European Journal of Pharmaceutical Sciences 120 (2018) 152–161

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Preclinical characterization and clinical evaluation of tacrolimus eye drops

Andrea Luaces-Rodríguez[a,b,1], Rosario Touriño-Peralba[c,1], Iria Alonso-Rodríguez[a], Xurxo García-Otero[a], Miguel González-Barcia[a,b,d], María Teresa Rodríguez-Ares[c], Laura Martínez-Pérez[c], Pablo Aguiar[e], Noemí Gómez-Lado[e], Jesús Silva-Rodríguez[e], Michel Herranz[e], Álvaro Ruibal-Morell[e], María Jesús Lamas[b,d], Francisco J. Otero-Espinar[a,*], Anxo Fernández-Ferreiro[a,b,d,**]

[a] Department of Pharmacology, Pharmacy and Pharmaceutical Technology and Industrial Pharmacy Institute, Faculty of Pharmacy, University of Santiago de Compostela (USC), Santiago de Compostela, Spain
[b] Clinical Pharmacology Group, University Clinical Hospital, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain
[c] Ophthalmology Department, University Clinical Hospital Santiago de Compostela (SERGAS), Santiago de Compostela, Spain
[d] Pharmacy Department, University Clinical Hospital Santiago de Compostela (SERGAS), Santiago de Compostela, Spain
[e] Molecular Imaging Group, University Clinical Hospital, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain

ARTICLE INFO

Keywords:
Corneal residence time
Cytotoxicity
Effectiveness
Eye drops
Stability
Tacrolimus

ABSTRACT

Severe allergic ocular diseases as atopic keratoconjunctivitis can induce corneal damage due to inflammatory substances released from giant papillae. Tacrolimus eye drops are one of the current therapeutic alternatives for its treatment. This work is aimed at developing and characterizing a 0.03% tacrolimus ophthalmic formulation, which was introduced in three types of vehicles (BBS, PVA and Hyaluronic Acid). For this, we have performed in vitro (stability studies) and in vivo assays (corneal permanence time measured directly by Positron Emission Tomography) of three potential formulations. Next, the best formulation was selected, and its toxicological profile and clinical effectiveness have been evaluated. The biopermanence studies (direct measurements and PET/CT) showed that the formulations with PVA and Hyaluronic Acid present more retention time on the ocular surface of rats than PBS. From the stability study, we have determined that tacrolimus with PVA in cold storage is the best option. Tacrolimus with PVA has shown lower cytotoxicity than cyclosporine at early times. On the other hand, the pilot study performed has shown significant improvements in patients, with no noticeable adverse reactions. Based on stability, biopermanence, safety and clinical effectiveness studies, we concluded that tacrolimus-PVA eye drops are a suitable candidate for its clinical application in inflammatory ophthalmology diseases.

1. Introduction

Severe allergic ocular diseases as atopic keratoconjunctivitis (AKC) or vernal keratoconjunctivitis (VKC) can induce corneal damage due to inflammatory substances released from giant papillae (Bielory and Bielory, 2016; Christensen et al., 2017). Moreover, the inflammation has been shown to be a key factor in the pathogenesis of other pathologies as dry eye, a pathology that affects thousands of people around the world (Lienert et al., 2016), or other complex ocular diseases as uveitis (Ghasemi, 2017). The pathogenesis involving these diseases is probably multifactorial; however, inflammation plays a major role in which Th2-driven mechanism with the involvement of mast cells, eosinophils, and lymphocytes has been suggested (Bonini et al., 2004).

Maintaining an appropriate eye health is essential to down-regulate the ocular immune response, preserve the integrity of the ocular architecture and decrease the associated symptomatology (Egwuagu et al., 2015). The current treatment of AKC is based on the use of topical antiallergic agents and corticosteroids, but the chronic use of topical corticosteroids may increase intraocular pressure and susceptibility to opportunistic infections (Whitcup and Azar, 2017). The second line of treatment is based on topical immunosuppressive drugs as cyclosporine

* Correspondence to: F. J. Otero-Espinar, Faculty of Pharmacy, University of Santiago de Compostela (USC) Pharmacology, Pharmacy and Pharmaceutical Technology Department, Seminario de Estudos Galegos s/n, E-15782 Santiago de Compostela, Spain.
** Correspondence to: A. Fernández-Ferreiro, Pharmacy Department, University Clinical Hospital Santiago de Compostela, A Choupana s/n, E- 15706 Santiago de Compostela, Spain.
E-mail addresses: francisco.otero@usc.es (F.J. Otero-Espinar), anxordes@gmail.com (A. Fernández-Ferreiro).
[1] The authors contributed equally: Rosario Touriño-Peralba and Andrea Luaces-Rodríguez.

https://doi.org/10.1016/j.ejps.2018.04.038
Received 7 November 2017; Received in revised form 2 April 2018; Accepted 24 April 2018
Available online 26 April 2018
0928-0987/

and tacrolimus. Cyclosporine and tacrolimus are not structurally related, however both bind with high affinity to immunophilin proteins that are present in most cells and the drug-receptor complex inhibits calcineurin. Cyclosporine binds to cyclophilins while tacrolimus binds to FK506 binding protein (FKBP). The formed complex of tacrolimus-FKBP-12, calcium, calmodulin and calcineurin inhibit the phosphatase activity of calcineurin. This lead to an inhibition of the translocation of some transcription factors (NF-AT), which ultimately decrease the transcription for genes which encode IL-2, IL-3, IL-4, IL-5, GM-CSF and TNF-, all of which cause the reduction of T-cell activation (Greiner and Dick, 2016). Its mechanism of action has promoted its use in pathologies such as corneal graft rejection (Joseph et al., 2007; Sloper et al., 2001), inflammatory conjunctival and corneal diseases ("Phase III Study of 0.1% Tacrolimus(FK506) Ophthalmic Suspension in Patients With Vernal Keratoconjunctivitis - Full Text View - ClinicalTrials.gov," 2014; Shoughy, 2017), uveitis (Ishioka et al., 1994; Mochizuki et al., 1993) or graft-versus-host disease (Magalhaes et al., 2013).

The use of ophthalmic cyclosporine has been described for several decades, yet only a few products have been successful in reaching the pharmaceutical market place. On the other hand, when it is necessary to use higher concentrations of cyclosporine, the Hospital Pharmacy Department (HPD) is responsible for its formulation as a sterile pharmaceutical compound (Lallemand et al., 2017; Stonecipher et al., 2016). Unlike cyclosporine, tacrolimus eye drops are not marketed and all its use rests in the elaboration in HPD. Nowadays, most of the topical ophthalmic solutions manufactured in these departments are based in vehicles with short retention time in corneal surface and consequently the need of frequent to obtain a sustainable benefit (Luaces-Rodríguez et al., 2017; Ribeiro et al., 2016). Several types of tacrolimus formulations as ointments (Dumrongkigchaiporn et al., 2004; Pastor-Clerigues et al., 2016), emulsions (Uno et al., 1997), liposomes (Pleyer et al., 1993), dextran conjugates (Yura et al., 1999) or cyclodextrins complexation (Hajiahmadi et al., 2017) have been described by other authors; however, if these are not synthesized to be marketed, their elaboration in HPD is complicated.

The use of new vehicles can be a feasible alternative that could help to overcome the high ocular clearance of conventional eye drops (Almeida et al., 2014). The increase of patient's compliance and the establishment of appropriate dosing schedules are key factors for improving the treatment of many ophthalmic pathologies (Patel et al., 2013). It is also important to determine safety as well as the stability of the new formulations in order to be administered to patients.

Nowadays, there are few studies on tacrolimus eye drops characterization, probably due to the short time as a therapeutic option. In this regard, the aim of this work is to develop and characterize a 0.03% tacrolimus ophthalmic formulation. For this, we have performed in vitro (stability studies) and in vivo (corneal permanence time measured directly by Positron Emission Tomography) assays of three potential formulations. Next, the best formulation has been selected. Its toxicological profile has been tested in vitro and its clinical effectiveness has been evaluated in patients.

2. Materials and methods

2.1. Preparation of formulations

50 mL of each three ophthalmic formulations with 0.03% (300 μg/mL) tacrolimus were prepared. Three millilitres of 5 mg/mL tacrolimus (Prograf® ampoules) were added into 47 mL of each vehicle. The first formulation (TBS) used BSS® (Balanced Salt Solution, Alcon®) as vehicle. The second formulation (TLI) used Liquifilm® (Allergan®; Composition: 1.4% Polyvinyl alcohol, Sodium chloride, Sodium phosphate dibasic, Sodium phosphate monobasic, Benzalkonium chloride, Edetate disodium and Purified water). The third formulation (THA) used hyaluronic acid (Acofarma®, Spain. Molecular weight 1.5 × 106–2.0 × 106 Da) dissolved in BSS® to obtain a mucoadhesive hyaluronic acid hydrogel at 0.4%.

Table 1
Grading scales for objective clinical signs clinical signs.

Signs	Score	Definition
Conjunctiva	3	Important dilatation of vessels
	2	Moderate dilatation of vessels
	1	Mild dilatation vessels
	0	None
Papillae	3	Giant (> 1 mm and elevated[a])
	2	Moderate (0.5–0.9 mm and flats
	1	Mild (0.1–0.4 mm and flats)
	0	None
Limbus	3	9 or more dots
Trantas'dots	2	5–8 dots
	1	1–4 dots
	0	None
Corneal sings	3	Shields ulcer or corneal erosion
	2	Exfoliation superficial punctate keratitis
	1	Superficial punctate keratitis
	0	None

[a] Elevated papillae in ½ or more of the upper palpebral conjunctiva.

2.2. Stability study

Three batches of each formulation (TBS, TLI, THA) were prepared for each storage condition. The formulations were stored in three different temperatures: room temperature (20 ± 2 °C), refrigerated (2 to 8 °C) and frozen (−15 to −20 °C), in all cases protected from light. A sample from each formulation and from each preservation condition was withdrawn immediately after preparation and at days 3, 7, 15, 30, 60 and 90. Osmolality, pH, microbiological control growth and concentration of drug were measured. All samples were also visually inspected for any macroscopic changes (e.g., colour, turbidity, precipitation). t90 was calculated in order to characterize the stability properties. t90 is the time that the concentration of tacrolimus in the formulations is maintained above the 90% of the initial concentration.

2.2.1. Quantification of tacrolimus amount

The determination of tacrolimus was performed using an Agilent 1260 series HPLC system (Agilent Technologies, USA) equipped with Diode Array Detector HS, a solvent delivery quaternary pump system, maximum pressure of 400 bar and an autosampler with thermostat. The software model OpenLAB CDS 3D UV (PDA) was used for the data processing. The analysis was performed in an isocratic method. The column used was a ZORBAX Eclipse Plus C18 (4,6 × 50 mm 5 μm) and at a temperature of 60 °C. The mobile phase was water-acetonitrile (35:65 *v*/v) using a flow rate of 1.5 mL.min^{-1}. A wavelength of 210 nm was employed for the quantification of tacrolimus. The volume of the injected sample was 10 μL and the retention time was 3.3 min. Each sample of each formulation (100 μL) was blended using a Vortex Mixer RSLAB-6PRO with 900 μL of water (HPLC grade) and then incorporated to the HPLC to determine the drug concentration. Frozen and refrigerated samples were kept at room temperature for 10 min before quantification. Each sample was assayed in triplicate. The analytical method was validated according to International Conference on Harmonisation guideline recommendations (International Conference on Harmonisation (ICH), 1996).

2.2.2. Osmolality, pH and microbiological control growth

The pH measurements were performed with a HI5221 HANNA® pHmeter and the osmolality was measured with a MicroOsmometer Fiske Model 210. In order to study the microbiological stability, 1 ml of each formulation were added in blood agar plates, sabouraud agar and liquid thioglycate medium. These samples were grown at 37 °C for a period of 48 h, 15 days and 10 days respectively. At the end of each incubation period, microbiological growth was observed and

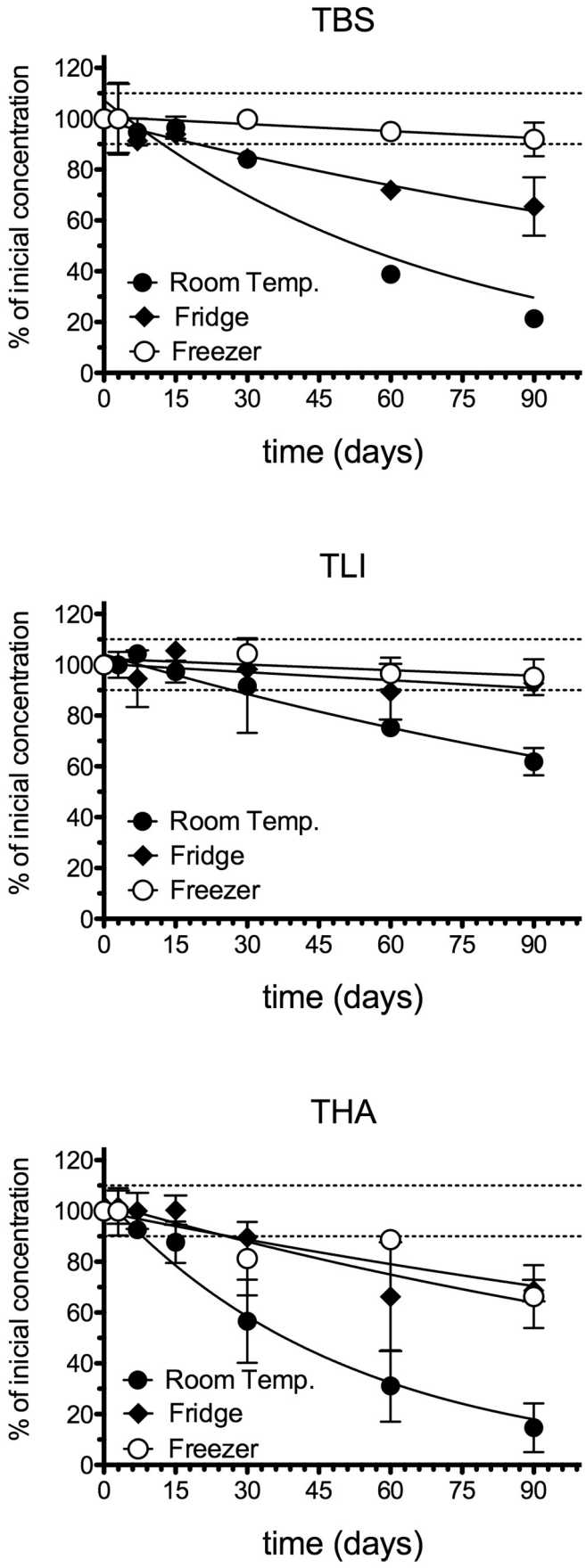

Fig. 1. Representation of the decrease in tacrolimus concentration for the three conserved temperature conditions. (a) Decrease in tacrolimus concentration over time for the formulation TBS in the three conservation conditions. (b) Decrease in tacrolimus concentration over time for the formulation TLI in the three conservation conditions. (c) Decrease in tacrolimus concentration over time for the formulation THA in the three conservation conditions.

Table 2
The t90 of three tacrolimus eye drops obtained by interpolation of the calculated regression line (percentage of remaining tacrolimus concentration versus time).

	K (h^{-1})	t_{90} (days)	R^2
TBS			
Room temperature	0.343 ± 0.610	7.4	0.9247
Refrigerated	0.115 ± 0.005	21.9	0.9652
Frozen	0.023 ± 0.002	109.8	0.9136
TLI			
Room temperature	0.130 ± 0.012	19.5	0.9622
Refrigerated	–	> 90	< 0.45
Frozen	–	> 90	< 0.45
THA			
Room temperature	0.472 ± 0.004	5.35	0.9823
Refrigerated	0.128 ± 0.202	19.8	0.9057
Frozen	0.092 ± 0.029	27.4	0.7894

determined.

2.2.3. Statistical analysis

The margins set as indicated in Pharmaceutical Codex (1994) have been established, with the expiration of the formulation being established when the concentration of active ingredient has been reduced by 10% with respect to the initial concentration. The percentage of drug unaltered vs time was fitted to a first order kinetics using GraphPad Prism® v.5.0b and the degradation constant k and the t90 was calculated. On the other hand, monitoring has been performed in order to observe if there have been changes in pH or osmolality. Microbiological stability is considered adequate provided no microbial growth is detected in the cultured samples. Finally, with respect to the descriptive characteristics of the product, it will not be considered acceptable if abnormal macroscopic changes are observed.

2.3. In vivo evaluation of the residence time on the ocular surface

In vivo studies were carried out on male Sprague-Dawley rats with an average weight of 250 g supplied by the animal facility at the University of Santiago de Compostela (Spain). The animals were kept in individual cages with free access to food and water on a room under controlled temperature (22 ± 1 °C) and humidity (60 ± 5%) and with day-night cycles regulated by artificial light (12/12 h). The animals were treated according to the guidelines for laboratory animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011; The Association for Research in Vision and Ophthalmology, 2014). Experiments were approved by the Galician Network Committee for Ethics Research following the Spanish and European Union (EU) rules (86/609/CEE, 2003/65/CE, 2010/63/EU, RD 1201/2005 and RD53/2013).

The Positron Emission Tomography (PET) and Computerized Tomography (CT) procedures for conducting the radiolabeling of the formulations and the quantitative ocular permanence study are described in our previous works (Fernández-Ferreiro et al., 2017). Briefly, PET and CT images were acquired using the Albira PET/CT Preclinical Imaging System (Bruker Biospin, Woodbridge, Connecticut, United States). Anesthetized animals were positioned into the imaging bed and 7.5 µL of each formulation labelled with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) were instilled into the conjunctival sac eye using a micropipette.

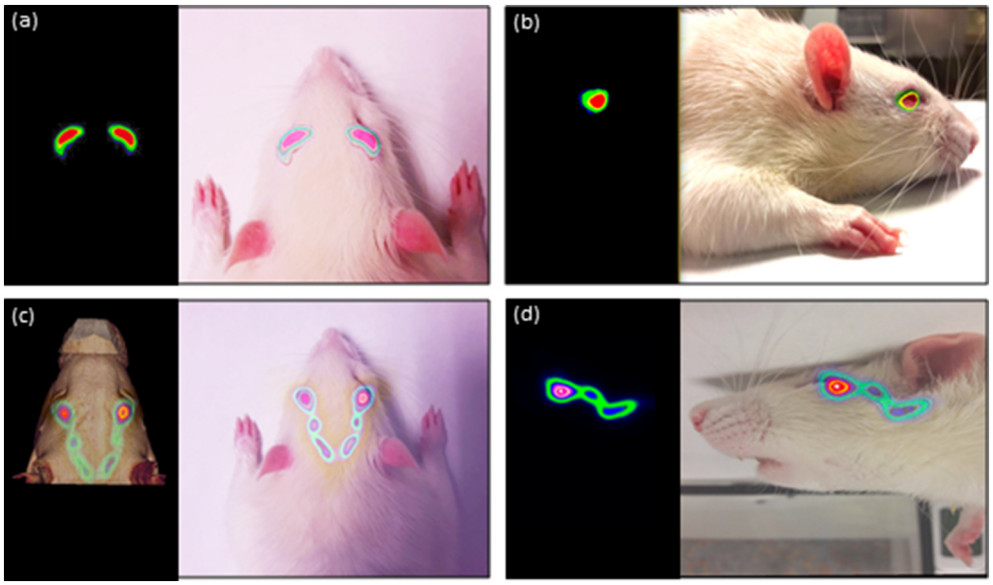

Fig. 2. Fusion PET-CT-Real images of the rat's head in which the formulation remains on the ocular surface after instillation [(a) and (b)] subsequently observed as it is eliminated by the nasolacrimal ducts [(c) and (d)]. Axial (a) and sagittal (b) PET image and fusion PET- real images of the rat 10 min post-administration. Axial (c) fusion PET- CT image and Sagittal (d) fusion PET-real images of the rat 90 min post-administration.

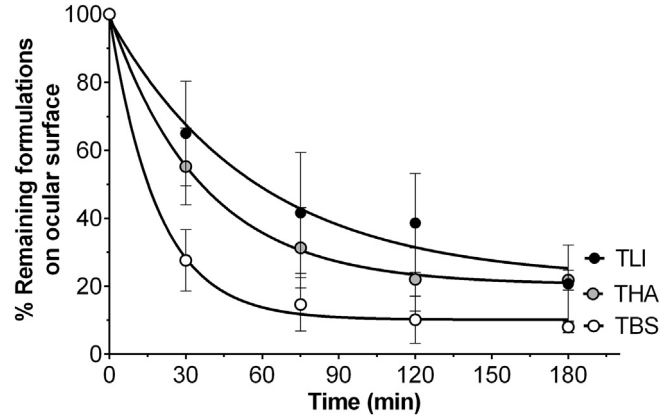

Fig. 3. Clearance rate of the formulations (TLI, THA, TBS) from the ocular surface determined by PET.

Table 3
Parameters obtained by the fitting of the % formulation remaining on ocular surface obtained by PET imaging.

Formulation	$t_{1/2\ (min)}$	R^{2}*	AUC_0^{∞} (mg/L·min)[a]	MRT (min)[a]
TLI	77.1 ± 31.4	0.9782	10,720.7 ± 4576.7	111.3 ± 45.2
THA	51.6 ± 20.6	0.9993	7160.4 ± 2804.7	74.7 ± 29.7
TBS	20.7 ± 8.0	0.9978	2920.1 ± 1078.1	29.9 ± 11.6

[a] Determination coefficient of the fitting to mono exponential decay equation.

After the instillation, static PET frames at different times were acquired. Three animals (6 eyes) were tested for each formulation. Regions of Interest (ROIs) were manually drawn containing the signal on each eye. The ROIs were replicated on the different frames over time and the results were corrected for radioactive decay. Afterwards, graphical representations of radioactivity versus time were obtained.

Fitting of the remaining formulation vs time to a monoexponential decay equation using a single compartmental model was performed using pKSolver (Zhang et al., 2010). Non-Compartmental Analysis was also performed calculating the mean residence time (MRT) and the total area under the curve (AUC) of the % remaining of formulations vs time.

2.4. Cytotoxicity assay

The best tacrolimus formulation was selected based on its major stability and residence ocular time. TLI was tested and compared to another anticalcineurinic drug used commonly in eye drops, cyclosporine (Restasis®). In order to study cell viability, we have used Normal Human Primary Corneal Epithelial Cells (HCE) obtained from ATCC® and maintained in an incubator (37 °C and 5% CO_2 saturation). HCE cells were kept in **corneal epithelial cell growth culture medium without** fetal bovine serum. All experiments were performed between steps 7 and 8. A xCELLigence Real-Time Cell Analyzer System (RTCA) (ACEA Biosciences, San Diego, CA) was used for real time monitoring of the cell culture growth. The methodology of the study has been described in detail in our previous works (Fernández-Ferreiro et al., 2015a, 2016). Briefly, Cell index (CI) (Xing et al., 2006) was used to represent the number of cells based on the measured electric impedance. Three thousand cells per well (16 wells *E*-plates®) were incubated for 24 h. Subsequently, the original culture medium was aspirated and different concentrations of TLI formulation (7.5 µg/mL, 15 µg/mL, 22 µg/mL and 37.5 µg/mL) and Restasis® eye drops (12 µg/mL, 25 µg/mL, 37.5 µg/mL and 50 µg/mL) were added to different wells. The obtained data were represented as dose response curves versus time and Normalized Cell Index (NCI) (Atienza et al., 2005). NCI is defined by the following equation: $NCI = CI_{(t)} / CI_{(t\ of\ the\ dose)}$; in which $CI_{(t)}$ is CI at time "t", and $CI_{(t\ of\ the\ dose)}$ is CI at the time when the drugs were added. Furthermore, IC50 changes over time for each tested formulation were calculated; IC50 represents the eye drops concentration that produces a decrease in the NCI of 50% and this parameter is obtained directly from the dose response curves (Ceriotti et al., 2007).

2.5. Pilot study of clinical use of TLI eye drops

A pilot study was conducted with the TLI eye drops characterized in previous preclinical studies in order to check its efficacy and safety. The prospective observational clinical study was performed in 16 eyes of 8 patients with severe ocular disease that include AKC and VKC, diagnosed by a qualified ophthalmologist. This was performed at the outpatient clinic of the Department of Ophthalmology (University Clinical Hospital of Santiago de Compostela). The study is adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board/Ethics Committee of the Ethical Committee of Clinical Research of Galicia.

All patients were previously treated with other topical agents as

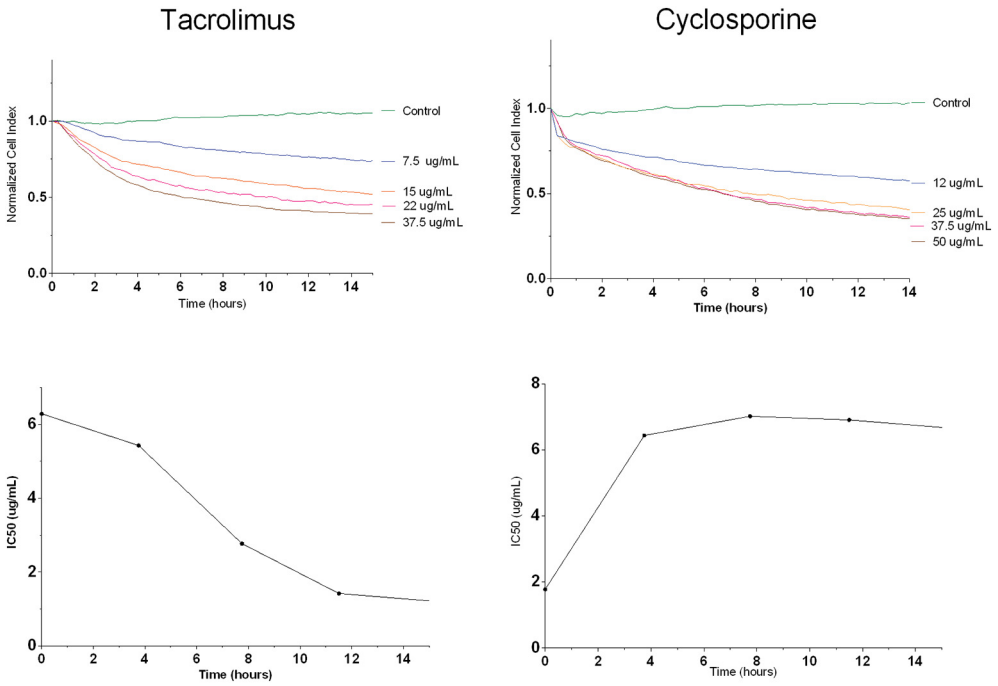

Fig. 4. Cytotoxicity kinetics and evolution of the CI50 over time of tacrolimus, TLI formulation (left) and cyclosporine eye drops, Restasis® (right).

cyclosporine which had been ineffective. After a washout period of 7 days for eye drops and the steroids gradually removed, the patients were instructed to administrated 0.03% TLI eye drops twice a day. Baseline data collected for each patient included demographic parameters as age and sex. Objective and subjective symptoms and quality-of-life questionnaires (EQ-5) were collected before and after 6 months with TLI eye drops. Ocular signs and symptoms of the patients based on the score previously used by Fukushima (Fukushima et al., 2014). The objective signs studied were: palpebral and bulbar conjunctiva, limbus, and corneal involvement. Each of them was scored on a four-point scale (0 = none; 1 = mild; 2 = moderate; 3 = severe) (Table 1). Five subjective symptoms (itching, discharge, lacrimation, foreign body sensation and photophobia) were evaluated by Visual Analog Scale (VAS) which assess each symptom in each eye. The patient was asked to rate each ocular symptom by placing a vertical mark on the horizontal line to indicate the level of discomfort. The VAS scored 0 mm (none = 0%) to 100 mm (very severe = 100%) (Hawker et al., 2011). Finally, the European Quality of Life-5 Dimensions (EQ-5D) questionnaire allowed us to determine the health-related quality of life variation due to the use of TLI eye drops (Wu et al., 1997). Changes from baseline in total score for objective and subjective clinical signs at initial and end of treatment were compared by non-parametric U de Mann-Whitney test.

Safety was assessed from ocular findings, visual acuity, adverse events and laboratory test results. All adverse events observed by the ophthalmologist or reported by patients during the six months of treatment were recorded. For laboratory tests, blood samples were obtained from a cubital vein after seven weeks of treatment. The blood concentration of tacrolimus was measured by chemiluminescent microparticle immunoassay (ARCHITECT i1000SR, Abbot®) which is widely used for quantitative determination of immunosuppressive drugs in biological fluids. We also assessed the pain-discomfort caused by TLI instillation through measurement scale score (Hicks et al., 2001).

3. Results

3.1. Stability assay

Fig. 1 shows the stability of tacrolimus in the different storage conditions plotted vs time. Data were fitted to the first order kinetics to characterize the process and the result of the fitting is included in Table 2. All data showed good agreement to the first order kinetics, characteristic of the degradation of drug in dissolution. In the case of the curves obtained for TLI in fridge and freezer storage, a very low determination coefficient (R^2) was obtained, indicating that the tacrolimus stability has not a linear dependence on the storage time and that drug is stable almost for 90 days. Also, TBS stored frozen (−20 °C) is stable at least throughout the 90-day study period. Room temperature has been shown to be the worst-preserving condition. TLI eye drops showed the best stability for all the storage conditions.

Regarding pH measurements, all formulations showed a suitable pH for their ophthalmic administration (around 7.5). However, all of them showed a high osmolality (higher than 1000 mOsm/Kg). No significant variations of pH and osmolarity were observed in the formulations during the studied period, therefore the preservation conditions had no influence on them. Adequate sterile preservation of all formulations has been observed in all the studied conditions. No microorganism growth was detected in any of the studied formulations. Finally, it should be noted that no macroscopic abnormalities have been observed in any of the formulations over the study time.

3.2. In vivo evaluation of the biopermanence time on the ocular surface

The ocular residence time of the tacrolimus formulations was calculated by using small-animal PET imaging in rats. Fig. 2 shows the clearance of the formulations from the eye. CT images show the external anatomy of the rat's head and PET images show the distribution of the formulations. A solid signal at initial times after instillation was observed in the eye for all formulations, but one hour after instillation, the radiotracer signal is detected on the nasolacrimal duct and in the nasal cavity due to the clearance of part of the formulations from the lacrimal sac into the nasal cavity.

In Fig. 3 it can be observed that after 30 min of contact, 64% of the TLI remains in the ocular surface, while 55% of THA and only 27% of TBS remains (significant differences between TBS and THA or TLI for $\alpha < 0.05$). Data were fitted to a mono exponential model dependent on time and the pharmacokinetic parameters obtained by the fitting to the

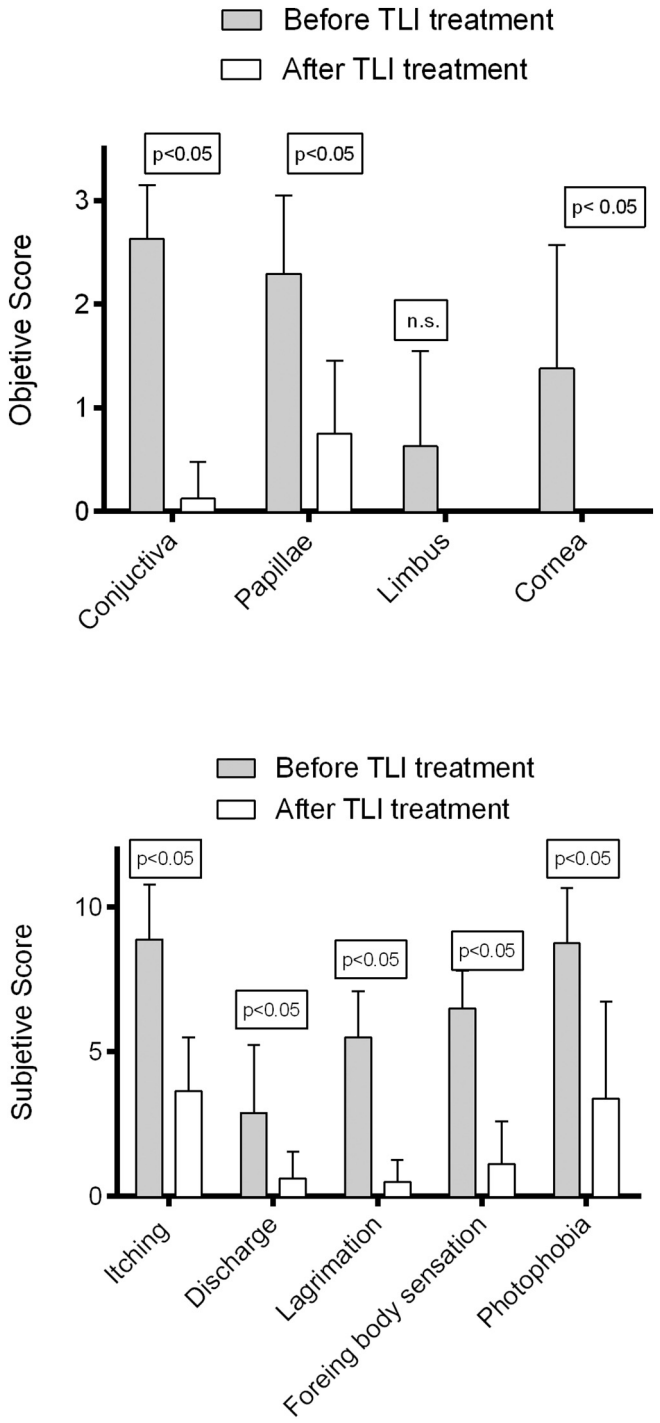

Fig. 5. Mean change in total score for objective (a) and subjective (b) signs in atopic keratoconjunctivitis (AKC) patients. Significance of differences was evaluated by non-parametric U de Mann-Whitney test before and after 6 months of TLI eye drops.

model are shown in Table 3. We obtained an average half-life time and a mean residence time of 77.1 and 111.3 min in the case of the TLI. The values for THA are 51.6 / 74.7 min and 20.7 / 29.9 min for TBS. One-way ANOVA for AUC and MRT gives significant differences between TLI and TBS ($\alpha < 0.05$).

3.3. Cytotoxicity assay

Tacrolimus and cyclosporine showed similar toxicity kinetics, causing gradual cell death over time; they can even eliminate the half population (NCI = 0.5) of HCE with the highest concentrations tested at prolonged times (Fig. 4). The IC50 for both formulations was significantly lower than the original concentration of eye drops (OCT) usually used. In the case of tacrolimus formulation (TLI), initially the IC50 is 6.29 µg/ml, which exceeds 47 times the OCT (300 µg/ml). The IC50 decreases progressively with the time, becoming more toxic, reaching IC50 values of 5.43 µg/ml (OCT exceeds 55 times this value) at 4 h and 1.19 µg/ml (OCT exceeds 252 times this value) at 15 h. By contrast, initially, Restasis® present less IC50 value (1.76 µg/ml) than tacrolimus, which exceeds 284 times the OCT value (500 µg/ml). With prolonged contact, IC50 increase progressively with the time, becoming less toxic, reaching IC50 values of 6.44 µg/ml from 4 h (OCT exceed 77 times this value) and 6.67 µg/ml from 15 h (OCT exceed 74 times this value).

These results indicate that at initial and intermediate times (< 4 h), the HCE tolerate better the formulation of tacrolimus (TLI) than Restasis® eye drops; however, in prolonged contact times, the cells in contact with Restasis® survive better than those in contact with TLI.

3.4. Clinical effectiveness of TLI eye drops

Male patients predominated in the study population, accounting for 87.5% of the participants (7/8) and the median age was 13 years [range 8–43]. Mean total score for objective clinical signs at baseline was 6.63 ± 2.67 and this value decreases statistically significantly ($p < 0.001$) after six months of treatment, reaching total values of 1.13 ± 0.99. Fig. 5 shows changes of severity of conjunctiva, papillae, limbus and cornea. Treatment with TLI showed statistically significantly greater improvement in all parameters tested, except in the limbus. The subjective symptoms have also significantly changed ($p < 0.001$) from mean total score of 32.50 ± 3.42 to 9.25 ± 3.91 after TLI treatment. In the Fig. 5 it is possible to observe the changes in each one of the parameters studied.

Fig. 6 shows the most outstanding macroscopic changes of some patients before and after treatment with TLI. The survey of subjective perception of improvement of quality of life shows a clear improvement over all aspects consulted, as can be seen in the Table 4. In addition, this can be seen in the quantitative assessments about their health status, going from average values of 3.43 ± 1.34 in the basal state to 8.56 ± 2.12, after 6 months of treatment. Finally, it should be noted that no adverse effects nor tacrolimus systemic absorption have been detected after six months of treatment in all patients. Nevertheless, 7 of the 8 patients showed mild eye itch after the instillation of TLI with a mean score of 3 ± 1.8.

4. Discussion

The development of new ophthalmic topical vehicles for increasing the permanence of drugs on the ocular surface is important to improve adherence (Thompson et al., 2015). Therefore, new methodologies are required to provide precise information on the residence time of a formulation on the ocular surface (Gomes et al., 2011). In this study, we propose the use of two vehicles commonly used in ophthalmology to increase the permanence of eye drops, hyaluronic acid and polyvinyl alcohol (Davies et al., 1991; Mochizuki et al., 2008). We have used a novel methodology based on PET technology to determine the biopermanence of tacrolimus eye drops developed with these two vehicles (THA, TLI) and to compare it with corneal residence time of classic eye drops (TBS). PET studies allow the quantitative analysis of the pharmacokinetic profile of the ophthalmic formulations and the calculation of the elimination constant, the half-life and the zero and first moment pharmacokinetic parameters AUC_0^{∞} and MRT. All the pharmacokinetic parameters indicate a significant increase in the ocular retention time of the THA and TLI compared to the TBS acquisitions. The results from the ocular biopermanence assays show that both vehicles are mucoadhesive

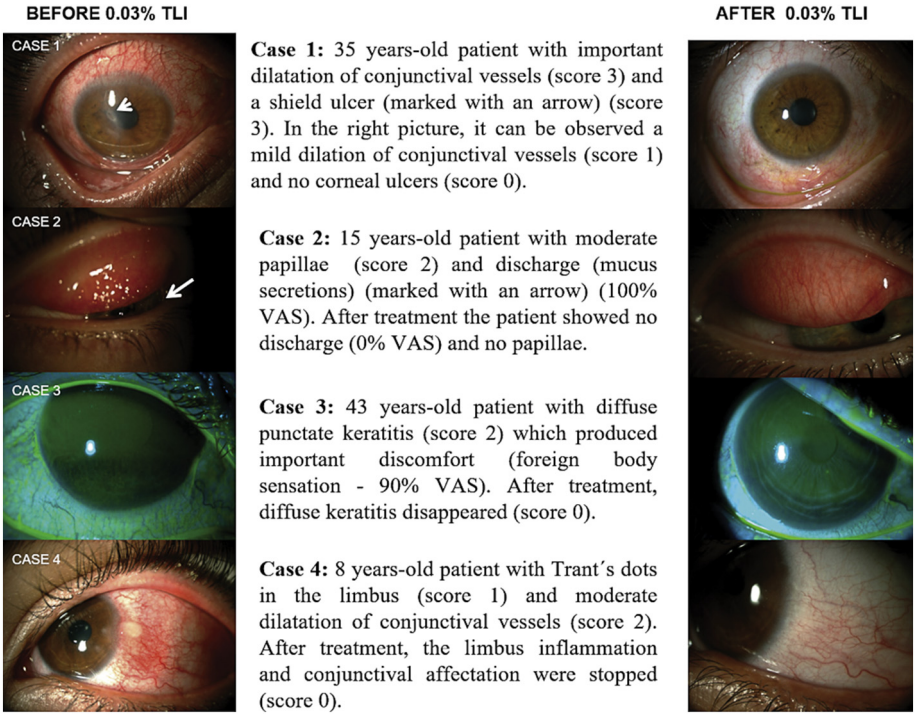

Fig. 6. Representative images of some patients before and after treatment with TLI.

Table 4
Variation in the number of patients according to dimensions and health-related quality of life indicators considered in the EQ-5D questionnaire before and after TLI treatment.

Parameters evaluated	Before	After
Mobility		
I have no problems in walking about	1	8
I have some problems in walking about	7	0
I am confined to bed	0	0
Self-Care		
I have no problems with self-care	8	8
I have some problems washing or dressing myself	0	0
I am confined to wash or dress myself	0	0
Usual activities		
I have no problems with performing my usual activities	2	8
I have some problems with performing my usual activities	5	0
I am unable to perform my usual activities	1	0
Pain/discomfort		
I have no pain or discomfort	2	5
I have moderate pain or discomfort	4	3
I have extreme pain or discomfort	2	0
Anxiety/Depression		
I am not anxious or depressed	5	8
I am moderately anxious or depressed	2	0
I am extremely anxious or depressed	1	0

and have an adequate consistence to remain on the ocular surface for a long time. Finally, the results indicate that the ocular retention time was slightly higher for TLI than THA. PVA has a mechanism based on the interdiffusion of polymer chains across the bioadhesive interface that produces entanglements and physical bonds between the polymer and the substrate. The intimate contact and the presence of hydroxyl-radicals in the polymer can promote the establishment of weak interactions with the mucin layer (i.e. hydrogen bonds) (Peppas and Mongia, 1997).

Hyaluronic acid has been shown to be a useful excipient to increase the biopermanence of many eye drops and artificial tears (Snibson et al., 1992), however, in our case the biopermanence is greater with polyvinyl alcohol. This fact has already been observed by other authors, where the combination of tacrolimus with hyaluronic decreases biopermanence (Zeng et al., 2016). We have observed that THA eye drops instilled in animals cause them to continuously flicker, unlike the other two formulations (Video Supporting information).

Stability studies of pharmaceutical compounds are essential to ensure drug efficacy (Gauthier et al., 2013; Yuan et al., 2009). In order to determine the concentration of active principle over time and in different preservation conditions we have adapted a HPLC method previously described by other authors (Wan et al., 2010). We have shown that temperature is an important factor in the stability under storage of tacrolimus ophthalmic formulations, and that TLI is the more stable formulation, obtaining similar results to those described by other authors (Ezquer-Garin et al., 2017).

In this way, we have determined that eye drops kept in a refrigerator have a half-life of > 12 weeks. This allows programming the preparation of the eye drops every 12 weeks. Therefore, patients are given 12 bottles of TLI for this period of time and they keep them refrigerated. Period after opening at room temperature is stablished at 7 days, both for reasons of physical and chemical stability and for prevention of microbiological contamination.

Once determined that TLI is the formulation with greater biopermanence and more stable, its innocuity and clinical effectiveness were assessed. One of the advantages of RTCA is the determination of IC50 in real-time (Fernández-Ferreiro et al., 2015b). These measurements allow understanding the dynamic toxicity behavior and the range of exposure time (Chen et al., 2012). As we have observed from the ocular biopermanence studies, the ocular surface is in contact with high drug concentrations only for short periods of time. For this reason, knowing the initial cytotoxicity is crucial (Kumar et al., 2011). We have observed that TLI in contact with the cells for < 4 h is more tolerated than cyclosporine. It should be noted that the expressed cytotoxicity is initially attributed to the active principle of the eye drops. However, this is a limitation since the formulations tested contain excipients that could have their own cytotoxicity. TLI contains polyvinyl alcohol (Saarinen-Savolainen et al., 1998), benzalkonium chloride (Bonniard et al., 2016) and ethanol (Oh et al., 2013); and Restasis®, contains

cytotoxic excipients as castor oil (Said et al., 2007) or polysorbate (Ayaki et al., 2008). For this reason, it would be interesting to design new ophthalmic formulations with the lowest possible cytotoxic potential. To our knowledge, this was the first assay that determine the cytotoxicity of tacrolimus eye drops in human corneal epithelial cells. Despite the cytotoxic potential of these formulations, we did not find appreciable ocular adverse reactions in the patients included in the study, obtaining similar results to other authors (Ebihara et al., 2012; Shoughy et al., 2016; Yuan et al., 2012).

Nowadays, tacrolimus concentrations used in clinical practice are very variable (range from 0.01% to 0.1%) (Dumrongkigchaiporn et al., 2004; Fukushima et al., 2014; Moscovici et al., 2012; Shoughy et al., 2016; Zanjani et al., 2017) and usually the concentration used is lower than in cyclosporine eye drops (Jeng and Holsclaw, 2011; Parrilha et al., 2015; Stonecipher et al., 2016). Probably the lower concentration of tacrolimus used is because this drug is 50–100 times more potent than cyclosporine (de Paulis et al., 1991; Nishino et al., 2002; Vichyanond and Kosrirukvongs, 2013). In our pilot study, we evaluated the efficacy and safety of TLI, which is formulated at 0.03%. Although tacrolimus and cyclosporine possess similar suppressive effects on cell-mediated and humoral immune responses (Kawazu et al., 1999), we observed that many of the patients who do not adequately respond to cyclosporine have done so to tacrolimus. Probably the lack of response of one to the other is multifactorial, although the different mechanism of action could be one of the causes. Tacrolimus binds to the immunophilin protein FKBP12 and this process inhibits phosphatase activity of calcineurin. This mechanism of action is similar to the one of cyclosporine, with the only difference being the cytoplasmic binding partner. In addition, in the inhibition of T cell signalling, tacrolimus appears to affect dendritic cell function by interfering with MHC class II-restricted antigen presentation after binding to another FKBP (Greiner and Dick, 2016). Concretely, tacrolimus blocks the early phase of T-cell activation, thus inhibiting both T-lymphocyte signal transduction and IL-2 transcription. It also inhibits the release of histamine from mast cells and affects prostaglandin synthesis (Liu et al., 1991; Ruzicka et al., 1999). Regarding the safety of tacrolimus eye drops, adequate tolerability has been observed, with no appreciable adverse reactions nor systemic absorption. The majority of patients presented a good toleration of the formulations. It was just reported some mild degree of ocular irritation immediately after instillation, probably caused by the high osmolarity of the formulation and the presence of some excipients as ethanol. The data from our clinical pilot study are consistent with those published previously by other authors, where the efficacy and safety of tacrolimus in severe allergic conjunctival diseases have been guaranteed (Al-Amri et al., 2016; Attas-Fox et al., 2008; Kheirkhah et al., 2011; Ohashi et al., 2010; Wakamatsu et al., 2011).

A new multidisciplinary scenario appears that must be explored at galenic level, with the contribution of universities and health research institutes in order to study in depth the formulation of eye drops with high permanence in the cornea that can include non-toxic concentrations of drugs. Until tacrolimus eye drops are marketed, Pharmacy Departments must offer suitable pharmaceutical compounds for clinical use. Based on the stability, biopermanence, safety studies and clinical effectiveness, TLI is a suitable candidate for clinical application in inflammatory ophthalmology diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2018.04.038.

Acknowledgments

A.F-F. acknowledges the support of Instituto de Salud Carlos III (Rio Hortega research grant CM15/00188). A.L-R acknowledges the financial support of the Xunta de Galicia and the European Union (European Social Fund - ESF).

References

Al-Amri, A.M., Mirza, A.G., Al-Hakami, A.M., 2016. Tacrolimus ointment for treatment of vernal keratoconjunctivitis. Middle East Afr. J. Ophthalmol. 23, 135. http://dx.doi.org/10.4103/0974-9233.164616.

Almeida, H., Amaral, M.H., Lobão, P., Lobo, J.M.S., 2014. In situ gelling systems: a strategy to improve the bioavailability of ophthalmic pharmaceutical formulations. Drug Discov. Today 19, 400–412. http://dx.doi.org/10.1016/j.drudis.2013.10.001.

Atienza, J.M., Zhu, J., Wang, X., Xu, X., Abassi, Y., 2005. Dynamic monitoring of cell adhesion and spreading on microelectronic sensor arrays. J. Biomol. Screen. 10, 795–805. http://dx.doi.org/10.1177/1087057105279635.

Attas-Fox, L., Barkana, Y., Iskhakov, V., Rayvich, S., Gerber, Y., Morad, Y., Avni, I., Zadok, D., 2008. Topical tacrolimus 0.03% ointment for intractable allergic conjunctivitis: an open-label pilot study. Curr. Eye Res. 33, 545–549. http://dx.doi.org/10.1080/02713680802149115.

Ayaki, M., Yaguchi, S., Iwasawa, A., Koide, R., 2008. Cytotoxicity of ophthalmic solutions with and without preservatives to human corneal endothelial cells, epithelial cells and conjunctival epithelial cells. Clin. Exp. Ophthalmol. 36, 553–559. http://dx.doi.org/10.1111/j.1442-9071.2008.01803.x.

Bielory, B., Bielory, L., 2016. Allergic Diseases of the Eye. SpringerLink, pp. 35–51. http://dx.doi.org/10.1007/978-3-319-30835-7_4.

Bonini, S., Coassin, M., Aronni, S., Lambiase, A., 2004. Vernal keratoconjunctivitis. Eye 18, 345–351. http://dx.doi.org/10.1038/sj.eye.6700675.

Bonniard, A.A., Yeung, J.Y., Chan, C.C., Birt, C.M., 2016. Ocular surface toxicity from glaucoma topical medications and associated preservatives such as benzalkonium chloride (BAK). Expert Opin. Drug Metab. Toxicol. 12, 1279–1289. http://dx.doi.org/10.1080/17425255.2016.1209481.

Ceriotti, L., Ponti, J., Broggi, F., Kob, A., Drechsler, S., Thedinga, E., Colpo, P., Sabbioni, E., Ehret, R., Rossi, F., 2007. Real-time assessment of cytotoxicity by impedance measurement on a 96-well plate. Sensors Actuators B Chem. 123, 769–778. http://dx.doi.org/10.1016/j.snb.2006.10.024.

Chen, H., Cui, L., Jiang, X.-Y., Pang, Y.-Q., Tang, G.-L., Hou, H.-W., Jiang, J.-H., Hu, Q.-Y., 2012. Evaluation of the cytotoxicity of cigarette smoke condensate by a cellular impedance biosensor. Food Chem. Toxicol. 50, 612–618. http://dx.doi.org/10.1016/j.fct.2011.11.040.

Christensen, L., McDonnell, J.T., Singh, J., 2017. Ocular manifestations of allergic and immunologic diseases. SpringerLink 51–78. http://dx.doi.org/10.1007/978-3-319-18389-3_3.

Davies, N.M., Farr, S.J., Hadgraft, J., Kellaway, I.W., 1991. Evaluation of mucoadhesive polymers in ocular drug delivery. I. Viscous solutions. Pharm. Res. 8, 1039–1043.

Dumrongkigchaiporn, P., Kosrirukvongs, P., Tantimongkolsuk, C., Jirapongsananuruk, O., Visitsunthorn, N., Vichyanond, P., 2004. Vernal keratoconjunctivitis. J. Allergy Clin. Immunol. 113, S178. http://dx.doi.org/10.1016/j.jaci.2004.01.077.

Ebihara, N., Ohashi, Y., Fujishima, H., Fukushima, A., Nakagawa, Y., Namba, K., Okamoto, S., Shoji, J., Takamura, E., Uchio, E., Miyazaki, D., 2012. Blood level of tacrolimus in patients with severe allergic conjunctivitis treated by 0.1% Tacrolimus ophthalmic suspension. Allergol. Int. 61, 275–282. http://dx.doi.org/10.2332/allergolint.11-OA-0349.

Egwuagu, C.E., Sun, L., Kim, S.-H., Dambuza, I.M., 2015. Ocular inflammatory diseases: molecular pathogenesis and immunotherapy. Curr. Mol. Med. 15, 517–528.

Ezquer-Garin, C., Ferriols-Lisart, R., Alós-Almiñana, M., 2017. Stability of tacrolimus ophthalmic solution. Am. J. Health-Syst. Pharm. AJHP Off. J. Am. Soc. Health-Syst. Pharm. 74, 1002–1006. http://dx.doi.org/10.2146/ajhp160169.

Fernández-Ferreiro, A., Santiago-Varela, M., Gil-Martínez, M., Parada, T.G.-C., Pardo, M., González-Barcia, M., Piñeiro-Ces, A., Rodríguez-Ares, M.T., Blanco-Mendez, J., Lamas, M.J., Otero-Espinar, F.J., 2015a. Ocular safety comparison of non-steroidal anti-inflammatory eye drops used in pseudophakic cystoid macular edema prevention. Int. J. Pharm. 495, 680–691. http://dx.doi.org/10.1016/j.ijpharm.2015.09.058.

Fernández-Ferreiro, A., Santiago-Varela, M., Gil-Martínez, M., Parada, T.G.-C., Pardo, M., González-Barcia, M., Piñeiro-Ces, A., Rodríguez-Ares, M.T., Blanco-Mendez, J., Lamas, M.J., Otero-Espinar, F.J., 2015b. Ocular safety comparison of non-steroidal anti-inflammatory eye drops used in pseudophakic cystoid macular edema prevention. Int. J. Pharm. 495, 680–691. http://dx.doi.org/10.1016/j.ijpharm.2015.09.058.

Fernández-Ferreiro, A., González-Barcia, M., Gil-Martínez, M., Santiago Varela, M., Pardo, M., Blanco-Méndez, J., Piñeiro-Ces, A., Lamas Díaz, M.J., Otero-Espinar, F.J., 2016. Evaluation of the in vitro ocular toxicity of the fortified antibiotic eye drops prepared at the hospital pharmacy departments. In: Farm. Hosp. Organo Of. Expresion Cient. Soc. Espanola Farm. Hosp. 40. pp. 352–370. http://dx.doi.org/10.7399/fh.2016.40.5.10416.

Fernández-Ferreiro, A., Silva-Rodríguez, J., Otero-Espinar, F.J., González-Barcia, M., Lamas, M.J., Ruibal, A., Luaces-Rodriguez, A., Vieites-Prado, A., Sobrino, T., Herranz, M., García-Varela, L., Blanco-Mendez, J., Gil-Martínez, M., Pardo, M., Moscoso, A., Medín-Aguerre, S., Pardo-Montero, J., Aguiar, P., 2017. Positron emission tomography for the development and characterization of corneal permanence of ophthalmic pharmaceutical formulations. Invest. Ophthalmol. Vis. Sci. 58, 772–780. http://dx.doi.org/10.1167/iovs.16-20766.

Fukushima, A., Ohashi, Y., Ebihara, N., Uchio, E., Okamoto, S., Kumagai, N., Shoji, J., Takamura, E., Nakagawa, Y., Namba, K., Fujishima, H., Miyazaki, D., 2014. Therapeutic effects of 0.1% tacrolimus eye drops for refractory allergic ocular diseases with proliferative lesion or corneal involvement. Br. J. Ophthalmol. 98, 1023–1027. https://doi.org/10.1136/bjophthalmol-2013-304453.

Gauthier, A.-S., Rival, B., Sahler, J., Fagnoni-Legat, C., Limat, S., Guillaume, Y., Delbosc, B., 2013. Galenic and analytic development of tacrolimus 0.06% eye drops. J. Fr. Ophtalmol. 36, 408–413. http://dx.doi.org/10.1016/j.jfo.2012.03.019.

Ghasemi, H., 2017. Roles of IL-6 in ocular inflammation: a review. Ocul. Immunol.

Inflamm. 0, 1–14. http://dx.doi.org/10.1080/09273948.2016.1277247.
Gomes, C.M., Abrunhosa, A.J., Ramos, P., Pauwels, E.K.J., 2011. Molecular imaging with SPECT as a tool for drug development. Adv. Drug Deliv. Rev. 63, 547–554. http://dx.doi.org/10.1016/j.addr.2010.09.015.
Greiner, K., Dick, A.D., 2016. Tacrolimus. SpringerLink 379–384. http://dx.doi.org/10.1007/978-3-540-75387-2_31.
Hajiahmadi, F., Derkhshan, A., Nikandish, M., Malaekeh-Nikouei, B., 2017. Preparation of tacrolimus ophthalmic solution using cyclodextrins. J. Birjand Univ. Med. Sci. 24, 19–27.
Hawker, G.A., Mian, S., Kendzerska, T., French, M., 2011. Measures of adult pain: visual analog scale for pain (VAS pain), numeric rating scale for pain (NRS pain), McGill pain questionnaire (MPQ), short-form McGill pain questionnaire (SF-MPQ), chronic pain grade scale (CPGS), short Form-36 bodily pain scale (SF-36 BPS), and measure of intermittent and constant osteoarthritis pain (ICOAP). Arthritis Care Res. 63, S240–S252. http://dx.doi.org/10.1002/acr.20543.
Hicks, C.L., von Baeyer, C.L., Spafford, P.A., van Korlaar, I., Goodenough, B., 2001. The faces pain scale – revised: toward a common metric in pediatric pain measurement. Pain 93, 173–183. http://dx.doi.org/10.1016/S0304-3959(01)00314-1.
International Conference on Harmonisation (ICH), 1996. [WWW Document]. http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.
Ishioka, M., Ohno, S., Nakamura, S., Isobe, K., Watanabe, N., Ishigatsubo, Y., Tanaka, S., 1994. FK506 treatment of noninfectious uveitis. Am J. Ophthalmol. 118, 723–729.
Jeng, B.H., Holsclaw, D.S., 2011. Cyclosporine a 1% eye drops for the treatment of subepithelial infiltrates after adenoviral keratoconjunctivitis. Cornea 30, 958–961. http://dx.doi.org/10.1097/ICO.0b013e31820cd607.
Joseph, A., Raj, D., Shanmuganathan, V., Powell, R.J., Dua, H.S., 2007. Tacrolimus immunosuppression in high-risk corneal grafts. Br. J. Ophthalmol. 91, 51–55. http://dx.doi.org/10.1136/bjo.2006.097428.
Kawazu, K., Yamada, K., Nakamura, M., Ota, A., 1999. Characterization of cyclosporin a transport in cultured rabbit corneal epithelial cells: P-glycoprotein transport activity and binding to cyclophilin. Invest. Ophthalmol. Vis. Sci. 40, 1738–1744.
Kheirkhah, A., Zavareh, M.K., Farzbod, F., Mahbod, M., Behrouz, M.J., 2011. Topical 0.005% tacrolimus eye drop for refractory vernal keratoconjunctivitis. Eye (Lond.) 25, 872–880. http://dx.doi.org/10.1038/eye.2011.75.
Kumar, S., Karki, R., Meena, M., Prakash, T., Rajeswari, T., Goli, D., 2011. Reduction in drop size of ophthalmic topical drop preparations and the impact of treatment. J. Adv. Pharm. Technol. Res. 2, 192–194. http://dx.doi.org/10.4103/2231-4040.85540.
Lallemand, F., Schmitt, M., Bourges, J.-L., Gurny, R., Benita, S., Garrigue, J.-S., 2017. Cyclosporine a delivery to the eye: a comprehensive review of academic and industrial efforts. Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Pharm. Verfahrenstechnik EV 117, 14–28. http://dx.doi.org/10.1016/j.ejpb.2017.03.006.
Lienert, J.P., Tarko, L., Uchino, M., Christen, W.G., Schaumberg, D.A., 2016. Long-term natural history of dry eye disease from the Patient's perspective. Ophthalmology 123, 425–433. http://dx.doi.org/10.1016/j.ophtha.2015.10.011.
Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., Schreiber, S.L., 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66, 807–815.
Luaces-Rodríguez, A., Díaz-Tomé, V., González-Barcia, M., Silva-Rodríguez, J., Herranz, M., Gil-Martínez, M., Rodríguez-Ares, M.T., García-Mazás, C., Blanco-Mendez, J., Lamas, M.J., Otero-Espinar, F.J., Fernández-Ferreiro, A., 2017. Cysteamine polysaccharide hydrogels: study of extended ocular delivery and biopermanence time by PET imaging. Int. J. Pharm. 528, 714–722. http://dx.doi.org/10.1016/j.ijpharm.2017.06.060.
Magalhaes, O.A., Marinho, D.R., Kwitko, S., 2013. Topical 0.03% tacrolimus preventing rejection in high-risk corneal transplantation: a cohort study. Br. J. Ophthalmol. 97, 1395–1398. https://doi.org/10.1136/bjophthalmol-2013-303639.
Mochizuki, M., Masuda, K., Sakane, T., Ito, K., Kogure, M., Sugino, N., Usui, M., Mizushima, Y., Ohno, S., Inaba, G., 1993. A clinical trial of FK506 in refractory uveitis. Am J. Ophthalmol. 115, 763–769.
Mochizuki, H., Yamada, M., Hato, S., Nishida, T., 2008. Fluorophotometric measurement of the precorneal residence time of topically applied hyaluronic acid. Br. J. Ophthalmol. 92, 108–111. http://dx.doi.org/10.1136/bjo.2007.121533.
Moscovici, B.K., Holzchuh, R., Chiacchio, B.B., Santo, R.M., Shimazaki, J., Hida, R.Y., 2012. Clinical treatment of dry eye using 0.03% tacrolimus eye drops. Cornea 31, 945–949. http://dx.doi.org/10.1097/ICO.0b013e31823f8c9b.
National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011. Guide for the care and use of laboratory animals. In: The National Academies Collection: Reports funded by National Institutes of Health, 8th ed. National Academies Press (US), Washington (DC).
Nishino, K., Fukushima, A., Okamoto, S., Ohashi, Y., Fukata, K., Ozaki, A., Ueno, H., 2002. Suppression of experimental immune-mediated blepharoconjunctivitis in Brown Norway rats by topical application of FK506. Graefes Arch. Clin. Exp. Ophthalmol. Albrecht Von Graefes Arch. Klin. Exp. Ophthalmol. 240, 137–143.
Oh, J.Y., Yu, J.M., Ko, J.H., 2013. Analysis of ethanol effects on corneal epithelium. Invest. Ophthalmol. Vis. Sci. 54, 3852–3856. http://dx.doi.org/10.1167/iovs.13-11717.
Ohashi, Y., Ebihara, N., Fujishima, H., Fukushima, A., Kumagai, N., Nakagawa, Y., Namba, K., Okamoto, S., Shoji, J., Takamura, E., Hayashi, K., 2010. A randomized, placebo-controlled clinical trial of tacrolimus ophthalmic suspension 0.1% in severe allergic conjunctivitis. J. Ocul. Pharmacol. Ther. Off. J. Assoc. Ocul. Pharmacol. Ther. 26, 165–174. http://dx.doi.org/10.1089/jop.2009.0087.
Parrilha, L.R., Nai, G.A., Giuffrida, R., Barbero, R.C., Padovani, L.D.F., Pereira, R.H.Z., Silva, D.A., Silva, M.C.A., Diniz, M.S., Andrade, S.F., Parrilha, L.R., Nai, G.A., Giuffrida, R., Barbero, R.C., Padovani, L.D.F., Pereira, R.H.Z., Silva, D.A., Silva, M.C.A., Diniz, M.S., Andrade, S.F., 2015. Comparison of 1% cyclosporine eye drops in olive oil and in linseed oil to treat experimentally-induced keratoconjunctivitis sicca in rabbits. Arq. Bras. Oftalmol. 78, 295–299. http://dx.doi.org/10.5935/0004-2749.20150078.
Pastor-Clerigues, A., Serrano, A., Milara, J., Marti-Bonmati, E., Lopez-Perez, F.J., Garcia-Montanes, S., Sanfeliu, J., Saval-Victoria, A.C., Cortijo, J., 2016. Evaluation of the ocular tolerance of three Tacrolimus topical pharmaceutical preparations by bovine corneal opacity and permeability test. Curr. Eye Res. 41, 890–896. http://dx.doi.org/10.3109/02713683.2015.1082187.
Patel, A., Cholkar, K., Agrahari, V., Mitra, A.K., 2013. Ocular drug delivery systems: An overview. World J. Pharmacol. 2, 47–64. http://dx.doi.org/10.5497/wjp.v2.i2.47.
de Paulis, A., Cirillo, R., Ciccarelli, A., Condorelli, M., Marone, G., 1991. FK-506, a potent novel inhibitor of the release of proinflammatory mediators from human Fc epsilon RI+ cells. J. Immunol. Baltim. Md 1950 (146), 2374–2381.
Peppas, N.A., Mongia, N.K., 1997. Ultrapure poly(vinyl alcohol) hydrogels with mucoadhesive drug delivery characteristics. Eur. J. Pharm. Biopharm. 43, 51–58. http://dx.doi.org/10.1016/S0939-6411(96)00010-0.
Phase III Study of 0.1% Tacrolimus(FK506) Ophthalmic Suspension in Patients With Vernal Keratoconjunctivitis - Full Text View - ClinicalTrials.gov [WWW Document], (2014) URL https://clinicaltrials.gov/ct2/show/NCT00567762 (accessed 8.17.17).
Pleyer, U., Lutz, S., Jusko, W.J., Nguyen, K.D., Narawane, M., Rückert, D., Mondino, B.J., Lee, V.H., Nguyen, K., 1993. Ocular absorption of topically applied FK506 from liposomal and oil formulations in the rabbit eye. Invest. Ophthalmol. Vis. Sci. 34, 2737–2742.
Ribeiro, M.V.M.R., Ribeiro, L.E.F., Ribeiro, Ê.A.N., Ferreira, C.V., Barbosa, F.T., Ribeiro, M.V.M.R., Ribeiro, L.E.F., Ribeiro, Ê.A.N., Ferreira, C.V., Barbosa, F.T., 2016. Adherence assessment of eye drops in patients with glaucoma using 8 item Morisky score: a cross sectional study. Rev. Bras. Oftalmol. 75, 432–437. http://dx.doi.org/10.5935/0034-7280.20160087.
Royal Pharmaceutical Society of Great Britain Dept of Pharmaceutical, 1994. The Pharmaceutical Codex: Principles and Practice of Pharmaceutics, Twelfth, Edition. ed. Pharmaceutical Press, London.
Ruzicka, T., Assmann, T., Homey, B., 1999. Tacrolimus: the drug for the turn of the millennium? Arch. Dermatol. 135, 574–580.
Saarinen-Savolainen, P., Järvinen, T., Araki-Sasaki, K., Watanabe, H., Urtti, A., 1998. Evaluation of cytotoxicity of various ophthalmic drugs, eye drop excipients and cyclodextrins in an immortalized human corneal epithelial cell line. Pharm. Res. 15, 1275–1280.
Said, T., Dutot, M., Christon, R., Beaudeux, J.-L., Martin, C., Warnet, J.-M., Rat, P., 2007. Benefits and side effects of different vegetable oil vectors on apoptosis, oxidative stress, and P2X7 cell death receptor activation. Invest. Ophthalmol. Vis. Sci. 48, 5000–5006. http://dx.doi.org/10.1167/iovs.07-0229.
Shoughy, S.S., 2017. Topical tacrolimus in anterior segment inflammatory disorders. Eye Vis. 4. http://dx.doi.org/10.1186/s40662-017-0072-z.
Shoughy, S.S., Jaroudi, M.O., Tabbara, K.F., 2016. Efficacy and safety of low-dose topical tacrolimus in vernal keratoconjunctivitis. Clin. Ophthalmol. Auckl. NZ 10, 643–647. http://dx.doi.org/10.2147/OPTH.S99157.
Sloper, C.M., Powell, R.J., Dua, H.S., 2001. Tacrolimus (FK506) in the management of high-risk corneal and limbal grafts. Ophthalmology 108, 1838–1844.
Snibson, G.R., Greaves, J.L., Soper, N.D., Tiffany, J.M., Wilson, C.G., Bron, A.J., 1992. Ocular surface residence times of artificial tear solutions. Cornea 11, 288–293.
Stonecipher, K.G., Torkildsen, G.L., Ousler, G.W., Morris, S., Villanueva, L., Hollander, D.A., 2016. The IMPACT study: a prospective evaluation of the effects of cyclosporine ophthalmic emulsion 0.05% on ocular surface staining and visual performance in patients with dry eye. Clin. Ophthalmol. Auckl. NZ 10, 887–895. http://dx.doi.org/10.2147/OPTH.S101627.
The Association for Research in Vision and Ophthalmology, 2014. Statement for the Use of Animals in Ophthalmic and Visual Research [WWW Document]. http://www.arvo.org/About_ARVO/Policies/Statement_for_the_Use_of_Animals_in_Ophthalmic_and_Visual_Research/.
Thompson, A.C., Thompson, M.O., Young, D.L., Lin, R.C., Sanislo, S.R., Moshfeghi, D.M., Singh, K., 2015. Barriers to follow-up and strategies to improve adherence to appointments for care of chronic eye diseases. Investig. Opthalmology Vis. Sci. 56, 4324. http://dx.doi.org/10.1167/iovs.15-16444.
Uno, T., Yamaguchi, T., Li, X.K., Suzuki, Y., Hashimoto, H., Harada, Y., Kimura, T., Kazui, T., 1997. The pharmacokinetics of water-in-oil-in-water-type multiple emulsion of a new tacrolimus formulation. Lipids 32, 543–548.
Vichyanond, P., Kosrirukvongs, P., 2013. Use of cyclosporine a and tacrolimus in treatment of vernal keratoconjunctivitis. Curr Allergy Asthma Rep 13, 308–314. http://dx.doi.org/10.1007/s11882-013-0345-0.
Wakamatsu, T.H., Tanaka, M., Satake, Y., Dogru, M., Fukagawa, K., Igarashi, A., Fujishima, H., 2011. Eosinophil cationic protein as a marker for assessing the efficacy of tacrolimus ophthalmic solution in the treatment of atopic keratoconjunctivitis. Mol. Vis. 17, 932–938.
Wan, Y.-D., Tang, X.-L., Ye, C.-T., 2010. Determination of tacrolimus ophthalmic emulsion by HPLC. J. Chin. Pharm. Sci. 45, 221–223.
Whitcup, S.M., Azar, D.T. (Eds.), 2017. Pharmacologic Therapy of Ocular Disease, Handbook of Experimental Pharmacology. Springer International Publishing, Cham.
Wu, A.W., Jacobson, D.L., Berzon, R.A., Revicki, D.A., van der Horst, C., Fichtenbaum, C.J., Saag, M.S., Lynn, L., Hardy, D., Feinberg, J., 1997. The effect of mode of administration on medical outcomes study health ratings and EuroQol scores in AIDS. Qual. Life Res. 6 0–0. https://doi.org/10.1023/A:1026471020698.
Xing, J.Z., Zhu, L., Gabos, S., Xie, L., 2006. Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity. Toxicol. in Vitro 20, 995–1004. http://dx.doi.org/10.1016/j.tiv.2005.12.008.
Yuan, J., Zhai, J., Chen, J., Ye, C., Zhou, S., 2009. Preparation of 0.05% FK506 suspension eyedrops and its pharmacokinetics after topical ocular administration. J. Ocul.

Pharmacol. Ther. Off. J. Assoc. Ocul. Pharmacol. Ther. 25, 345–350. http://dx.doi.org/10.1089/jop.2008.0125.

Yuan, J., Zhai, J., Huang, X., Zhou, S., Chen, J., 2012. Ocular safety and pharmacokinetics study of FK506 suspension eye drops after corneal transplantation. J. Ocul. Pharmacol. Ther. 28, 153–158. http://dx.doi.org/10.1089/jop.2011.0108.

Yura, H., Yoshimura, N., Hamashima, T., Akamatsu, K., Nishikawa, M., Takakura, Y., Hashida, M., 1999. Synthesis and pharmacokinetics of a novel macromolecular prodrug of Tacrolimus (FK506), FK506-dextran conjugate. J. Control. Release Off. J. Control. Release Soc. 57, 87–99.

Zanjani, H., Aminifard, M.N., Ghafourian, A., Pourazizi, M., Maleki, A., Arish, M., Shahrakipoor, M., Rohani, M.R., Abrishami, M., Khafri Zare, E., Barzegar Jalali, F., 2017. Comparative evaluation of Tacrolimus versus interferon alpha-2b eye drops in the treatment of vernal Keratoconjunctivitis: a randomized, double-masked study. Cornea 36, 675–678. http://dx.doi.org/10.1097/ICO.0000000000001200.

Zeng, W., Li, Q., Wan, T., Liu, C., Pan, W., Wu, Z., Zhang, G., Pan, J., Qin, M., Lin, Y., Wu, C., Xu, Y., 2016. Hyaluronic acid-coated niosomes facilitate tacrolimus ocular delivery: Mucoadhesion, precorneal retention, aqueous humor pharmacokinetics, and transcorneal permeability. Colloids Surf. B: Biointerfaces 141, 28–35. http://dx.doi.org/10.1016/j.colsurfb.2016.01.014.

Zhang, Y., Huo, M., Zhou, J., Xie, S., 2010. PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft excel. Comput. Methods Prog. Biomed. 99, 306–314. http://dx.doi.org/10.1016/j.cmpb.2010.01.007.