA and stabilizers in case of F2 [54]. The high values of%EE and%DL were expected due to high viscosity of organic phase, as increased viscosity could prevent the TAC diffusion from inner organic phase towards the outer aqueous phase [54]. Moreover, high lipophilicity of TAC confirmed increased values of encapsulation and loading [55] in the presence higher concentrations of stabilizers in the present study.

4.3. Freeze-drying and stability

To provide the physical stability and prolonged shelf life, the developed PLGA-NPs were freeze-dried. The stability results indicated that TAC loaded lyophilized PLGA-NPs were stable at room temperature up to 30 days in freeze-dried state. No obvious alterations in various selected characterization parameters were noted in the stored PLGA-NPs and the results were concluded (Table 2). The size of PLGA-NPs had almost no or little change, which might be due to the stabilizers protection on PLGA shell [36].

4.4. Transcorneal permeation

Amount of TAC permeated (*i.e.* permeation flux, J) and apparent permeability (P_{app}) was calculated by accounting 0.636 cm^2 corneal cross sectional area and 6.9 mL of release medium with 300 $\mu g\,mL^{-1}$ of initial TAC-concentration (*i.e.* 180 µg in 600 µL). The pH of TAC-AqS and PLGA-NPs (F2) was suitable for ophthalmic use; however they are slightly differ from the pH of tear fluid but these can be easily buffered by the tear fluid (good buffering capacity). In case of suspension most of TAC was permeated up to 1st h of study while the sustained release of TAC from the excised cornea was observed in case of NPs. The permeated amount of TAC was slightly decreased from suspension state in the late hour of study, which indicated that all the drugs were release in initial hours and due to maintenance of sink condition with STF after each sampling there was dilution in the receptor compartment. High levels of TAC could reach in corneal epithelia when the two formulations of TAC were placed on the corneal surface. Higher

Table 2
Effect of storage on particle size, zeta-potential, polydispersity, encapsulation and loading of TAC from PLGA-NPs for 1 month at 25 ± 2 °C temperature (mean ± SD, n = 3).

Storage time	Particle size (nm)	Polydispersity index	Zeta-potential (mV)	Encapsulation efficiency (% EE)	TAC loading (% DL)
At initial (at the time when formulations were developed)					
F1 (PVA:Pluronic-F68; 0.5: 1.0)	277.59 ± 19.57	0.169 ± 0.008	−11.83 ± 1.82	82.38 ± 3.72	13.23 ± 2.89
F2 (PVA:Pluronic-F68; 1.0: 1.0)	164.53 ± 12.52	0.108 ± 0.004	−15.56 ± 2.41	83.35 ± 4.52	15.92 ± 2.75
After 10 days					
F1 (PVA:Pluronic-F68; 0.5: 1.0)	280.27 ± 8.26	0.172 ± 0.006	−11.06 ± 0.98	81.12 ± 3.01	13.05 ± 1.08
F2 (PVA:Pluronic-F68; 1.0: 1.0)	169.24 ± 7.95	0.114 ± 0.005	−15.01 ± 1.23	82.35 ± 2.95	15.01 ± 1.21
After 30 days					
F1 (PVA:Pluronic-F68; 0.5: 1.0)	285.25 ± 10.65	0.176 ± 0.009	−10.08 ± 1.42	79.28 ± 2.09	12.65 ± 1.25
F2 (PVA:Pluronic-F68; 1.0: 1.0)	173.18 ± 8.97	0.119 ± 0.003	−14.54 ± 1.72	78.23 ± 2.87	14.76 ± 1.31

permeation of TAC was expected since the donor and receptor components were positioned vertically.

From the plots of amount permeated *versus* time (Fig. 2) and their values (Table 3), it was observed that NPs facilitated sustained and controlled release of TAC as compared to TAC-AqS. This might be endorsed due to smaller sized NPs were having enough surface area and hence crossed the intracellular junctions of the corneal epithelia. The results of transcorneal permeation indicated that the cumulative amount of TAC permeated was 100.83 $\mu g\,cm^{-2}$ at 1st h (from TAC-AqS), and then the amount of drug remained almost unchanged (102.68 $\mu g\,cm^{-2}$) till 4th h of study. While the permeated amount of drug was only 28.48 $\mu g\,cm^{-2}$ at 1st h (from F2) and the permeation of drug gradually increased with time (98.74 $\mu g\,cm^{-2}$ at 4th h) in a sustained manner.

The molecular mass of TAC (804.03 $g mol^{-1}$) and its octanol/water partition coefficient (LogP 2.7) suggest that TAC is lipophilic, which indicated relatively easier corneal epithelial passage of TAC as compared to hydrophilic molecules [56] because the corneal epithelial surface is lipophilic in nature. The dissociation constant values (pKa1 = 2.94; pKa2 = 9.95 and pKa3 = 14.07) of TAC suggesting the availability of larger fraction of TAC in unionized state at mentioned pH values in Table 3, which might promote a high transcorneal passage of TAC from TAC-AqS during initial hours.

4.5. Ocular irritation prospective of PLGA-NPs

The ocular irritation prospective of PLGA-NPs (F2) was evaluated in rabbit eyes, taking saline as control. No any sign of physical discomfort was noted (score 0) during long term and acute irritation study in the eyes of experimental rabbits. After frequent administration of F2, very low impatience and irritation was observed in two animals (Table 4). The treated eyes of all the animals did not show any mucoidal discharge after F2 instillation. Our analysis indicated no much difference in observed ocular severity among the animals. Overall, the ocular irritation experimental observations indicated the comparative safety of the TAC-loaded PLGA-NPs and it was non-irritant for rabbit eyes. In our experiment we found this was a satisfactory model for ocular irritation and the accuracies validated here are showing a possible approach for ocular irritation test. Making the dataset available (Table 4) allowing our analysis relatively impressive and strongly supports safety of the developed nanocarrier for ocular use [57].

4.6. UPLC–MS adopted method

The analyte (TAC) was successfully analyzed in the presence of endogenous materials in aqueous humor samples indicated the selectivity of the adopted methods [40,45]. The symmetrical peaks of TAC were found with 0.91 min R_t. No any interference was observed with TAC peaks throughout the 3 min run time. The chromatograms of blank aqueous humor (Fig. 3a–a'), aqueous humor spiked with 20 ng mL^{-1} of TAC and 50 ng mL^{-1} of IS (Fig. 3b–b') and aqueous humor sample obtained 6 h post instillation of F2 spiked with 50 ng mL^{-1} of IS (Fig. 3c–c') were compared to show the specificity and selectivity of the chosen and slightly modified analysis method. The R_t values of TAC and CsA (IS) were around 0.91 and 1.09 min respectively, and no interferences were observed because of endogenous materials or formulation ingredients during TAC and CsA analysis in aqueous humor samples, indicating the selectivity of the adopted and modified methods.

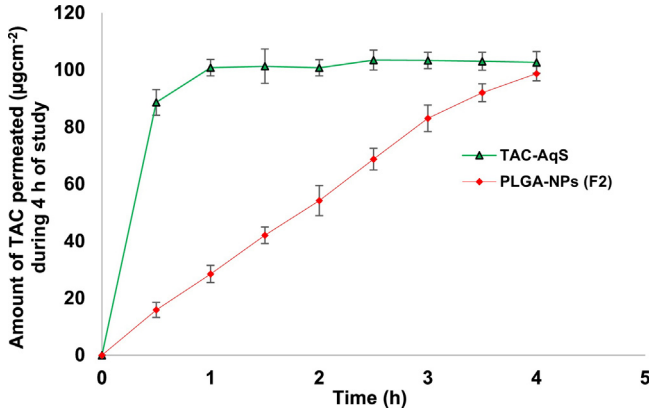

Fig. 2. Transcorneal permeation of TAC from TAC-AqS and PLGA-NPs (F2) (mean ± SD, $n = 3$) where the available corneal area was 0.636 cm^2.

4.7. Tacrolimus biodistribution in rabbit's aqueous humor and ocular tissues

The adopted UPLC–MS method was successfully used to analyze TAC in ocular tissues and aqueous humor of rabbit after topical application of TAC-AqS and PLGA-NPs (F2). Usually, the transcorneal permeation and absorption are considered as the main trail for high molecular weight drugs available to intraocular tissues, though, the non-corneal absorption pathway like conjunctiva also adds sufficient permeation of drugs [56]. So, there was a need to quantify the absorbed drug in to conjunctiva and cornea. After a single topical ocular instillation of the 0.03% TAC containing PLGA-NPs (F2) has shown maximum TAC concentration in cornea at 24th h of post instillation as compared to TAC-AqS. At 24th h of post-instillation, TAC concentration from TAC-AqS in corneal and conjunctival tissues were decreased to 2.53 ± 1.83 and 7.27 ± 4.26 ngg^{-1} respectively, while F2 was able to maintain the TAC concentrations (56.11 ± 7.73 ngg^{-1} in cornea and 65.68 ± 8.77 ng. g^{-1} in conjunctiva). These concentrations of TAC from NPs would be sufficient to modify the local immune responses to suppress the inflammatory and dry-eye conditions, to treat other ocular autoimmune diseases and to prevent the corneal transplant rejection. The high quantity of TAC above the therapeutic level even at 24 h in cornea and conjunctiva from F2 advising that these clinically related ocular tissues may act as reservoir for TAC delivery in to eyes [41]. A high accumulation of TAC (65.68 ngg^{-1}) was found in conjunctiva, which is required and anticipated because the conjunctiva is considered as the target tissues for any pharmacological effect of a drug. These findings suggested that the distribution of TAC in the rabbit corneal epithelium was very less from TAC-AqS than that of the NPs (F2). This indicated that accumulation of TAC from AqS in cornea and its elimination from the corneal tissues are quicker as compared to TAC-loaded NPs (F2), as evidenced by the transcorneal permeation parameters. Thus, we can conclude that good efficacy of TAC can be achieved from NPs and hence it might be considered as advantageous system for ocular delivery of TAC over its conventional delivery systems.

TAC concentrations quantified in aqueous humors collected at different time points and calculated pharmacokinetic parameters are illustrated in Fig. 4 and Table 5, respectively. TAC concentration was detected in aqueous humor up to 12 h only in case of TAC-AqS treated group thereafter TAC was undetected that justifying the rapid and fast precorneal loss of TAC in aqueous form. The TAC was sufficiently quantified in aqueous humor samples even at 24th h following topical application of F2 in to eyes of first group of animals. F2 has shown significant ($p < 0.05$) bioavailability of TAC as compared to TAC-AqS. A noticeable, 2.70-fold greater $AUC_{0\text{-}24h}$ was detected with F2 than that of TAC-AqS. The $t_{1/2}$ of TAC from F2 was 1.77-fold greater which was significantly high and no significant difference in peak aqueous-humor drug concentration (C_{max}) between the two applied formulations was found.

Table 3
Transcorneal permeation parameters for TAC from TAC-AqS and PLGA-NPs (F2), where concentration of TAC was 0.03% w/v, (mean ± SD, $n = 3$).

Parameters	PLGA-NPs (F2)	TAC-AqS
Cumulative amount permeated at 1st h ($\mu g\,cm^{-2}$)	28.48 ± 3.01	100.83 ± 2.87
Cumulative amount permeated at 2nd h ($\mu g\,cm^{-2}$)	54.19 ± 5.30	100.78 ± 2.83
Cumulative amount permeated at 3rd h ($\mu g\,cm^{-2}$)	83.07 ± 4.65	103.37 ± 2.84
Cumulative amount permeated at 4th h ($\mu g\,cm^{-2}$)	98.74 ± 2.56	102.68 ± 3.82
pH of the formulations	6.87 ± 0.48	7.21 ± 0.56
Steady-state flux, J ($\mu g\,cm^{-2}\,h^{-1}$)	9.31	38.77
Permeability coefficient, P ($cm\,h^{-1}$)	1.55×10^{-2}	6.06×10^{-2}

Table 4
"Weighted scores obtained for the severity of ocular irritation experiments by TAC-loaded PLGA-NPs (F2)".

Lesion	Score for Rabbit 1	Score for Rabbit 2	Score for Rabbit 3	Score for Rabbit 4	Score for Rabbit 5	Score for Rabbit 6
Cornea						
"i. Opacity-Degree of density (area which is most dense is taken for reading)"						
"Scattered or diffuse area – details of iris clearly visible"	0	1	0	1	1	0
"Easily discernible translucent areas, details of iris slightly obscured"	2	0	2	0	0	2
"Opalescent areas, no details of iris visible, size of pupil barely discernible"	0	0	0	0	0	0
"Opaque, iris invisible"	0	0	0	0	0	0
"ii. Area of cornea involved"						
"One quarter (or less) but not zero"	0	0	0	0	0	0
"Greater than one quarter but less than one half"	0	0	0	0	0	0
"Greater than one half but less than three quarters"	0	0	0	0	0	0
"Greater than three quarters up to whole area"	4	4	4	4	4	4
"Total score obtained = (i × ii × 5)" =	**40**	**20**	**40**	**20**	**20**	**40**
Iris						
i. Values						
"Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)"	0	1	1	0	1	1
"No reaction to light, hemorrhage; gross destruction (any one/all of these)"	1	0	0	1	0	0
"Total score obtained = (i × 5)" =	**5**	**5**	**5**	**5**	**5**	**5**
Conjunctiva						
"i. Redness (refers to palpebral conjunctiva only)"						
"Blood vessels definitely injected above normal"	0	1	1	0	1	0
"More diffuse, deeper crimson red, individual vessels not easily discernible"	2	0	0	2	0	2
"Diffuse beefy red"	0	0	0	0	0	0
ii. Chemosis						
"Any swelling above normal (includes nictitating membrane)"	1	0	1	1	0	1
"Obvious swelling with partial eversion of the lids"	0	2	0	0	2	0
"Swelling with lids about half closed"	0	0	0	0	0	0
"Swelling with lids about half closed to completely closed"	0	0	0	0	0	0
iii. Discharge						
"Any amount different from normal (does not include small amount observed in inner canthus of normal animals)"	1	0	1	0	1	0
"Discharge with moistening of the lids and hairs just adjacent to the lids"	0	2	0	2	0	2
"Discharge with moistening of the lids and considerable area around the eye"	0	0	0	0	0	0
"Total score obtained = (i + ii + iii) × 2" =	**8**	**10**	**6**	**10**	**8**	**10**

The significantly high values of pharmacokinetic parameters such as $AUC_{0\text{-inf}}$, $AUMC_{0\text{-inf}}$ and $MRT_{0\text{-inf}}$ (Table 5) suggesting the prolonged corneal and conjunctival retention of F2 which promoted the higher transcorneal uptake of TAC as compared to TAC-AqS. Being an anionic polymer, PLGA is not mucoadhesive, but because of relatively smaller particle sizes of PLGA-NPs, F2 was retained on the epithelial surfaces of cornea and conjunctiva of rabbit eyes [9]. Stabilizers like, PVA (HLB = 18) and Pluronics F-68 were used to stabilize the PLGA-NPs, both are swellable hydrophilic macromolecule and high HLB-value of PVA (reducing the inter-surface tension) were the reasons for prolonged retention of PLGA-NPs on ocular surface [8,58]. Moreover, these stabilizers provided good spreading propensity to PLGA-NPs, which supported the enhanced corneal and precorneal retention of NPs which in turn enhanced their transcorneal uptake [58].

The results of increased pharmacokinetic parameters for F2 are indicating that the corneal and conjunctival epithelial surfaces are capable of endocytosis. The adsorptive and receptor-mediated endocytosis on corneal surfaces involve energetic and saturable transcorneal uptake processes, which depend on binding to specific or nonspecific binding sites and receptors on corneal surfaces, respectively. The smaller size molecules are generally transported through receptor-mediated endocytosis process [9]. Furthermore, Pluronics-F68 blocks P-glycoprotein-efflux pump activity by reducing the ATP availability, so would sensitize the inflamed cells and would enhance the TAC effects on such inflamed ocular cells and tissues by double or triple fold [49]. Thus, we assume that PLGA-NPs uptake in ocular region may occurred through adsorption-mediated endocytosis process. TAC concentration versus time plot (Fig. 3) was satisfactory to complete the analysis of pharmacokinetic data from TAC-NPs and its aqueous suspension. The deliberated pharmacokinetic parameters of the two preparations from less variable obtained data from minimum numbers of animals were reliably established. Hence, the prepared

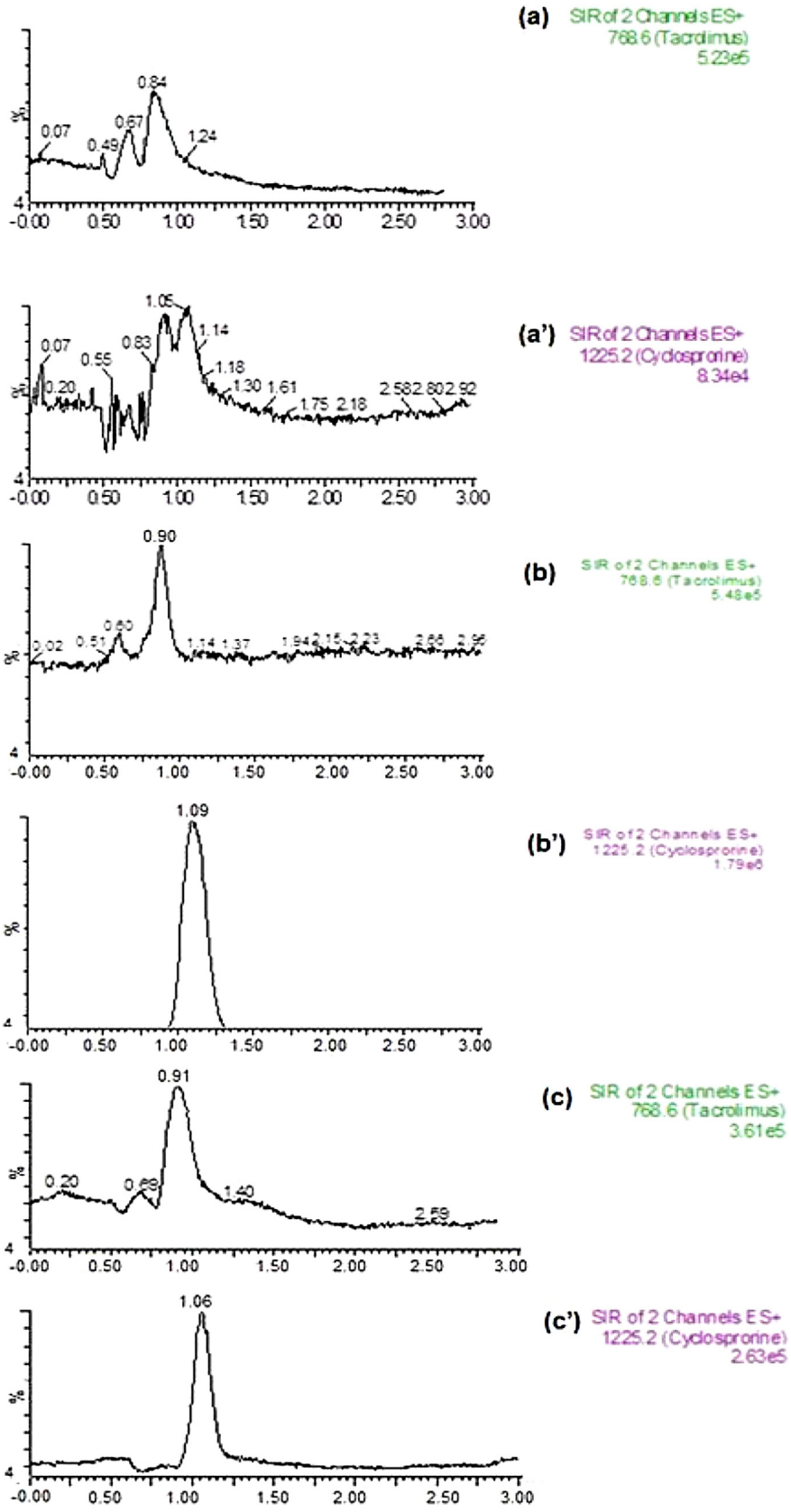

Fig. 3. Representative chromatogram of TAC (a) and CsA as internal standard IS (a') in blank aqueous humor; in aqueous humor spiked with 20 ng mL^{-1} of TAC (b) and 50 ng mL^{-1} of CsA as IS (b'); in aqueous humor samples obtained at 6 h after dosing from one rabbit eye, TAC (c) and spiked with 50 ng mL^{-1} of IS (c').

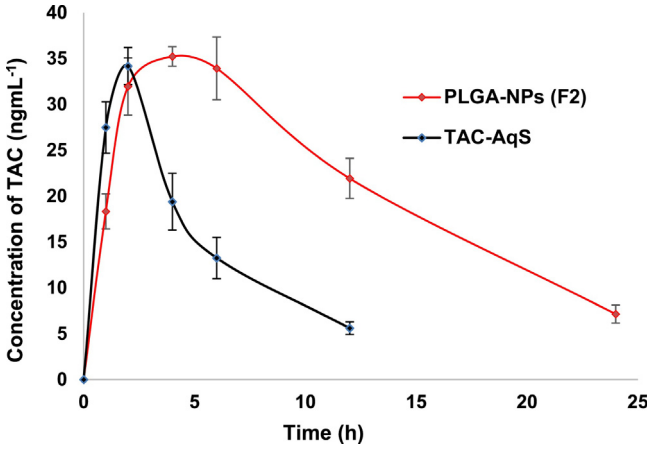

Fig. 4. Pharmacokinetic profile of TAC following a single dose (15 μg) topical administration of F2 and TAC-AqS (data were expressed in mean ± SD, $n = 3$).

Table 5
Pharmacokinetic parameters of TAC in aqueous humor after topical administration of two products (mean ± SD, $n = 3$, for each time point).

Parameter with units	TAC-AqS	PLGA-NPs (F2)
$t_{1/2}$ (h)	4.576 ± 0.298	8.104 ± 1.086
T_{max} (h)	2.0 ± 0.0	4.666 ± 1.154
C_{max} (ng mL^{-1})	35.831 ± 3.157	35.902 ± 1.932
$AUC_{0\text{-}24h}$ (ng mL^{-1} h)	189.863 ± 23.361	512.748 ± 25.889
$AUC_{0\text{-}inf}$ (ng mL^{-1} h)	226.818 ± 26.917	597.171 ± 48.596
$AUC_{0\text{-}t/0\text{-}inf}$	0.836 ± 0.013	0.860 ± 0.026
$AUMC_{0\text{-}inf}$ (ng mL^{-1} h^2)	1497.802 ± 186.185	7644.607 ± 1307.63
$MRT_{0\text{-}inf}$ (h)	6.607 ± 0.299	12.743 ± 1.128

PLGA-NPs would be successful carrier for topical and might be intravitreal distribution of TAC in different above mentioned ocular disease conditions.

5. Conclusion

On the basis of the data it can be concluded that the F2 formulation is the best among the lot of five optimized PLGA-NP formulations, in terms of characterization parameters, transcorneal permeation and stability. The Draize's test did not reveal any irritant or corrosive effects of the F2 and indicates its nontoxic nature when administered topically into the eyes. Comparing the pharmacokinetic data, F2 has shown high TAC ocular bioavailability than that of TAC-AqS. Higher availability of TAC from F2 was postulated because of prolonged retention of NPs in ocular region. The NPs size dependency along with the sufficient transcorneal uptake of TAC indicated that PLGA-NPs can be used successfully for enhanced absorption and sustained release of lipophilic drugs in to eyes. The developed PLGA-NPs for TAC delivery would have potential in controlled drug release in various ocular anterior/posterior segment inflammations, dry eye conditions, ocular autoimmune diseases and some retinal diseases where intravitreal injection are required. Conclusively, good efficacy of TAC can be achieved from PLGA-NPs and would be considered as advantageous carrier system for TAC topical ocular delivery over its conventional dosage forms.

Conflict of interest

The authors declare no conflict of interest.

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