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A polymeric aqueous tacrolimus formulation for topical ocular delivery

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ABSTRACT

Tacrolimus (TAC) suspension is used to treat moderate to severe atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC). The objectives of this study were to formulate the hydrophobic compound TAC (TAC) in an aqueous eye drop formulation and study its ocular biodistribution on topical ocular application to a healthy rabbit model, with the overall aim of using the formulation to treat AKC and VKC. A thin-film hydration method was used to encapsulate TAC within the chitosan-based amphiphile: N-palmitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycolchitosan (Molecular Envelope Technology – MET) in an aqueous formulation. The formulation was characterized, and its stability studied under three storage conditions for one month. The ocular distribution of the formulation was studied in healthy rabbits and the ocular tissues and the whole blood analyzed by LC-MS/MS. A 200 nm nanoparticle formulation (MET-TAC) containing 0.1 ± 0.002% w/v TAC was produced with viscosity, osmolarity and pH within the ocular comfort range, and the formulation was stable on refrigeration for one month. On topical application, the TAC concentrations in rabbit cornea and conjunctiva one hour after dosing were 4452 ± 2289 and 516 ± 180 ng/g of tissue, respectively. A topical ocular aqueous TAC eye drop formulation has been prepared with the ability to deliver sufficient drug to the relevant ocular surface tissues.

1. Introduction

Allergic conjunctivitis affects 6–30% of the general population in Europe, with 25% of cases involving severe and persistent disease (Leonardi et al., 2015). Allergic ocular symptoms affect 40% of the US population at least once in their lifetime, with a prevalence rate of 29.7% (Singh et al., 2010). The majority of allergic conjunctivitis patients (55–81%) suffer from seasonal allergic conjunctivitis, while the more severe forms of the disease: atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC) affect 4–39% of allergic conjunctivitis patients, depending on geographical location; with particularly high numbers of VKC (39% of allergic conjunctivitis patients) and AKC (39% of allergic conjunctivitis patients) in Brazil (Leonardi et al., 2015). VKC affects children, resolves around puberty and is more prevalent in boys with a prevalence of 1.16–10.55 per 10,000 of the general population in Western Europe (Bremond-Gignac et al., 2008) and 18% in Nigerian primary school children (Duke et al., 2016). VKC is a sight-threatening disease with no overall gold standard form of therapy (Addis and Jeng, 2018). AKC is also a sight-threatening condition which affects adults mostly and is usually present as a co-morbidity with atopic dermatitis (Guglielmetti et al., 2010), with 67.5% of atopic dermatitis patients diagnosed with AKC in one Japanese study (Dogru et al., 1999). Notwithstanding the rare diseases of AKC and VKC, an estimated 25% of ocular allergy patients have frequent episodes (more than four times a week) for more than four weeks and are classed as having a severe disease which impacts negatively on their quality of life (Leonardi et al., 2015). Patients with severe disease are treated with anti-allergy drugs, and corticosteroids and treatments are frequently inappropriate (Leonardi et al., 2015). Prolonged steroid use is associated with glaucoma (Kersey and Broadway, 2006) and hence is not ideal especially with the younger patients.

TAC acts by binding to FK506 binding protein forming a complex which inhibits calcineurin (Thomson et al., 1995). This inhibition of calcineurin suppresses dephosphorylation of nuclear factor activated T-cells, resulting in suppression of the interleukin 2 gene, interferon-gamma and interleukin 4 and interleukin 5; ultimately inhibiting the proliferation of T-cells (Zhai et al., 2011). TAC also inhibits the release of histamine from mast cells (Sengoku et al., 2000). These mechanisms contribute to the effectiveness of TAC in allergic conjunctivitis. In a 56 patient randomized controlled trial involving severe allergic

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conjunctivitis patients who were not responding to anti-allergy drugs and corticosteroids (n = 28 patients per arm), there was a significant drop in the objective sign score in the treatment arm (Talymus 0.1% - a TAC suspension - twice daily) when compared to the placebo arm after 1 month of treatment, with a significant decrease in the objective signs score observed one week into the treatment course (Ohashi et al., 2010). There were significant changes in the giant papillae and corneal involvement scores with Talymus 0.1% (Ohashi et al., 2010). A visual analogue scale was used to assess seven subjective symptoms, and five of these symptoms (itching, discharge, hyperaemia, lacrimation and foreign body sensation) were also significantly reduced in the treatment arm after 4 weeks of treatment (Ohashi et al., 2010). A subsequent 1436 patient prospective observational study revealed that the total signs and symptoms score reduced significantly from baseline one month after dosing with Talymus 0.1% twice daily, and that giant papillae and corneal lesions were also significantly reduced (Fukushima et al., 2014). Both studies reported ocular irritation as the main side effect with mild ocular irritation affecting 46.2% of patients in the 56 patient study and a transient burning sensation affecting 3.2% of patients in the 1436 patient study (Fukushima et al., 2014), although general ocular adverse events affected 6.2% of patients in the 1436 patient study (Fukushima et al., 2014). Additionally, a hospital compounded TAC formulation (TAC capsules – Prograf – formulated in a balanced salt solution) containing 0.01% TAC was found to be effective in reducing the signs and symptoms of VKC in a 62 patient study (Shoughy et al., 2016). Talymus is approved in Japan (Takamura et al., 2017; Talymus Product Information Sheet, 2014). A cationic emulsion of CsA (Vekacia®, 0.1% w/v), has been granted orphan drug status by the European Commission for the treatment of VKC (Kersey and Broadway, 2006; Thomson et al., 1995). However, Talymus is reported to provide a therapeutic response in allergic conjunctivitis patients not responding to CsA (Fukushima et al., 2014). There is thus substantial evidence of the efficacy of TAC eye drops in severe allergic conjunctivitis.

In order to overcome the limitations associated with the use of TAC, such as ocular irritation and pain (Fukushima et al., 2014) transient blurring of vision due to the opacity of the formulation (Addis and Jeng, 2018) and plasma exposure (Ebihara et al., 2012), a known non-irritant, mucoadhesive ocular penetration enhancer - N-palmitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycolchitosan (Molecular Envelope Technology – MET) has been used (Qu et al., 2006; Siew et al., 2012) to formulate the hydrophobic drug TAC within positively charged nanoparticles, with the formulation presenting as a clear liquid. This formulation may be useful in the treatment of AKC and VKC.

2. Experimental details

2.1. Materials

TAC powder (MW 804.02 g/mol) and ascomycin (ASC) powder (MW 792.02 g/mol) were purchased from Generon Ltd. (Slough, UK). MET was supplied by Nanomerics Ltd. (London, UK). Water, Acetonitrile, Methanol and Formic Acid LC-MS Grade solvents were purchased from (VWR, Leicestershire, UK). All other chemicals and reagents used were of analytical grade.

2.2. Drug formulation studies

A thin-film hydration method was used to encapsulate TAC within MET nanoparticles. This method was previously reported to encapsulate hydrophobic compounds (Sahu et al., 2011). TAC powder was dissolved in absolute ethanol (0.1% w/v). MET was dispersed in filtered deionized water (1.0% w/v). Both preparations were mixed and placed in a Savant Vacuum Evaporator (ThermoFisher Scientific, Waltham, USA) at 45 °C and spun under vacuum for 5 h until a thin, dry film was formed. The dry film was rehydrated with glycerol (2.6% w/v) as an osmotic agent (Heaton et al., 1986) and mixed vigorously for 30 min to disperse the film in the solvent. A transparent liquid was obtained, thus producing a clear formulation of MET-TAC with a nominal drug concentration of (0.1% w/v). The formulation was adjusted to a pH of 7.0 in a calibrated pH meter using 1.0 M NaOH, and a simulated sterile filtration step was carried out using a 0.22 μm sterile filter. The formulation was then analyzed for drug content, particle size distribution, zeta potential distribution, viscosity and osmolarity.

2.2.1. High-performance liquid chromatography (HPLC) analysis of TAC

A HPLC method was developed for TAC using an Agilent 1220 infinity chromatographic system fitted with a vacuum degasser, quaternary pump, auto-sampler, column compartment with a thermostat and an ultraviolet (UV) detector (Agilent technologies, Berkshire, UK). A gradient method (Table 1) was developed using a mobile phase consisting of acetonitrile, phosphoric acid (0.1%), a reversed-phase onyx monolithic C18 (Phenomenex Inc., Torrance, USA) column (100 × 4.6 mm; particle size, 5 μm). The column temperature was maintained at 50 °C, and the UV detector was set at a wavelength of 215 nm. The flow rate was 1.0 mL/min, and the injection volume was 10.0 μL, the run time was 7 min.

2.2.2. Particle size and zeta potential measurements

The particle size distribution and particle zeta potential of the formulation were determined on a Malvern ZetaSizer Nano ZS (Malvern Panalytical, Malvern, UK).

The size distribution analysis was performed using dynamic light scattering at a backscattering angle of 173° and a temperature of 25 °C. An aliquot of the sample (100 μL) was placed in a disposable plastic cuvette and was subsequently loaded into the instrument without any dilution. The particle size was reported as intensity distribution, which describes the relationship between light scattering intensity and the particle hydrodynamic diameter (Stetefeld et al., 2016). The mean size of the individual peaks and their corresponding percentages were determined and recorded as mean ± SD of three independent measurements.

The zeta potential is the electrokinetic potential in a colloidal system and measures the surface particle charge in a given medium (Gumustas et al., 2017). This parameter was obtained via the electrophoretic light scattering technique. An aliquot of the sample (600 μL) was loaded into folded capillary cells (zeta cells, polycarbonate cell with gold-plated electrodes; Malvern Instruments, DTS1060C) and measured at 25 °C, 40 V. The results were presented as mean ± SD, and the resulting data were analyzed using the DTS (Version 4.2) software, Malvern Instruments Ltd. (Malvern, UK).

2.2.3. Osmolarity measurements

The osmolarity of the formulation (100 μL) was determined using Roebling Milliosmol Osmometer (Geminibv, Apeldoorn, NL) coupled with a digital display and a freezing needle. The machine was calibrated before each measurement with 300 mOsm/Kg reference standards solution (Reagecon Diagnostics Ltd., Clare, IE). The measurements were conducted in triplicate.

2.2.4. Viscosity measurements

The viscosity of the formulation was measured utilizing m-VROC viscometer (Rheosense Inc., San Ramon, USA). Samples (500 μL) were

Table 1
HPLC gradient condition of TAC.

Time (min)	0.1% H_3PO_4 in H_2O **Solvent A (%)**	Acetonitrile **Solvent B (%)**
0.00	40	60
4.00	5	95
5.50	5	95
7.00	40	60

inserted into the measuring cell using a 0.5 mL syringe with extreme care to avoid air bubble formation. The viscosity was measured at three different shear rates (5,000 s^{-1}, 7,500 s^{-1} and 10,000 s^{-1}) at 25 °C.

2.2.5. Stability measurements

The stability of the MET-TAC formulation was determined by measuring the physicochemical properties over 30 days at different storage conditions and relative humidities (RH). Formulations were stored at room temperature (25 ± 2 °C / 60 ± 5% RH), under refrigeration temperature (5 ± 3 °C) and under accelerated conditions (40 ± 2 °C / 75 ± 5% RH). The storage stability conditions were chosen according to the International Conference on Harmonization (ICH) guidelines (Nezlin, 2003; Niazi, 2003), and the measurements were done in triplicate.

Samples (750 μL) were withdrawn from the formulation at each storage condition immediately after preparation and at days 7 and 30 of storage. The samples were analyzed to determine if the encapsulated drug concentration, size distribution, zeta potential, pH, osmolarity and viscosity were altered upon storage. The samples were also visually inspected for any macroscopic changes, including precipitation, turbidity and colour changes.

Statistical analysis was performed using two-way ANOVA with Tukeý's post-test. All data were expressed as mean ± SD. ($p < 0.05$) was considered statistically significant.

2.3. In vivo study

2.3.1. Animals

New Zealand white male rabbits weighing between 2.0 and 3.0 kg were obtained from Envigo (Huntingdon, UK) and were acclimatized for at least seven days before the experiment. The rabbits had free access to water and food throughout the study. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. All animal studies were ethically reviewed and performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.3.2. Bioanalytical LC-MS/MS assay

2.3.2.1. Preparation of working standard solutions. TAC stock solution was prepared at a concentration of 100 μg/mL in methanol (MeOH). TAC working stock solutions were prepared by serially diluting TAC stock solutions into MeOH to obtain TAC working stock solutions at a range of concentrations (0.0167–33.4 μg/mL). TAC working standards were prepared by serially diluting TAC working stock solutions into MeOH to obtain the TAC working standards ranging from a concentration (0.5–1000 ng/mL). Ascomycin was used as the internal standard (IS). A stock solution of the IS was freshly prepared at a concentration of 100 ng/mL in methanol.

2.3.2.2. Preparation of standard and quality control curves. Working standard solutions were prepared to obtain an individual standard curve in each of the rabbits' eyes blank tissues, and in the cornea, conjunctiva, sclera, choroid-retina, aqueous humor, vitreous humor, and whole blood. Tissues were homogenized according to the following protocol. Briefly, the solid frozen tissue was cut into small pieces with scissors and ground to a fine powder with a mortar and pestle placed in dry ice, and the absolute tissue mass was weighed. Aqueous samples (aqueous humor, vitreous humor, and whole blood) were transferred into a sterile polypropylene tube, and the absolute mass was weighed. Normal saline (500 μL) was added, and samples were vortex-mixed for 5 min. The mixtures were then homogenized using probe sonication (MSE Soniprep 150 sonicator) from MSE UK Ltd. (London, UK) at 50% of its maximum output for 25–50 s in an ice bath. The homogenized samples were spiked with TAC working standards (50 μL) in order to generate the standard curves. Also, samples were spiked with the internal standard ascomycin in MeOH (60 μL). Methanol (1060 μL) was added to precipitate the proteins.

The quality control standard curves were generated to evaluate the recovery rate and the matrix effect on drug extraction. Blank samples were prepared in a similar manner to tissue's standard curve. Briefly, TAC working standard samples (50 μL) were added to normal saline (500 μL) to generate the standard curves. Samples were spiked with ascomycin in MeOH (60 μL). Methanol (1060 μL) was added to mimic the extraction protocol.

The mixtures were then vortexed for 5 min and centrifuged (5000 g × 10 min at 4 °C, Hettich Mikro 200R, Tuttlingen, Germany). An aliquot (1 mL) of the centrifuged homogenate supernatant was evaporated to dryness within the speed vac at 45 °C and spun under vacuum in the evaporator for at least 2 h. The residue was reconstituted in the LC-MS/MS mobile phase (100 μL) and vortex-mixed for 5 min. The sample was once again centrifuged (2000 g × 2 min at 4 °C) to precipitate any tissues. Following this, the sample (80 μL) was transferred to HPLC vials. Ten μL of the reconstituted sample was injected into the LC-MS/MS system.

2.3.2.3. Chromatography. Samples were analyzed using an Agilent 6400 Series Triple Quadrupole LC/MS system (Agilent technologies, Berkshire, UK) comprising a degasser (HiP Degasser 1260/G4225A), a binary pump (HiP 1260 binary pump/G1312B), an autosampler (HiP sampler 1260/ G1367E), a column oven (G1316A) and a triple-quadrupole mass spectrometer (G6460A). An Agilent MassHunter Workstation Software was used for system control, data acquisition and data processing.

A sensitive LC-MS/MS method was applied to determine the concentration of TAC in the eye tissue homogenates and blank tissue samples. Samples (10 μL) were chromatographed over an XBridge BEH C8 XP column (2.5 μm, 2.1 mm × 50 mm) equipped with a Vanguard Cartridge Holder guard column from Waters Limited (Herts, UK) and at a temperature of 50 °C, with the mobile flow rate of 0.5 mL/min. The runtime was 4 min, followed by a 1-minute post run time. The mobile phase was formic acid (0.1% v/v) in water and acetonitrile containing (LC-MS grade solvents) in the following gradient conditions (Table 2).

TAC and ascomycin were monitored by positive electrospray ionization on an Agilent jet stream ion source with ionization source parameters as outlined (Table 3). Samples were scanned using multiple reaction monitoring mode for transitions of TAC *m/z* [M+Na]+ (826.3 → 415.2), and for ascomycin *m/z* [M+Na]+ (814.2 → 604.1), respectively.

2.3.3. MET-TAC pharmacokinetics animal study

New Zealand white albino male rabbits weighing between 2.0 and 3.0 kg, were acclimatized for not less than seven days before the experiments. The rabbits had free access to water and food throughout the study. Twenty-five μL of the MET-TAC 0.1% w/v formulation was administered to both eyes. Briefly, the lower eyelid was gently pulled away from the eye globe and using a calibrated micropipette, 25 μL of the formulation was applied in the lower conjunctival cul-de-sac. After dosing, the upper and lower eyelids were hand-held together for approximately 5 s to permit the formulation to come into contact with

Table 2
LC-MS/MS mobile phase composition of TAC and internal standard.

Time (min)	0.1% FA in H_2O **Solvent A (%)**	0.1% FA in ACN **Solvent B (%)**
0.00	60	40
0.50	60	40
1.00	0	100
3.00	0	100
4.00	60	40

Table 3
LC-MS/MS source parameters for TAC and ascomycin.

Parameter	TAC (Analyte)	Ascomycin (Internal standard)
Capillary voltage (V)	4000	4000
Gas temperature (°C)	300	300
Gas flow (L/min)	9	9
Sheath gas heater (°C)	325	325
Sheath gas flow (L/min)	12	12
Nebuliser (psi)	45	45
Fragmentor (V)	300	300
Collision energy (V)	50	45
Precursor ion (*m/z*)	826.3	814.2
Product ion (*m/z*)	415.2	604.1

the cornea. The number of blinks in the next 60 s was recorded. Subsequently, after predetermined time points (1, 2, 8 h, n = 4 each), the blood sample was withdrawn (1–3 mL) through the marginal ear vein using a 23-gauge butterfly needle. After blood collection, the rabbit was culled with an IV over-dose injection of 20% w/v phenobarbital (5 mL) through the marginal ear vein using a 25-gauge butterfly needle. The eye globe was enucleated using sterilized scissors, washed twice with 0.9% w/v normal saline and dried on a filter paper. Subsequently, the various tissues were dissected, rinsed twice with 0.9% w/v normal saline and dried on a filter paper. The eye tissues were harvested in the following order to minimize cross-contamination: conjunctiva, aqueous humor, vitreous humor, lens, iris/ciliary body, cornea, choroid-retina, sclera. The harvested dried tissues were immediately stored in ice (2–5 h after dissection), and eventually stored at −80 °C until further analysis could be performed. The aqueous humor was withdrawn using a 26-gauge needle attached to a 2 mL syringe, while vitreous humor was aspirated using a 23-gauge needle attached to a 2 mL syringe. All tissue dissections were performed using sterilized tools. Disposable scalpels, tweezers and scissors were used as necessary. Any other sharp tools were disinfected with 70% ethanol and washed with 0.9% w/v normal saline before moving to the following tissue. All tissues were rinsed twice with 0.9% w/v normal saline before being added to a pre-weighed tube.

For TAC extraction, a volume of the tissue homogenates (500 μL) and aqueous samples were used. Briefly, to extract TAC, the protein precipitation method was used. To all the tissue homogenates, an aliquot of the IS (100 ng/mL, 60 μL) was added and vortexed for 5 min. Methanol (1110 μL) was added to precipitate the proteins and extract the TAC. Mixtures were then vortexed for 5 min and centrifuged (5000*g* × 10 min at 4 °C). Aliquots (1 mL) of the centrifuged homogenate supernatant were evaporated to dryness within the speed vac at 45 °C and spun under the vacuum evaporator for at least 2 h. The residues were reconstituted in the LC-MS/MS mobile phase (100 μL) and vortex-mixed for 5 min. The samples were again centrifuged (2000*g* × 2 min at 4 °C) to precipitate any tissues. An aliquot of the resulting supernatants (80 μL) were transferred to HPLC vials. Ten μL of the reconstituted samples were injected into the LC-MS/MS system.

3. Results and discussions

3.1. Drug formulation studies

A reverse-phase HPLC method was developed and used to quantify TAC drug content in the formulation. A calibration curve was constructed by plotting the average peak area of TAC areas against concentration. The method developed showed linearity of TAC in a concentration range (62.5–1000 μg/mL). The straight-line equation (peak area = 5667.3 [TAC] + 11.85, $r^2 = 0.9996$) was used to quantify the concentration of the encapsulated TAC in the formulation (Table 4).

In Table 4, y = peak area ratio (analyte/ internal standard) and x = concentration of analyte. The measured lower limit of quantification (LLOQ) for TAC was 62.5 μg/mL. The calculated limit of detection (LOD) and limit of quantification (LOQ) were 13.46 μg/mL, and 40.80 μg/mL, respectively. The LOD and LOQ were calculated according to ICH guidelines (ICH, 2006) as follows:

$$\boldsymbol{LOD} = 3.3\boldsymbol{\sigma}/\boldsymbol{S} \quad (1)$$

$$\boldsymbol{LOQ} = 10\boldsymbol{\sigma}/\boldsymbol{S} \quad (2)$$

where σ is the standard deviation of the response, and S is the slope of the calibration curve.

We manufactured 0.1% w/v of TAC eye drops as this concentration has been tested in patients with severe allergic conjunctival diseases (Abeysiri et al., 2010; Fujishima et al., 2010). The solvent evaporation method approach utilizing the MET polymer was used to formulate the lipophilic drug TAC. The level of palmitoylation and quaternization (mole%) of MET can be altered to meet the specific requirements of the formulation (Chooi et al., 2014). In this study, we chose MET with similar levels of palmitoylation (18%) and quaternization (18%) to balance the lipophilicity and hydrophilicity of the polymer. We adopted this strategy in order to overcome the corneal barrier functions (Baba et al., 2011; Li et al., 2015). The corneal epithelium is lipophilic, which will restrict the movement of hydrophilic compounds, whereas the stroma is hydrophilic and will limit the movement of lipophilic compounds. This dual nature of the cornea acts as a strong barrier for drug entry into the ocular globe. Therefore, compounds must possess both hydrophilic and lipophilic properties to overcome the corneal barrier.

The encapsulation method showed a high drug content of aqueous TAC in the formulation after filtration (0.1 ± 0.002% w/v) with reproducible results over three independent experiments.

The particle size and particle surface potential were determined for the prepared formulations. The formulations possessed two nanoparticle populations after filtration. The main peak population (~200 nm) was presumed to be the encapsulated TAC in the formulation. The second peak population with smaller particle sizes (~10 nm) was assumed to be empty MET micelles as has been reported previously (Qu et al., 2006). MET possess an overall positive charge due to the presence of quaternary ammonium groups on their surface (Lalatsa et al., 2012) and as such, we obtained a positive zeta potential value with the MET-TAC formulation (16 ± 4.6 mV) over three independent experiments.

The viscosity of the formulation was reported as 1.7 ± 0.003 mPa.s at 25 °C from three independent experiments. All prepared formulations were examined for pH and osmolarity. The pH of the formulation was adjusted to 7.1 ± 0.1 with 1.0 M NaOH. Topical ophthalmic drops require an osmolarity of 270–340 mOsm/L (Dutescu et al., 2015) to be within the ocular comfort range. Our formulation has an osmolality value within the range tolerable by the eye (327 ± 3.05 mOsm/kg).

3.2. Stability measurements

The stability of the MET-TAC formulations was determined by measuring the physicochemical properties over 30 days. Table 5 shows the properties of all formulations upon storage at different conditions for one month. After 30 days of storage, the formulation stored in the fridge displayed excellent drug content stability compared to formulations stored at room temperature and 40 °C at all time points ($p < 0.05$). The

Table 4
TAC HPLC assay parameters.

Parameter	Quantification range (μg/mL)	Equation of the straight line	r^2	Limit of detection (μg/mL)	Limit of quantification (μg/mL)
Value	62.5–1000	y = 5667.3x + 11.85	0.9996	13.46	40.80

Table 5
Parameters of MET-TAC stored at the fridge (5 ± 3 °C), room temperature (25 ± 2 °C), and at an accelerated condition (40 ± 2 °C) for 30 days.

Storage condition	Parameters	Day 0	Day 7	Day 30
5 ± 3 °C	**Drug concentration (mg/mL)**	0.99 ± 0.002	1.00 ± 0.001^{ns}	0.94 ± 0.049 *
	pH	7.1 ± 0.1	6.8 ± 0.01 #	6.9 ± 0.1 #
	Main peak size (nm)	235 ± 64	234 ± 16^{ns}	373 ± 7 *
	Peak intensity (%)	(49 ± 5%)	(53 ± 2%)	(61 ± 2%)
	Second peak size(nm)	13 ± 1	11 ± 1^{ns}	13 ± 2 *
	Peak intensity (%)	(48 ± 2%)	(47 ± 2%)	(39 ± 2%)
	Zeta potential (mV)	+16 ± 4.6	+21 ± 4.2^{ns}	+19 ± 2.4^{ns}
	Osmolarity (mOsm/kg)	327 ± 3.1	319 ± 3.1^{ns}	326 ± 10.02^{ns}
	Viscosity (mPa.s)	1.7 ± 0.003	1.6 ± 0.04 #	1.5 ± 0.01 #
25 ± 2 °C 60 ± 5% RH	**Drug concentration (mg/mL)**	0.99 ± 0.002	0.87 ± 0.004 #	0.41 ± 0.006 #
	pH	7.1 ± 0.1	6.7 ± 0.02 #	6.5 ± 0.05 #
	Main peak size (nm)	235 ± 64	194 ± 17^{ns}	174 ± 11^{ns}
	Peak intensity (%)	(49 ± 5%)	(40 ± 2%)	(38 ± 1%)
	Second peak size(nm)	13 ± 1	12 ± 1^{ns}	12 ± 1^{ns}
	Peak intensity (%)	(48 ± 2%)	(60 ± 2%)	(62 ± 1%)
	Zeta potential (mV)	+16 ± 4.6	+21 ± 3.1^{ns}	+24 ± 2.3^{ns}
	Osmolarity (mOsm/kg)	327 ± 3.1	314 ± 4.5 *	314 ± 3.1 *
	Viscosity (mPa.s)	1.7 ± 0.003	1.5 ± 0.02 #	1.7 ± 0.01^{ns}
40 ± 2 °C 75 ± 5% RH	**Drug concentration (mg/mL)**	0.99 ± 0.002	0.27 ± 0.002 #	0.03 ± 0.001 #
	pH	7.1 ± 0.1	6.8 ± 0.01 #	6.3 ± 0.01 #
	Main peak size (nm)	235 ± 64	156 ± 12^{ns}	218 ± 52^{ns}
	Peak intensity (%)	(49 ± 5%)	(34.43 ± 2.19)	(33.97 ± 3.12%)
	Second peak size(nm)	13 ± 1	12 ± 1^{ns}	13 ± 1^{ns}
	Peak intensity (%)	(48 ± 2%)	(65.57 ± 2.19%)	(65.33 ± 3.10%)
	Zeta potential (mV)	+16 ± 4.6	+18 ± 3.1^{ns}	+23 ± 3.5^{ns}
	Osmolarity (mOsm/kg)	327 ± 3.1	308 ± 2.5 #	310 ± 4.5 #
	Viscosity (mPa.s)	1.7 ± 0.003	1.4 ± 0.03 #	1.6 ± 0.01 #

ns = not significant, * = $p < 0.05$, # = $p < 0.005$.

TAC in the formulation stored at refrigeration temperature (5 ± 3 °C) displayed a similar drug content at day 0 (0.99 ± 0.002 mg/mL) as compared to day 7 (1.0 ± 0.01 mg/mL, $p > 0.05$), but different than day 30 (0.94 ± 0.049 mg/mL, $p < 0.05$).

MET-TAC formulations stored at room temperature and under accelerated conditions showed significant changes in drug content at days 7, and 30 compared to day 0 ($p < 0.05$). The TAC concentration at (40 ± 2 °C / 75 ± 5% RH) fell substantially from (0.99 ± 0.002 mg/mL) to (0.04 ± 0.001 mg/mL) after 30 days ($p < 0.05$), indicating that almost 95% of the drug was lost from its original content.

Trasi et al. showed that TAC was physically unstable when prepared with ethanol at an elevated temperature and stored for 4 weeks at (40 ± 2 °C / 75 ± 5% RH), as TAC reverts to its crystalline form on storage (Trasi et al., 2017a). Peterka et al. reported the sensitivity of an amorphous form of TAC at an elevated temperature (50 °C / 75% RH) (Peterka et al., 2019). The proportion of TAC impurities in amorphous material increased with higher moisture content absorbed on the surface, thus providing reaction media or acting as a reactant for the chemical degradation of the TAC amorphous material (Peterka et al., 2019).

The MET-TAC formulation was prepared using ethanol, and the ethanol was evaporated using the thin-film hydration method. TAC in the formulation was precipitated at an elevated temperature, and this precipitation could be due to TAC returning to its crystal nature upon storage at accelerated storage conditions (40 ± 2 °C / 75 ± 5% RH).

The optimum pH for eye drops equals that of tear fluid and is 7.4 (Baranowski et al., 2014). If the pH value gets outside the acceptable range that is tolerated by eye 6.6–7.8 (Garcia-Valldecabres et al., 2004), the patient may feel discomfort, there may be irritation, and the drug bioavailability may decrease because of increased tearing (Banik, 2011). All prepared formulations were examined for pH. The pH of the formulation was adjusted to 7.1 ± 0.1 with 1.0 M NaOH. The MET-TAC formulation stored in the fridge showed a pH value within an acceptable range (6.8–7.1). The pH of the MET-TAC formulation stored at room temperature showed a reduction in the pH after 30 days of storage (6.5 ± 0.05, $p < 0.05$). The pH of the MET-TAC formulation stored at (40 ± 2 °C) showed a reduction in the pH after 30 days of storage (6.3 ± 0.01, $p < 0.05$) as compared to day 0.

The size and surface potential were determined for the prepared formulations. The MET-TAC formulation possessed two nanoparticle populations after filtration. The average size of the main peak population following filtration was 235 ± 64 nm, and the corresponding percentage intensity was 49 ± 4.6%. The size of the smaller population was 13 ± 0.5 nm, with a percentage intensity of 48 ± 2.0%. There were no significant differences in the formulation particle size at day 0 to day 7, and 30 days of storage at room temperature and at accelerated conditions ($p > 0.05$). The change in the formulation particle size when the formulation was stored in the fridge at day 30 compared to day 0 was significant ($p < 0.05$). However, the sample remained clear of any visual precipitation. It has been reported that in aqueous environments, MET self-assembles to form polymeric micelles with a particle size between 5 and 30 nm (Serrano et al., 2015). The size distribution results showed a bimodal distribution and this bimodal size is believed to be due to an equilibrium being established between drug-filled nanoparticles and empty micelles (Qu et al., 2006; Serrano et al., 2015).

The zeta potential of the formulations was measured. There were no significant differences in the formulation zeta potential on day 0, day 7, and day 30 irrespective of the storage conditions ($p > 0.05$), indicating good physical stability of the nanoformulation due to electrostatic repulsion of individual particles (Joseph and Singhvi, 2019). The positive charge of the particles is desirable to prevent particle aggregation, promote colloidal stability and facilitate electrostatic interactions with the negatively charged sialic acid residues of mucin in the eye surface (Silva et al., 2017) and MET is known to be mucoadhesive in the gastrointestinal tract (Siew et al., 2012).

The tears have tonicity equivalent to 0.9% w/v NaCl solution (Cholkar et al., 2015) (300 mOsm/L) (Iyamu and Enobakhare, 2019; Strandvik, 2009). Topical ophthalmic drops are required to be at an optimal osmolarity (~300 mOsm/L) to avoid adverse effects on the eye, with a mean osmolarity for healthy eyes ranging from 270 (CI ± 261–309 mOsm/L) to 328 mOsm/L (CI ± 279–365 mOsm/L) (Baenninger et al., 2018). Our formulations are isotonic and have an osmolarity value range of 308–327 mOsm/kg, which is within the range that is tolerable for the eye. Hypertonic and hypotonic formulations can induce a burning sensation after instillation, leading to excessive lacrimation and consequently, drug removal from the ocular surface (Awwad et al., 2017). Clinical evaluation of TAC eye drops with high osmolality values of more than 1000 mOsm/kg has shown that these formulations induce ocular irritation in patients following the instillation of the formulation into the eyes (Luaces-Rodríguez et al., 2018).

The viscosity of the formulations at day 0 was 1.7 ± 0.003 mPa.s at 25 °C. The viscosity of the formulation is about twice that of plain water

(0.89 mPa.s at 25 °C) (Korson et al., 1969), due to the presence of the MET polymer in the formulation (Qu et al., 2006). Clearance from the eye is reduced with the more viscous eye drop formulations (Gagliano et al., 2018). Zhu et al. reported that a viscosity value (4.4 mPa.s) of Newtonian fluids is required to reduce the tear drainage rate (Zhu and Chauhan, 2008). Other studies showed that eye drops with a viscosity of 10 mPa.s is required to maintain the precorneal residence time in human eyes (Rahman et al., 2012; Zaki et al., 1986). MET is a quaternary ammonium and thus positively charged glycol chitosan based amphiphile that is a known mucoadhesive (Siew et al., 2012). It is conceivable that this positively charged polymer would electrostatically bind to negatively charged sialic acid residues of mucin in the eye surface, thus, promoting the ocular drug absorption through intimate contact with the corneal surface and the diffusion of the cargo into ocular tissues due, essentially to the chitosan mucoadhesive properties. As the MET-TAC formulation is intended for topical ocular administration in the eye, the slightly increased viscosity could be found to be essential to promoting a prolonged residence time in the eye in addition to the MET bioadhesive properties (Siew et al., 2012). However, a nanoparticles formulation attached with fluorescent dye to confirm the prolonged residence time has not been carried out.

Fig. 1 showed the results of the visual inspection of all formulations upon storage at three different conditions. There was a loss of the drug content for the formulations stored at ambient (25 ± 2 °C / 60 ± 5% RH) and accelerated (40 ± 2 °C / 75 ± 5% RH) conditions, and this drug loss can be observed with the precipitation of the formulation after 30 days (Fig. 1). The MET-TAC formulation stored in the fridge for 30 days shows a clear formulation compared to formulations stored at room temperature and under accelerated conditions. This finding was consistent with the findings of others. Rodriguez et al. demonstrated that TAC formulated with Liquifilm eye drops containing polyvinyl alcohol (0.03% w/v), did not degrade when stored for 90 days at low temperature, either in the fridge (5 ± 3 °C) or the freezer (−17.5 ± 2.5 °C) (Luaces-Rodríguez et al., 2018). They concluded that the TAC formulation stored at room temperature showed a considerable decline by 50% in the drug content (0.015% w/v) (Luaces-Rodríguez et al., 2018). We found a similar observation where TAC stored at room temperature showed a reduction in the drug content by ~50% after 30 days (0.99 mg/mL for day 0, and 0.41 mg/mL for day 30).

Based on these results, our formulation appeared to be stable against degradation when stored in the fridge. Thus, samples of TAC should be analyzed immediately or stored at low temperature (+4 °C).

3.3. In vivo study

3.3.1. Bioanalytical LC-MS/MS assay

The LC-MS/MS chromatograms for TAC and ascomycin are presented in (supplementary material, Fig. 1). Both TAC and ascomycin were ionized under the positive electrospray ionization for analyte quantification. The sodium adduct is employed as it was stable in the non-ammoniated mobile phase. The formation of the adduct is a standard ionization method in electrospray ionization mass spectrometry (Kruve and Kaupmees, 2017). It has been reported that sodium adduct formation tends to dominate in weakly acidic mobile phases containing 0.1% formic acid (Kruve and Kaupmees, 2017). Guan et al. observed the formation of [M+Na]+ in a mobile phase containing 0.1% formic acid (Guan et al., 2003). Utilizing a formic acid-containing mobile phase has the advantage that it never clogs the tubes and the seals (Earla et al., 2012). Also, the sodium adduct could have originated from glassware, stainless steel, or as an impurity in chemicals or solvents (Kruve and Kaupmees, 2017; Mortier et al., 2004). Also, the formation of sodium adducts in the analysis could be due to the addition of normal saline in the extraction process. Jurchen et al. reported the formation of a sodium adduct when sodium chloride was employed in the extraction analysis (Jurchen et al., 2003).

Multiple reactions monitoring mode was utilized to detect TAC and ascomycin. The precursor ions to product ions transitions of *m/z* [M+Na]+ (826.3 → 415.2) and *m/z* [M+Na]+ (814.2 → 604.1) were chosen for TAC and ascomycin, respectively, based on the most abundant product ions. The assay conditions had an adequate specificity for TAC, while no interfering peaks were observed at its retention time. The retention time was 2.11 and 2.10 min for TAC and ascomycin, respectively (supplementary material). The MRM transition of TAC used is similar to that used in a recent report (Siegl et al., 2019). Ascomycin was used as the internal standard because of its structural similarity to TAC, and its similar fragmentation pattern.

3.3.2. Preparation of standard and quality control curves

Working standards solutions were prepared to obtain a standard curve of TAC in the mobile phase and in each of the tissues: cornea, conjunctiva, sclera, choroid-retina, aqueous humor, vitreous humor, and whole blood.

Table 6 shows the assay parameters used to analyze the tissues. Individual calibration curves for the blank and each tissue obtained by plotting the peak area ratios (TAC/ internal standard, y) versus the analyte concentration (x). The calibration curves were linear for TAC in a concentration range of 0.5–250 ng/mL when extracted from all tissues, with an r^2 greater than 0.99.

TAC and ascomycin were extracted and separated from the blank and tissues matrices. The measured LLOQ for TAC was 0.5 ng/mL for the blank and all the tissues with a signal to noise ratio (SNR) ≥ 10 at the lower limit of quantification. The signal to noise ratio describes the

Table 6
Assay parameters of TAC in blank and ocular tissues.

Parameters	Equation of the straight line	r^2	Accuracy (%)	Precision (%)
Blank	y = 0.4936x + 2.5483	0.9939	–	–
Conjunctiva	y = 0.5766x + 0.9188	0.9980	114.25	10.65
Aqueous humor	y = 0.4865x + 1.5001	0.9973	98.92%	11.88
Vitreous humor	y = 0.4937x + 1.2581	0.9987	110.32	26.65
Cornea	y = 0.5369x + 1.528	0.9964	105.53	11.86
Choroid-retina	y = 0.4892x + 0.6221	0.9998	96.30	14.14
Sclera	y = 0.5126x + 2.1769	0.9942	99.33	4.92
Whole blood	y = 0.4389x + 0.8	0.9994	88.41	14.52
Average			101.87	13.52
SD			8.81	6.60

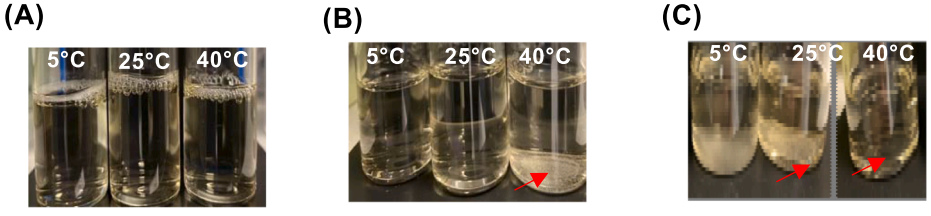

Fig. 1. MET-TAC visual inspection stability over 30 days of storage. Day 0, (B) Day 7, (C) Day 30. Left: Fridge (5 °C), Middle: Room temperature (25 ± 2 °C / 60 ± 5% RH), Right: Accelerated condition (40 ± 2 °C / 75 ± 5% RH).

extraction efficiency of the assay and quantifies the capability to remove all endogenous interfering components, which are usually present in the biological matrix (Earla et al., 2012). Generally, the SNR threshold of 10 is used for LC-MS analyses (Zhang, 2012).

Accuracy was examined by analyzing ocular tissues and the blank sample, in which the drug was dissolved in the mobile phase (Table 6). Accuracy was calculated by dividing the ratio of the peak area in the presence of matrix, to the peak area in the absence of matrix multiplied by 100 (Guideline on bioanalytical method validation, 2012). Accuracy of the method must be between 85% and 115% of the nominal value in all the standards, except at the LLOQ level, which is 80–120% according to the guidance for industry bioanalytical method validation in Food and Drug Administration guidelines (Bioanalytical Method Validation Guidance for Industry, 2018; Earla et al., 2012). The accuracy of all the standards is 101.87%; this is in line with the guideline on bioanalytical method validation in which a range ± 15% of the nominal value is acceptable for the non-zero calibrators, and between ± 20% at the LLOQ. The accuracy of all the standards is presented in (Table 6).

Precision was calculated using the coefficient of variation (CV) (standard deviation/mean) multiplied by 100. The precision of the method should be within 15% of the nominal concentration except at the LLOQ, which is within 20% (Bioanalytical Method Validation Guidance for Industry, 2018). The average CV of all the standards is 13.52%. These results demonstrate a CV within the acceptable value.

3.3.3. MET-TAC pharmacokinetics animal study

We examined the possible delivery to the front of the eye using MET-TAC (25 μL, 0.1% w/v). The ocular tissue distribution study was conducted in New Zealand White healthy rabbits following topical instillation of the formulation into the conjunctival cul-de-sac.

Topical single instillation of 0.1% w/v MET-TAC resulted in detectable and quantifiable TAC levels in the front of the eye tissues, i.e. cornea and conjunctiva (Fig. 2). TAC concentrations were also quantified in the aqueous humor. Moreover, TAC was detected in the choroid – retina, a posterior chamber eye tissues (Fig. 2). No TAC was discovered in the vitreous humor (LLOQ = 0.5 ng/mL) and the whole blood (LLOQ = 0.5 ng/mL) at all time points.

Fig. 2 shows the ocular drug distribution of TAC in a healthy rabbit

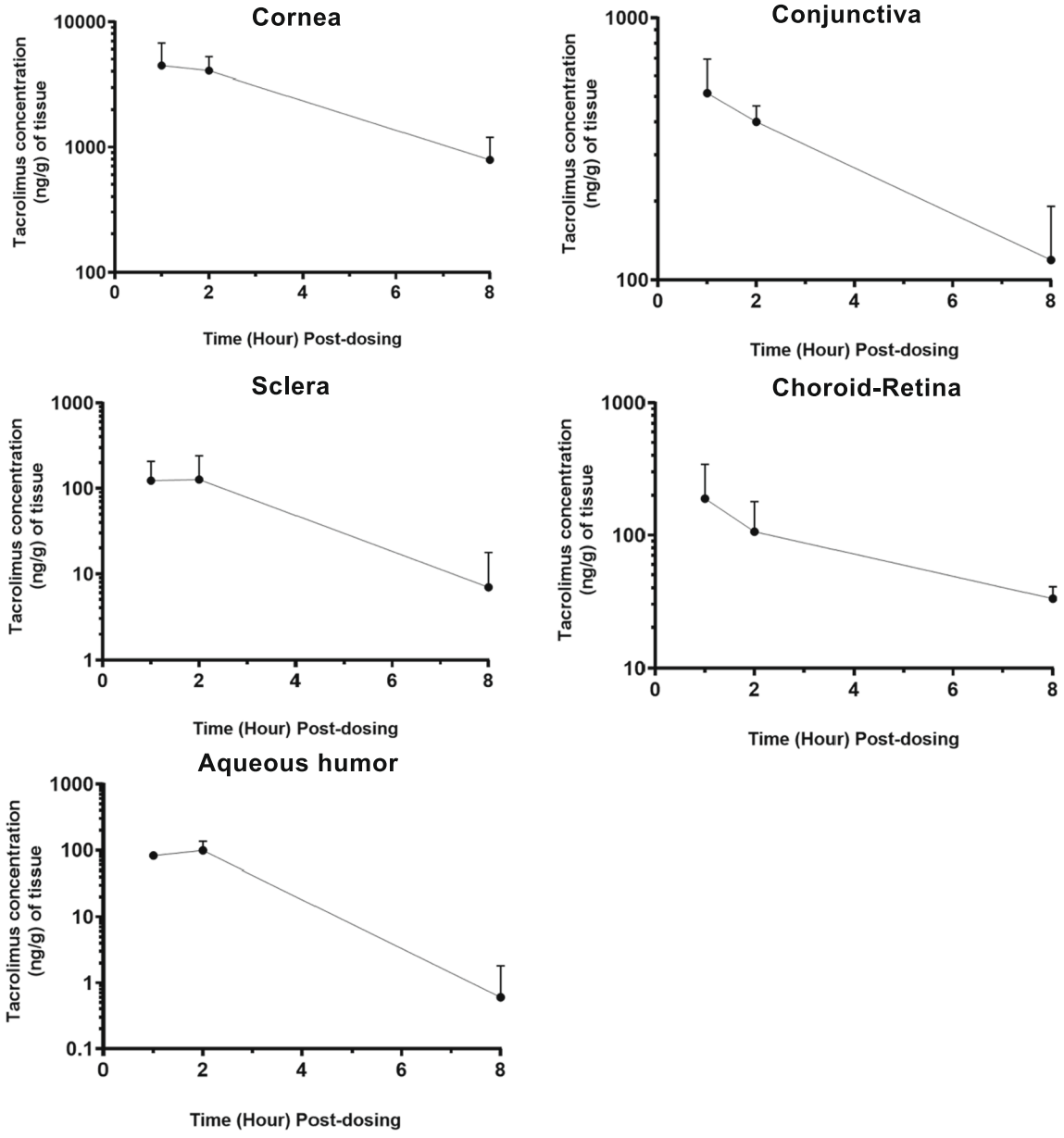

Fig. 2. In vivo TAC drug distribution in rabbit ocular tissues following the instillation of 25 μL of MET-TAC 0.1% w/v.

model, and the pharmacokinetic parameters are presented in (Table 7).

The Cmax of TAC in the cornea, conjunctiva and sclera after 1 h was 4452 ± 2289 ng/g, 516 ± 180 ng/g and 123 ± 84 ng/g of tissue, respectively with a tmax of 1 h in all tissues except the sclera where the tmax was 2 h. These tissue levels were achieved following a single ocular dose of 25 μg TAC in MET-TAC. A recent study reported TAC levels in pig corneas following multiple instillations of an aqueous formulation (50 μL, 0.07%) (Siegl et al., 2019). This formulation is based on Marinosolv technology containing glycyrrhizin (2% w/v), escin (0.03% w/v), dexpanthenol (5% w/v), EDTA (0.1% w/v), mannitol (2.7% w/v), iota-carrageenan (0.24% w/v), and propylene glycol (3% w/v) in a buffered solution. TAC was dissolved in propylene glycol, and the dissolved TAC added to the aqueous solution (Siegl et al., 2019). With two doses per day (70 μg TAC/day), and four doses per day (140 μg TAC/day), samples were collected one hour after the last dose, and the TAC Cmax was reported as ~4.0 μg/g of the cornea, irrespective of the dose (tmax = 1 h) (Siegl et al., 2019). Ultimately this corneal level obtained with a TAC dose of 70–140 μg/day is similar to the corneal level obtained with a TAC dose of 25 μg/day in the current study (Table 7). For comparison, the ocular corneal Cmax of TAC in eyes instilled four times with Talymus, an ophthalmic TAC suspension (50 μL, 0.1% w/v, 200 μg TAC/day), was <0.5 μg/g (tmax = 1 h) (Siegl et al., 2019). In the current work, the MET-TAC (25 μL, 0.1% w/v, 25 μg TAC) formulation resulted in a higher corneal TAC level than the 8-fold higher Talymus dose (25 μg versus 200 μg).

Another study reported the Cmax of TAC following a single ocular instillation in rabbits (30 μL, 0.1% w/v, 30 μg TAC/day) of TAC ophthalmic suspension (Fujita et al., 2008) with TAC levels in the cornea of ~300 ng/g, TAC levels in the conjunctiva of ~100 ng/g, and TAC levels in the sclera ~100 ng/g of tissue after 1 h, respectively (Fujita et al., 2008). Once again, the current MET-TAC formulation delivered higher levels to the cornea, conjunctiva and sclera even though the TAC doses were similar (25 μg versus 30 μg).

The nanoparticle MET-TAC formulation is clearly superior in delivering the drug to the cornea and conjunctiva when compared to suspensions. In a study comparing the drug delivery efficiency in steroid solutions, gels, and suspensions (Nourry et al., 2011), Nourry et al. found that eye drop suspensions delivered less drug when compared to solutions and gels (Nourry et al., 2011). The suspensions delivered variable levels of drug (23–99%) into each drop of the formulation, compared to solutions and gels, which released about 100% of the content of the drug in each drop (Nourry et al., 2011). This could explain the higher tissue levels measured with solutions when compared to the use of a suspension (Fujita et al., 2008; Siegl et al., 2019). It must be stated that a straightforward comparison between both formulations was not carried out. These TAC tissue levels from the MET-TAC 0.1% w/v formulation would be sufficient to modify the local immune responses, to suppress the inflammatory and dry-eye conditions, to treat other ocular autoimmune diseases and may be used to prevent the corneal transplant rejection (Kalam and Alshamsan, 2017).

Following the ocular administration of the MET-TAC (25 μg), the Cmax of TAC in the aqueous humor was 99 ± 37 ng/mL (tmax = 2 h), while no drug was detected in the vitreous humor. In a recent study, Abul Kalam et al. reported the level of TAC in the aqueous humor of rabbits eyes after a single instillation of TAC loaded ploy-lactide-co-glycolide (PLGA) nanoparticles in comparison to TAC aqueous suspension (50 μL, 0.03% w/v, 15 μg TAC) (Kalam and Alshamsan, 2017). The concentration of TAC in the aqueous humor was 20 ng/mL and 30 ng/mL, from the TAC-PLGA and TAC aqueous suspension, respectively (Kalam and Alshamsan, 2017). When adjusting for dose and assuming linearity, MET-TAC delivers an estimated 2-fold greater level of TAC in the aqueous humor when compared to the use of PLGA nanoparticles or a suspension. The PLGA nanoparticles described by Abul Kalam et al. had a net negative surface charge (Kalam and Alshamsan, 2017). In contrast, the MET-TAC formulation has a permanent positive charge, and this may explain the higher levels of TAC in the aqueous humor obtained with the MET-TAC formulation when compared to the PLGA nanoparticle formulation. TAC from MET-TAC (25 μL, 0.1% w/v, 25 μg TAC) was also delivered to the back of the eye on topical ocular administration with a Cmax of TAC in the choroid-retina of 189 ± 154 ng/g (tmax = 1 h). On administration of TAC suspension, the levels of TAC in the choroid-retina were < 10 ng/g of tissue after a single instillation of 30 μg TAC from the TAC ophthalmic suspension (Fujita et al., 2008). Siegl et al. have reported the Cmax level of TAC in the pig choroid was 100 ng/g, or 50 ng/g of tissue (tmax = 1 h), after two doses per day (70 μg TAC/day) or four doses per day (140 μg TAC/day), respectively (Siegl et al., 2019). In the same study, TAC Cmax in the pig retina was 60 ng/g and 90 ng/g of tissue (tmax = 1 h) after dosing 70 μg TAC/ day or 140 μg TAC/ day, respectively (Siegl et al., 2019).

We examined the blood exposure to TAC following ocular dosing. We reasoned that the conjunctival blood capillaries might lead to TAC being detected in the blood. A certain fraction of the drug may reach the systemic circulation via conjunctival vessels and nasolacrimal duct (Irimia et al., 2018). Thus, we examined the distribution of TAC in the whole blood of the treated rabbits at all time points. The distribution of TAC between whole blood to plasma is > 20:1; therefore, measurements of TAC is generally undertaken in the blood rather than plasma (Taylor et al., 1996). TAC was not detected in whole blood (LLOQ 0.5 ng/mL) at all time points. Fujita et al. reported the level of TAC in whole blood following 0.1% TAC suspension eye drops (~1 ng/g of tissue after 1-hour post-dosing (Fujita et al., 2008). Although the therapeutic level of TAC is (5–10 ng/mL) (Robles-Piedras and González-López, 2009), such a formulation tends to work locally in the eye, and it is not desirable for TAC to be in the systemic circulation, due to the possibility of drug adverse events. Our results demonstrated an absence of TAC in the blood, which is a preferable outcome for the management of allergic ocular diseases.

TAC has a molecular weight of 804.02 Da (Trasi et al., 2017b), and a partition coefficient (log *P*) of 2.74 (Fujita et al., 2008). We hypothesize that such a lipophilic drug, when presented to the cornea in molecular form (e.g. when released from MET nanoparticles) may partition into the cornea through the epithelium and be gradually released into the aqueous humor through the stroma and endothelium (Fujita et al., 2008). It is conceivable that the positively charged nanoparticles would allow an electrostatic interaction with the negatively charged mucin from the ocular surface (Irimia et al., 2018). Due to the presence of the sialic acid residues from the terminal ends of the mucopolysaccharide chain, the mucin could interact electrostatically (Irimia et al., 2018) with the cationic MET particles. MET nanoparticles have a permanent positive charge, due to the presence of quaternary ammonium groups on the surface of the nanoparticles (Hecq et al., 2015; Kanwal et al., 2019; Serrano et al., 2015). Thus, we postulated that MET would be electrostatically attached to the negatively charged membrane in particulate form and prolong the corneal and conjunctival retention of TAC, and may lead to slow diffusion of the drug from the cornea to the aqueous humor (Agrahari et al., 2016).

We have shown that MET was able to promote the permeation of the insoluble TAC into ocular tissues and to deliver the drug to the cornea and conjunctiva (target tissues) in a concentration higher than its therapeutic levels (5–10 ng/mL) (Robles-Piedras and González-López, 2009). These results indicate that MET may be used as a novel carrier for

Table 7
Pharmacokinetics parameters of TAC after single ocular instillation of MET-TAC ophthalmic formulation solution in rabbits.

Pharmacokinetic parameters	Cornea	Conjunctiva	Sclera	Choroid-Retina	Aqueous humor
C_{max} (ng/g tissue)	4452 ± 2289	516 ± 180	123 ± 84	189 ± 154	99 ± 37
T_{max} (hr)	1	1	2	1	2
AUC_{0-8} (ng.hr/g)	21,026	2277	587	660	430

TAC and overcomes both the static and dynamic barriers to reach the ocular tissues. The exact mechanism of how MET delivered TAC to the back of the eye tissues is not clear, and further studies to confirm the mechanistic pathway are required.

3.3.4. Ocular tolerability

The blink rate following dosing is an acceptable method of assessing acute ocular irritation, although other parameters such as erythema and swelling are also used to assess ocular irritation (Carlisle and Digiovanni, 2015). In the first 60 s post-dosing after a single instillation of MET-TAC (25 μL, 0.1% w/v), the blink rate was assessed. After topical drop instillation, the upper and lower eyelids were hand-held together for approximately 5 s to permit the formulation to come into contact with the cornea, and the blink rate in the next 60 s was recorded. Normal saline was used as a control (n = 4). The average blink rate in both eyes in the normal saline group was recorded as 1.0 ± 0.41 blinks/min. Normal saline is frequently utilized as a control (Kalam and Alshamsan, 2017) in assessing the blink rate in rodents (Kaminer et al., 2011), and to evaluate the lacrimation characteristics in rabbits (Whittaker and Williams, 2015). The average blink rate in both eyes in all treatment groups was reported as (7.3 ± 1.32 blinks/ min, $p > 0.05$) (Fig. 3). Toshida et al. reported the blink rate / 3 min in healthy rabbits receiving a single instillation of 40 μL, 0.1% w/v CsA eye drops (Toshida et al., 2009). The blink rate of CsA-treated eyes (4.86 ± 0.86 blinks per 3 min) was significantly higher than that of vehicle-treated eyes (1.71 ± 0.42 blinks per 3 min) 3 h after instillation ($p < 0.05$) (Toshida et al., 2009). Thus, the blink rate data in rabbit treatment groups when compared to the human blink rate (17.6 ± 2.4 blinks/min during rest) (Bentivoglio et al., 1997; Kaminer et al., 2011), may suggest that there is a difference in the reaction to drugs between rabbits and humans because rabbits seem to show higher ocular sensitivity to instilled substances (Toshida et al., 2009), and there may be a more considerable contact time for drugs topically applied in the animal, due to its significantly lower blink rate (Maurice, 1995). It is noteworthy that a direct comparison to a standard irritant substance such as raw material or high concentrations of sodium lauryl sulphate (Bondi et al., 2015) has not been carried out. However, The number of blinks was taken as a preliminary investigation into the possible irritant nature of the formulation.

(Top) Number of blinks in rabbits following single instillation of MET-TAC (25 μL, 0.1% w/v); (Bottom) visual assessment of perceptible irritation of the MET-TAC formulation to rabbits' eyes. Time points: 1, 2, 8 h post-dosing (n = 4 rabbits, each group). Statistical analysis was performed on the blink rate with One-way ANOVA with Tukey's multiple comparison analysis. The value $p < 0.05$ was considered significant. The ocular irritation of the MET-TAC formulation was visually evaluated using the parameters of erythema and swelling (Fig. 3). There were no visible signs of erythema, tearing or swellingfollowing the ocular administration of MET-TAC. These data suggested that MET-TAC, while increasing the rabbit eye blink rate, does not cause dose-limiting ocular irritation to rabbits' eyes.

4. Conclusion

With the difficulties of applying topical ointment and suspensions

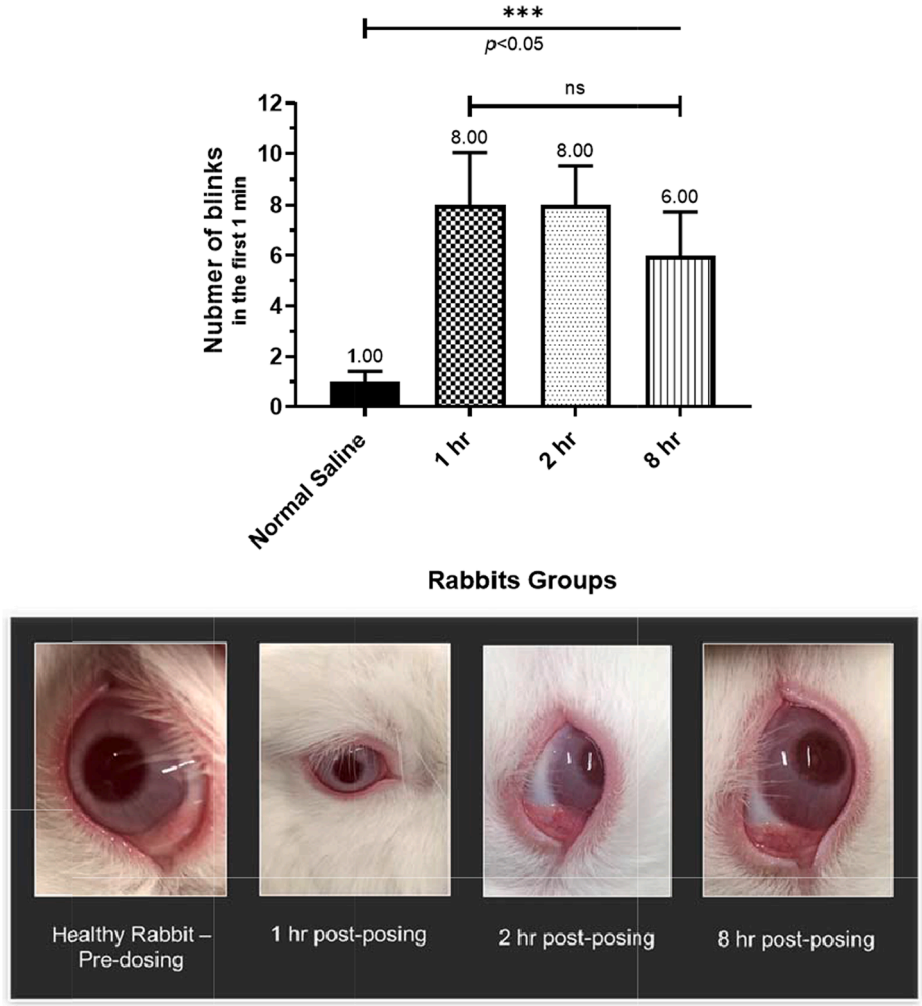

Fig. 3. Assessment of ocular tolerability of the MET-TAC formulation to rabbits' eyes.

forms of TAC in ocular allergic conditions, here, the utility of using a 0.1% w/v aqueous-based MET-TAC formulation has been demonstrated. The formulation showed a high drug content of TAC (0.1% w/v) that was stable for 30 days on refrigeration. The formulation was sterile filtered and within the optimum pH and tonicity, ideal for an eye drop formulation. A single topical dose of MET-TAC in healthy New Zealand White rabbits was able to deliver high levels of the drug to the cornea and conjunctiva (the target tissues), with a TAC concentration many folds higher than the therapeutically effective levels. This formulation may be useful in the treatment of AKC and VKC.

Credit author statement

Moutaz Y. Badr (MYB) performed most of the experiments and wrote the first draft of the manuscript, Nurul S. Abdulrahman (NSA) performed some of the stability experiments, Andreas. G. Schätzlein (AGS) helped design the study, Ijeoma. F. Uchegbu (IFU) led the study, designed the study and wrote part of the manuscript.

Declaration of Competing Interest

I.F. Uchegbu and A.G. Schatzlein are directors of Nanomerics Ltd. Apart from that, the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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