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Therapeutic alternatives research for the treatment of ocular inflammatory diseases

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TESE DE DOUTORAMENTO

THERAPEUTIC ALTERNATIVES RESEARCH FOR THE TREATMENT OF OCULAR INFLAMMATORY DISEASES

Xurxo García Otero

ESCOLA DE DOUTORAMENTO INTERNACIONAL DA UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

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Therapeutic alternatives research for the treatment of ocular inflammatory diseases

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Therapeutic alternatives research for the treatment of ocular inflammatory diseases

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SUMMARY

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SUMMARY

The quality of life of millions of people around the world is compromised by various eye conditions and pathologies. One of the most complex challenges for pharmacists is to achieve efficient and effective treatments for the eye, since its anatomy and physiology presents many protective mechanisms, which makes it a really complex organ to treat. For this reason, it is important that the bioavailability of the drug in those specific sites of action of the different ocular structures where treatment is needed is adequate.

Advances in drug delivery technologies have evolved over time, but major challenges remain in the treatment of ocular diseases. These ocular delivery systems must maintain therapeutic drug concentrations, reduce dosing frequency and cross the various ocular barriers.

The use of topical-ophthalmic instillation for the treatment of diseases affecting the anterior segment of the eye is the most desirable route of administration. These systems present several advantages as they are considered easy to handle, have high patient acceptance and are also economically cost-effective. However, they also present certain limitations in terms of low ocular bioavailability, this is due to the high tear turnover rate which equates to significant clearance through the nasolacrimal drainage, impacting on the constant repetition of frequent instillations. Therefore, much effort needs to be focused on addressing this problem. This solution involves the development of systems that increase the permanence on the ocular surface and facilitate entry into the tissues.

On the other hand, direct administration by intravitreal injections of drugs is useful in the treatment of ocular pathologies of the posterior segment of the eye. This route has some advantages compared to systemic administration by circumventing blood-ocular barriers, allowing higher drug levels to be reached in the ocular cavity and limiting systemic side effects. However, it also has some limitations since it is considered an invasive route that usually requires repeated injections, for this reason, it is necessary to know the intravitreal pharmacokinetics of the drugs in order to develop new sustained release systems, and thus reduce the number of administrations.

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The disadvantages of the topical-ophthalmic route and intravitreal injections are reflected in the treatment of ocular inflammatory pathologies affecting the anterior and posterior segment of the eye, such as uveitis in its broad spectrum or keratoconjunctivitis, pathologies on which this doctoral thesis focuses.

Uveitis is the term used for a very heterogeneous group of diseases that cause ocular inflammation. Because their origin and site of involvement can be very diverse, the treatment of these diseases requires different routes and strategies depending on the site and degree of involvement, so that a wide range of drugs and routes of administration are necessary for an efficient cure of the disease. Over time, the use of different families of drugs with therapeutic action have been studied, as well as the optimization and improvement of the routes of administration to reduce the possible systemic adverse effects caused by the drugs. The corticosteroid family is one of the most widely used because it produces favorable effects quickly. However, it is also common to find undesirable adverse effects in some ocular structures and systemic toxicity in long-term treatments. For this reason, new families of drugs have emerged that have demonstrated a therapeutic effect against these diseases, such as immunosuppressants (tacrolimus) or biologic drugs (adalimumab), both drugs studied in this doctoral thesis.

Chapter 1 contains a literature review that aims to give an overview of the main aspects involved in the pharmacokinetics of ocular drugs intended to treat different ocular diseases. First, the different factors involved in ocular drug delivery were analyzed, covering the different routes of entry into the eye. The physiological barriers and drug transport pathways were described in detail, and the advantages and disadvantages of the different routes of administration to the eye were also discussed.

Conventional routes of administration, such as topical or systemic, often present important limitations, either because of low ocular penetration or because of the occurrence of side effects linked to the dosage, among others. Therefore, new drug delivery systems (DDS) are needed to help prolong and adapt administration intervals in ocular pathologies. However, the development of these new systems is particularly challenging due to several aspects that need to be considered, such as pharmacokinetics, immunogenicity, biodegradation, tolerability and toxicity. The last few decades have seen an exponential increase in the design and development of new drug delivery systems for the treatment of ocular pathologies. Unfortunately, knowledge about the ability of these systems to deliver drugs into the eye remains scarce.

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There have also been great advances in the research and development of new alternative routes of administration to achieve drug concentrations in the

different parts of the eye. All of them have specific pharmacokinetic characteristics that make them useful for the treatment of specific ocular pathologies. However, these routes have shown various advantages and limitations, where the choice of one or the other depends not only on the pathology itself, but also on the pharmaceutical form, the drug used and the patient's adherence to treatment.

Chapter 2 includes the study of the development and efficacy of an ophthalmic formulation of tacrolimus 0.03% *(w/v)* based on the systemic commercial presentation (Prograf®) and introduced in three types of vehicles: Balanced Salt Solution (BSS), polyvinyl alcohol solution (PVA, Liquifilm®) and a hyaluronic acid solution.

For this purpose, *in vitro* (stability studies) and *in vivo* (permanence time in the cornea by Positron Emission Tomography) tests of the three possible formulations were carried out. The stability study at different temperature conditions was carried out for 90 days in freezing (-20 °C), refrigeration (2-8 °C) and at room temperature (20-25 °C). Osmolality, pH and drug concentration parameters were studied during this period. The only formulation that remained stable for 90 days under refrigeration and freezing conditions was that of the PVA vehicle, in BSS it only withstood freezing and in hyaluronic acid it was not preserved under any of the conditions. The pH and osmolality measurements remained constant with a pH of 7.5 and osmolality values too high (above 1000 mOsm/kg) compared to the physiological osmolality of the ocular surface. Regarding the study of ocular permanence by PET, a 3-hour follow-up was carried out after instillation of the eye drops in the eye, in which it was possible to verify that tacrolimus eye drops with PVA produced the least clearance, increasing the contact time on the ocular surface.

The best formulation (tacrolimus in PVA) was then selected, and its toxicological profile and clinical efficacy was evaluated in comparison with commercial cyclosporine eye drops (Restasis®). Tacrolimus in PVA showed less cytotoxicity than cyclosporine and was better tolerated by corneal epithelial cells in the early stages. On the other hand, a pilot study was performed in 8 patients in which significant improvements were shown in the patients, with no appreciable adverse reactions. However, 7 of the 8 patients showed a slight ocular itching after instillation of this tacrolimus eye drop, probably caused by the high osmolality of the formulation and the presence of excipients poorly adapted to the ocular route present in Prograf®.

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Based on the stability, ocular permanence, safety and clinical efficacy studies, it is possible to conclude that tacrolimus-PVA eye drops are a suitable candidate for clinical application in ophthalmic inflammatory diseases.

In order to solve the problems arising from the presence of poorly adapted excipients in Prograf® in **Chapter 3**, the design and development of two types of tacrolimus-containing ophthalmic formulations for the treatment of uveitis was addressed. Tacrolimus is an immunosuppressant drug characterized by very low solubility in water and low stability in aqueous media, for this reason the main objective was to achieve solubilization of the drug by combining different proportions of a derivative of β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin (HPβCD). In this way, an interaction was achieved between the drug and the cyclodextrin forming the complexation of tacrolimus in the hydrophobic cavity of HPβCD, generating an increase in the solubility of the drug. All this information was obtained by performing solubility, NMR and molecular modeling studies.

After having achieved the solubilization of tacrolimus, it was proceeded to test whether this complex could work in other aqueous solvents, as in the case of a BSS (Balanced Salt Solution) solution, and also in Liquifilm®, a lubricating tear containing polyvinyl alcohol (PVA). Different parameters for the optimization of the formulation were carried out, managing to fix the composition of each eye drop and the optimal tacrolimus solubilization time. These studies resulted in four definitive formulations: two formulations with 20% *(w/v)* HPβCD and 0.01% *(w/v)* tacrolimus using BSS as vehicle in the first case and Liquifilm® in the second, and two other formulations with 40% *(w/v)* HPβCD and 0.02% *(w/v)* tacrolimus, also in BSS and Liquifilm®.

Once the formulations were ready, physicochemical characterization was performed, determining parameters such as pH, osmolality, surface tension of the formulation and the force required to dispense a drop. These studies concluded that the formulations have an optimal pH range for the physiology of the eye and osmolality values of ≃320 mOsm/kg. This solved the osmolality problem existing in classical formulations prepared in the hospital pharmacy that had values above ≃1200 mOsm/kg.

Ocular irritation and toxicity studies were performed through the permeability and opacity test on bovine cornea and also with the test on the chorioallantoic membrane of fertilized eggs. In this way it was proved that none of the formulations generated the slightest indication of toxicity or irritation. Another important characteristic that had to be demonstrated was that tacrolimus remained stable in aqueous solution for a reasonable period of time. Therefore, a stability study was performed on the two eye drops containing the most tacrolimus, 0.02% *(w/v)* in BSS and Liquifilm®, which showed that the eye drops could be preserved for at least three months under refrigeration (4 ± 2 °C).

The residence time of an eye drop on the ocular surface is another extremely important parameter to increase the bioavailability of the drug in the therapeutic target. Therefore, *ex vivo* corneal mucoadhesion and *in vivo* corneal surface permanence time studies were performed using PET imaging. After analyzing the results, it was observed that HPβCD not only increases the solubility and stability of tacrolimus in solution, but also influences the mucoadhesion properties, since increasing the proportion of cyclodextrin improves the permanence time. The formulation with 40% *(w/v)* HPβCD, 0.02% *(w/v)* tacrolimus dissolved in Liquifilm® doubled the dwell time compared to the classical tacrolimus formulation.

Chapter 4 is based on the demonstration of the anti-inflammatory effect in an endotoxin-induced uveitis model in rats of the best formulation obtained in the previous chapter. For this purpose, 32 rats were divided into 4 groups of 8 rats each: (a) a group of untreated healthy rats, (b) a group of rats with untreated uveitis, (c) a group of rats with uveitis treated with the standard treatment of dexamethasone eye drops (Maxidex®) and (d) a group of rats with uveitis treated with the proposed TAC-HPβCD eye drops.

Uveitis was induced by inoculating 1 mg/kg of *Escherichia coli* lipopolysaccharide (LPS) diluted in 0.1 mL of BSS into the animal's right paw. The treated rats received the instillation of the eye drops 3h hours before the induction of the disease and every 3h until 24 h of study. Once the study period was over, the rats were euthanized with carbon dioxide to proceed to sample collection, in order to quantitatively analyze the presence of leukocytes in the aqueous humor, evaluate the ocular histology and finally perform PCR quantification of the expression of IL-6, IL-8, MIP-1α and TNFα levels in the eye.

The first thing that was proven was that the uveitis model was induced in an appropriate manner, which implies that after LPS administration there is an activation of TLRs, increasing levels of proinflammatory mediators, developing inflammation of the anterior uvea, choroid and retina and resulting in the breakdown of the blood-tumoral barrier, with exudation of leukocytes into the aqueous humor. This effect could be verified by significantly higher levels of TNFα, IL-6, IL-8, MIP-1α and leukocytes, as well as histological evaluation of the untreated uveitis group compared to the healthy group.

The significantly lower levels of TNFα, IL-6, MIP-1α and leukocytes, as well as the histological evaluation of the TAC-HPβCD eye drops compared to the diseased group confirms the efficacy of this formulation in the treatment of uveitis. Regarding the comparison between the efficacy of our formulation and standard treatment with dexamethasone, no statistically significant differences were found between the levels of proinflammatory cytokines in the TAC-HPβCD

group and the dexamethasone group, demonstrating that TAC-HPCD eye drops could be an alternative to topical corticosteroid therapy in the treatment of this model of uveitis and their translational clinical use should be studied in future studies.

Moreover, regarding TNFα mRNA expression, its reduction is statistically significant only between the TAC-HPβCD and uveitis groups, but not between the dexamethasone and uveitis groups, which is consistent with the mechanism of action of tacrolimus and could be especially beneficial in uveitis related to the increase of this factor, such as HLA B27-associated uveitis, sarcoid uveitis, and uveitis associated with Behçet's disease, among other non-infectious uveitis entities.

Adalimumab is an anti-TNFα drug approved for the treatment of uveitis by subcutaneous injection. This route of administration exposes patients to systemic adverse effects and makes it difficult to obtain therapeutic concentrations of the drug at the site of action due to the anatomic and physiologic barriers of the eye.

The use of molecular imaging techniques in combination with radiolabeling of antibodies allows not only the observation of their distribution pattern in the body, but also their quantification in real time. In this sense, Positron Emission Tomography (PET) represents a promising imaging tool for the non-invasive evaluation of antibody pharmacokinetics, allowing longitudinal studies in which each animal is followed over time. This clearly represents the advantage of reducing the number of animals, according to the 3Rs frameworks. Zirconium-89 (^{89}Zr) is a radionuclide that plays an important role in Immuno-Positron Emission Tomography (Immuno-PET) imaging techniques. Its long half-life (3.3 days) is favorable for assessing the *in vivo* distribution of monoclonal antibodies (mAb), being of great potential in monitoring antibody-based therapies. The Immuno-PET technique combines the sensitivity of PET with the specificity of the antibody, allowing to know its body distribution and pharmacokinetic behavior.

Since, as has been shown, increased TNFα expression plays an important role in ocular inflammatory diseases, in **Chapter 5** it was set out to study the ocular pharmacokinetic profile and the clearance and distribution in tissues of an intravitreally injected monoclonal antibody that acts by inhibiting TNFα receptors called adalimumab.

To achieve the objective of this study, adalimumab was conjugated with a chelating agent called deferoxamine whose function is to trap the ^{89}Zr nucleus. Once the conjugation was completed, the conjugated antibody was radiolabeled with ^{89}Zr with a maximum specific activity of 10 MBq/mg, achieving an optimal

radiolabeling result, since the radiochemical purity after ultrafiltration was 99.69%. Regarding the study design, two groups of rats were used: a control group of healthy rats (n=3; 6 eyes) and a group of rats with uveitis induced in the same way as in Chapter 4 (n=6, 12 eyes). Each of the animals was injected into the vitreous with 4 μL containing ≃1.74 MBq of ^{89}Zr-labeled adalimumab (in both eyes). MicroPET image acquisition was performed immediately after injection and at different time points throughout a 10-day study, at the same times blood samples were collected through the tail vein.

Quantitative analysis was performed on the PET images obtained, in them different regions of interest (ROIs) were drawn in the study organs (eyes, heart, liver, spleen and cervical lymph nodes) in order to quantify the radioactive activity emitted by ^{89}Zr-adalimumab and thus generate their kinetic curves. One and two-compartment models were used to fit the experimental data.

The ocular pharmacokinetics of the antibody was adjusted to a one-compartment model, showing an intraocular elimination half-life of 15.57 hours for healthy rats and 33.64 hours for rats with uveitis, implying that ^{89}Zr-adalimumab remained about twice as long in rats with the disease compared to healthy rats. These results show that, despite the fact that ocular clearance of a drug in an eye with inflammation would be expected to be faster due to the increased permeability of the ocular barriers, this is not the case here. The ability of adalimumab to selectively bind tumor necrosis factor is well known, so this result is consistent with the overproduction of TNFα from macrophages and other cytokines in the process of uveitis, which activates dendritic cells, initiating the inflammatory cascade in which Th1 and Th17 cells migrate and infiltrate the blood-retinal barrier causing damage.

The results of compartmental pharmacokinetic analysis in blood show different behavior between healthy and diseased animals. The activity versus time in healthy animals conforms to a two-compartment model showing a rapid uptake of the antibody from the eye simultaneously with the tissue distribution process. However, in the case of animals with uveitis, the activity versus time conforms better to a one-compartment model. This may be because the transfer process from the eye to the blood is much slower acting as a limiting step, so that the distribution process to the organs is not appreciated.

This study shows for the first time the ocular and blood pharmacokinetic analysis of adalimumab in a rat model of uveitis, providing valuable information for the development of new controlled release systems of adalimumab that allow spacing the administration of the drug and thus improve the quality of life of patients.

In **conclusion**, in this doctoral thesis different tacrolimus formulations for topical-ophthalmic administration have been proposed with great potential, supported by extensive preclinical studies in order to achieve a consistent basis as an alternative to other pharmacological treatments for ocular inflammatory diseases. This was confirmed with extensive *in vitro*, *ex vivo* and *in vivo* studies. Moreover, with regard to the study of intravitreal injections of adalimumab, this doctoral thesis has elucidated the intravitreal distribution and pharmacokinetics of the monoclonal antibody that will allow the development of new delivery systems.

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RESUMO

A calidade de vida de millóns de persoas en todo o mundo vese comprometida por causa de diferentes afeccións e patoloxías oculares. Un dos retos máis complexos para os farmacéuticos é conseguir tratamentos eficaces e efectivos para o ollo, xa que a súa anatomía e fisioloxía presenta moitos mecanismos de protección, o que fai que sexa un órgano realmente complexo de tratar. Por esta razón, é importante que a biodispoñibilidade do fármaco sexa adecuada naqueles lugares de acción específicos das diferentes estruturas oculares onde sexa necesario un tratamento.

Os avances alcanzados respecto ás tecnoloxías de administración de fármacos foron evolucionando co paso do tempo, aínda que quedan grandes desafíos no que se refire ó tratamento de enfermidades oculares. Estes sistemas de administración por vía ocular deben manter as concentracións terapéuticas do fármaco, reducir a frecuencia de dosificación e atravesar as diferentes barreiras oculares.

O uso da instilación tópico-oftálmica para o tratamento de enfermidades que afectan ó segmento anterior do ollo é a vía de administración máis desexable. Estes sistemas presentan varias vantaxes xa que considéranse fáciles de manexar, teñen unha aceptación alta por parte do paciente e tamén son rendibles economicamente. Non obstante, tamén presenta certas limitacións en canto á baixa biodispoñibilidade ocular, isto é debido á elevada taxa de renovación lacrimal o que equivale a un aclaramento importante a través do drenaxe nasolacrimal, repercutindo na repetición constante de instilacións frecuentes. Polo tanto, é necesario que moitos dos esforzos enfóquense en abordar este problema. Esta solución pasa por desenvolver sistemas que permitan aumentar a permanencia na superficie ocular e que faciliten a entrada nos tecidos.

Por outro lado, a administración directa mediante inxeccións intravítreas de fármacos é útil no tratamento de patoloxías oculares do segmento posterior do ollo. Esta vía presenta algunhas vantaxes en comparación ca administración sistémica eludindo as barreiras hemato-oculares, permitindo alcanzar niveis de fármaco máis elevados na cavidade ocular e limitando os efectos secundarios sistémicos. Sen embargo, tamén presenta algunha limitación xa que considérase

unha ruta invasiva que normalmente require inxeccións repetidas, por este motivo, é necesario coñecer a farmacocinética intravítrea dos fármacos para poder desenvolver novos sistemas de liberación sostida, e así reducir o número de administracións.

As desvantaxes da vía tópico-oftálmica e das inxeccións intravítreas vense reflexadas nos tratamentos de patoloxías inflamatorias oculares que afectan ó segmento anterior e posterior do ollo como poden ser a uveíte no seu amplo espectro ou a queratoconxuntivite, patoloxías nas que se centra esta tese de doutoramento.

A uveíte é o termo empregado para un grupo moi heteroxéneo de enfermidades que causan inflamación ocular. Debido a que a súa orixe e lugar de afectación poden ser moi diversos, para o tratamento destas enfermidades é necesario empregar diferentes vías e estratexias segundo o lugar e o grado de afectación, polo que un amplo abanico de fármacos e rutas de administración son necesarias para unha cura eficiente da enfermidade. Ó longo do tempo, foise estudando o uso de diferentes familias de fármacos con acción terapéutica, así como a optimización e mellora das vías de administración para reducir os posibles efectos adversos sistémicos producidos polos fármacos. A familia de corticosteroides é unha das máis empregadas xa que produce efectos favorables rapidamente, sen embargo, tamén é frecuente atopar efectos adversos non desexados nalgunhas estruturas oculares e toxicidade sistémica en tratamentos de longa duración. Por esta razón, xurdiron novas familias de fármacos que demostraron un efecto terapéutico fronte a estas enfermidades, como son o caso dos inmunosupresores (tacrolimus) ou fármacos biolóxicos (adalimumab), ambos fármacos estudados nesta tese de doutoramento.

O **capítulo 1** contén unha revisión bibliográfica que pretende dar unha visión xeral dos principais aspectos que interveñen na farmacocinética dos fármacos oculares destinados a tratar diferentes enfermidades oculares. En primeiro lugar, analizáronse os diferentes factores que interveñen na administración ocular de fármacos, incluíndo as diferentes rutas de entrada no ollo. As barreras fisiolóxicas e as vías de transporte dos fármacos foron detalladamente descritas, e tamén discutíronse as vantaxes e inconvenientes das diferentes vías de administración ó ollo.

As vías de administración convencionais, como a tópica ou a sistémica, normalmente presentan importantes limitacións, xa sexa pola baixa penetración ocular ou pola aparición de efectos secundarios ligados á posoloxía, entre outros. Polo tanto, son necesarios novos sistemas de administración de fármacos (DDS) que axuden a prolongar e adecuar os intervalos de administración nas patoloxías oculares. Sen embargo, o desenvolvemento destes novos sistemas é

especialmente complicado debido a varios aspectos que deben considerarse, como a farmacocinética, a inmunoxenicidade, a biodegradación, a tolerabilidade e a toxicidade. Nas últimas décadas observouse un aumento exponencial no deseño e desenvolvemento de novos sistemas de administración de fármacos destinados ó tratamento de patoloxías oculares. Desafortunadamente, os coñecementos sobre a capacidade destes sistemas para vehiculizar os fármacos no ollo continúan a ser escasos.

Deste xeito, producíronse grandes avances en canto á investigación e o desenvolvemento de novas vías de administración alternativas para acadar concentración de fármaco nas diferentes partes do ollo. Todas elas teñen características farmacocinéticas específicas que as fan adecuadas para o tratamento de patoloxías oculares concretas. Non obstante, estas vías mostraron diversas vantaxes e limitacións, onde a elección dunha ou outra depende, non só da propia patoloxía, senón da forma farmacéutica, do fármaco empregado e da adherencia do paciente ó tratamento.

O **capítulo 2** engloba o estudo da elaboración e eficacia dunha formulación oftálmica de tacrolimus ó 0,03% *(p/v)* a partir da presentación comercial sistémica (Prograf®) e introducida en tres tipos de vehículos: Balanced Salt Solution (BSS), solución de alcohol polivinílico (PVA, Liquifilm®) e unha solución de ácido hialurónico.

Para iso, realizáronse ensaios *in vitro* (estudos de estabilidade) e *in vivo* (tempo de permanencia na córnea mediante Tomografía de Emisión de Positróns) das tres posibles formulacións. O estudo de estabilidade a diferentes condicións de temperatura levouse a cabo durante 90 días en conxelación (-20 °C), refrixeración (2-8 °C) e a temperatura ambiente (20-25 °C). Os parámetros de osmolalidade, pH e concentración de fármaco estudáronse durante este período. A única formulación que se mantivo estable durante 90 días en condicións de refrixeración e conxelación foi a do vehículo de PVA, en BSS só aguantou en conxelación e en ácido hialurónico non se preservou en ningunha das condicións. As medidas de pH e de osmolalidade mantivéronse constantes cun pH de 7,5 e uns valores de osmolalidade demasiado altos (por encima de 1000 mOsm/kg) en comparación ca osmolalidade fisiolóxica da superficie ocular. En canto ó estudio da permanencia ocular mediante PET fíxose un seguimento de 3 horas trala instilación do colirio no ollo, no que se puido comprobar que o colirio de tacrolimus con PVA era o que mellor aclaramento producía, aumentando o tempo de contacto na superficie ocular.

A continuación, seleccionouse a mellor formulación (tacrolimus en PVA), e avaliouse seu perfil toxicolóxico e a súa eficacia clínica en comparación co colirio comercial de ciclosporina (Restasis®). O tacrolimus con PVA mostrou menor

citotoxicidade que a ciclosporina sendo mellor tolerado polas células epiteliais corneais nos primeiros tempos. Por outra parte, realizouse un estudo piloto en 8 pacientes no que se mostraron melloras significativas nos pacientes, sen reaccións adversas apreciables. Sen embargo, 7 dos 8 pacientes mostraron un leve proído ocular despois da instilación deste colirio de tacrolimus, seguramente provocado pola alta osmolalidade da formulación e a presencia de excipientes pouco adaptados á vía ocular presentes no Prograf®.

Baseándose nos estudos de estabilidade, permanencia ocular, seguridade e eficacia clínica, é posible concluír que o colirio de tacrolimus-PVA é un candidato adecuado para a súa aplicación clínica nas enfermidades inflamatorias oftalmolóxicas.

Coa finalidade de resolver os problemas derivados da presenza de excipientes pouco adaptados no Prograf® no **capítulo 3** abordouse o deseño e desenvolvemento de dous tipos de formulacións oftálmicas que conteñen tacrolimus para o tratamento da uveíte. O tacrolimus é un fármaco inmunosupresor que se caracteriza por ter unha moi baixa solubilidade en auga e unha baixa estabilidade en medio acuoso, por esta razón o obxectivo principal foi conseguir a solubilización do fármaco combinando diferentes proporcións dun derivado da β-ciclodextrina, a 2-hidroxipropil-β-ciclodextrina (HPβCD). Desta maneira, logrouse unha interacción entre o fármaco e a ciclodextrina formando a complexación do tacrolimus na cavidade hidrofóbica da HPβCD, xerando un aumento na solubilidade do fármaco. Toda esta información conseguiuse realizando estudos do diagrama de solubilidade, RMN e modelaxe molecular.

Tras conseguir a solubilización do tacrolimus procedeuse a comprobar se este complexo funcionaría noutros solventes acuosos, como no caso dunha solución de BSS (Balanced Salt Solution), e tamén en Liquifilm®, unha lágrima lubrificante que contén alcohol polivinílico (PVA). Diferentes parámetros para a optimización da formulación foron levados a cabo, conseguindo fixar a composición de cada colirio e o tempo óptimo de solubilización do tacrolimus. Destes estudos resultaron catro formulacións definitivas: dúas formulacións co 20% *(p/v)* de HPβCD e 0,01% *(p/v)* de tacrolimus utilizando como vehículo BSS no primeiro caso e Liquifilm® no segundo, e outras dúas formulacións co 40% *(p/v)* de HPβCD e 0,02% *(p/v)* de tacrolimus, tamén en BSS e Liquifilm®.

Unha vez que se axustaron as formulacións procedeuse á caracterización fisicoquímica, determinando parámetros como o pH, osmolalidade, tensión superficial da formulación e a forza necesaria para dispensar unha gota. Estes estudos concluíron que as formulacións teñen un rango de pH óptimo para a fisioloxía dos ollos e valores de osmolalidade de ≃320 mOsm/kg. De esta

maneira, solucionouse o problema de osmolalidade existente nas formulacións clásicas preparadas na farmacia hospitalaria que tiñan valores por encima de ≃1200 mOsm/kg.

Os estudos de irritación e toxicidade ocular foron realizados a través do test de permeabilidade e opacidade en córnea bovina e tamén co ensaio na membrana corioalantoidea de ovos fertilizados. Desta maneira comprobouse que ningunha das formulacións xerou o mínimo indicio de toxicidade ou irritación. Outra característica importante que se tiña que demostrar é que o tacrolimus seguise estable en solución acuosa durante un período razoable de tempo. Polo que se realizou un estudo de estabilidade nos dous colirios que máis tacrolimus tiñan, 0,02% *(p/v)* en BSS e Liquifilm®, con este ensaio demostrouse que os colirios podían preservarse polo menos tres meses en refrixeración (4 ± 2 °C).

O tempo de permanencia dun colirio sobre a superficie ocular é outro parámetro sumamente importante para aumentar a biodispoñibilidade do fármaco na diana terapéutica. Polo tanto, realizáronse estudos de mucoadhesion corneal *ex vivo* e de tempo de permanencia na superficie corneal *in vivo* mediante imaxe PET. Despois de analizar os resultados observouse que a HPβCD non só aumenta a solubilidade e estabilidade do tacrolimus en solución, senón que tamén inflúe nas propiedades de mucoadhesividade, xa que aumentando a proporción de ciclodextrina mellora o tempo de permanencia. A formulación co 40% *(p/v)* de HPβCD, 0,02% *(p/v)* de tacrolimus diluído en Liquifilm® duplicou o tempo de permanencia comparando ca formulación clásica de tacrolimus.

O **capítulo 4** basease na demostración do efecto antiinflamatorio nun modelo de uveíte inducido por endotoxina en ratas da mellor formulación obtida no capítulo anterior. Para iso dividíronse 32 ratas en 4 grupos de 8 ratas cada un: (a) un grupo de ratas sas non tratadas, (b) un grupo de ratas con uveíte non tratadas, (c) un grupo de ratas con uveíte tratadas co tratamento estándar de gotas oftálmicas de dexametasona (Maxidex®) e (d) un grupo de ratas con uveíte tratadas co colirio de TAC-HPβCD proposto.

A uveíte foi inducida inoculando 1 mg/kg de Lipopolisacárido de *Escherichia coli* (LPS) diluído en 0,1 mL de BSS na pata dereita do animal. As ratas con tratamento recibiron a instilación dos colirios 3 horas antes da indución da enfermidade e cada 3h ata cumprir as 24 h de estudo. Unha vez rematado o período de estudo, as ratas foron eutanasiadas con dióxido de carbono para proceder á recollida de mostras, e así poder analizar cuantitativamente a presenza de leucocitos no humor acuoso, avaliar a histoloxía ocular e por último realizar a cuantificación mediante PCR da expresión de niveis de IL-6, IL-8, MIP-1α e TNFα no ollo.

O primeiro que se comprobou foi que o modelo de uveíte induciuse de maneira adecuada, que supón que trala administración de LPS produciuse unha activación dos TLRs, aumentando os niveis de mediadores proinflamatorios, xerándose a inflamación da úvea anterior, a coroides e a retina e producíndose a rotura da barreira hematohumoral, con exsudación de leucocitos ó humor acuoso. Este efecto púidose comprobar gracias ós niveis significativamente maiores de TNFα, IL-6, IL-8, MIP-1α e leucocitos, así como a avaliación histolóxica do grupo con uveíte sen tratar en comparación co grupo san.

Os niveis significativamente máis baixos de TNFα, IL-6, MIP-1α e leucocitos, así como a avaliación histolóxica do colirio de TAC-HPβCD en comparación co grupo enfermo confirma a eficacia desta formulación no tratamento da uveíte. En canto á comparación entre a eficacia da nosa formulación e o tratamento estándar con dexametasona, non se atoparon diferencias estatisticamente significativas entre os niveis de citoquinas proinflamatorias do grupo de TAC-HPβCD e do grupo de dexametasona, o que demostra que os colirios de TAC-HPβCD poderían ser unha alternativa ó tratamento tópico con corticoides no tratamento deste modelo de uveíte e seu uso clínico traslacional debería estudarse en futuros estudos.

Ademais, no que respecta á expresión do ARNm do TNFα, a súa redución é estatisticamente significativa só entre o grupo de TAC-HPβCD e o de uveíte, pero non entre o grupo de dexametasona e o de uveíte, o cal é coherente co mecanismo de acción do tacrolimus e podería ser especialmente beneficioso en uveítes relacionadas co aumento deste factor, como a uveíte asociada a HLA B27, a uveíte sarcoide e a uveíte asociada á enfermidade de Behçet, entre outras entidades de uveíte non infecciosas.

Posto que, tal como se demostrou, o aumento da expresión do TNFα xoga un importante papel nas enfermidades inflamatorias oculares, no **capítulo 5** expúxose o estudo do perfil farmacocinético ocular e do aclaramento e distribución en tecidos dun anticorpo monoclonal inxectado intravítreamente que actúa inhibindo os receptores de TNFα chamado adalimumab.

O adalimumab é un fármaco anti-TNFα aprobado para o tratamento da uveíte por inxección subcutánea. Esta vía de administración expón ós pacientes a efectos adversos sistémicos e dificulta á obtención de concentracións terapéuticas do fármaco no lugar de acción debido ás barreiras anatómicas e fisiolóxicas do ollo.

O uso de técnicas de imaxe molecular en combinación co radiomarcaxe de anticorpos permite non só a observación de seu patrón de distribución no organismo, senón tamén a súa cuantificación en tempo real. Neste sentido, a Tomografía por Emisión de Positróns (PET) representa unha prometedora

ferramenta de imaxe para a avaliación non invasiva da farmacocinética dos anticorpos, permitindo realizar estudos lonxitudinais nos que se sigue a cada animal ó longo do tempo. Isto representa claramente a vantaxe de reducir o número de animais, segundo os marcos das 3Rs. O Zirconio-89 (^{89}Zr) é un radionúclido que xoga un papel importante nas técnicas de imaxe de Inmuno-Tomografía por Emisión de Positróns (Inmuno-PET). A súa longa vida media (3,3 días) é favorable para avaliar a distribución *in vivo* dos anticorpos monoclonais (mAb), sendo de gran potencial na monitorización de terapias baseadas en anticorpos. A técnica Inmuno-PET combina a sensibilidade do PET ca especificidade do anticorpo, permitindo coñecer a súa distribución corporal e seu comportamento farmacocinético.

Para lograr o obxectivo deste estudo o adalimumab conxugouse cun axente quelante chamado deferoxamina cuxa función é a de atrapar os núcleos de ^{89}Zr. Unha vez rematada a conxugación o anticorpo conxugado foi radiomarcado con ^{89}Zr cunha actividade específica máxima de 10 MBq/mg, conseguindo un resultado de radiomarcaxe óptimo, xa que a pureza radioquímica trala ultrafiltración foi do 99,69%. En canto ó deseño do estudo empregáronse dous grupos de ratas: un grupo control de ratas sas (n=3; 6 ollos) e un grupo de ratas con uveíte inducidas da mesma forma que no capítulo 4 (n=6, 12 ollos). Cada un dos animais foi inxectado no vítreo con 4 μL que contiñan ≃1,74 MBq de adalimumab marcado con ^{89}Zr (nos dous ollos). A adquisición de imaxes do microPET realizáronse inmediatamente despois da inxección e en diferentes puntos temporais ó longo dun estudo de 10 días, neses mesmos tempos recolléronse mostras de sangue a través da vena da cola.

O análise cuantitativo foi realizado nas imaxes PET obtidas, nelas debuxáronse diferentes rexións de interese (ROIs) nos órganos de estudo (ollos, corazón, fígado, bazo e ganglios linfáticos cervicais) co fin de cuantificar a actividade radioactiva emitida polo ^{89}Zr-adalimumab e así xerar as súas curvas cinéticas. Modelos mono- e bicompartimentais foron empregados para axustar os datos experimentais.

A farmacocinética ocular do anticorpo axustouse a un modelo mono-compartimental, mostrando unha semivida de eliminación intraocular de 15,57 horas para as ratas sas e de 33,64 horas para as ratas con uveíte, o que implica que o ^{89}Zr-adalimumab permaneceu ó redor de dúas veces máis nas ratas ca enfermidade en comparación cas sas. Estes resultados mostran que, pese a que é esperable que o aclaramento ocular dun fármaco nun ollo con inflamación sexa máis rápido debido ó aumento da permeabilidade das barreiras oculares, neste caso non ocorre isto. A capacidade do adalimumab de unirse selectivamente ó factor de necrose tumoral é ben coñecida, polo que este resultado é coherente ca sobreprodución de TNFα dos macrófagos e outras citoquinas no proceso de

uveíte, que activa as células dendríticas, iniciando a cascada inflamatoria na que as células Th1 e Th17 migran e se infiltran na barreira hemorretiniana causando danos.

Os resultados do análise farmacocinético compartimental en sangre mostran un comportamento diferente entre os animais sans e os enfermos. A actividade fronte ó tempo nos animais sans axústase a un modelo bicompartimental que mostra unha rápida captación do anticorpo dende o ollo simultaneamente ó proceso de distribución a tecidos. Sen embargo, no caso dos animais con uveíte, a actividade fronte ó tempo axústase mellor a un modelo dun compartimento. Isto pode deberse a que o proceso de transferencia do ollo á sangue é moito máis lento actuando como paso limitante, polo que non se aprecia o proceso de distribución ós órganos.

Este estudo mostra por primeira vez o análise farmacocinético ocular e sanguíneo de adalimumab nun modelo de uveíte en ratas aportando unha información moi valiosa para poder desenvolver novos sistemas de liberación controlada de adalimumab que permitan espazar a administración do fármaco e así mellorar a calidade de vida dos pacientes.

En **conclusión**, nesta tese de doutoramento propuxéronse diferentes formulacións de tacrolimus para a administración tópico-oftálmica cun gran potencial, apoiadas por amplos estudos preclínicos co fin de lograr unha base consistente como alternativa a outros tratamentos farmacolóxicos para as enfermidades inflamatorias oculares. Isto foi confirmado con amplos estudos *in vitro*, *ex vivo* e *in vivo*. Por outra parte, no que respecta ó estudo de inxeccións intravítreas de adalimumab, esta tese de doutoramento dilucidou a distribución e farmacocinética intravítrea do anticorpo monoclonal que permitirá o desenvolvemento de novos sistemas de administración.

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RESUMEN

La calidad de vida de millones de personas en todo el mundo se ve comprometida por causa de diferentes afecciones y patologías oculares. Uno de los retos más complejos para los farmacéuticos es conseguir tratamientos eficaces y efectivos para el ojo, ya que su anatomía y fisiología presenta muchos mecanismos de protección, lo que hace que sea un órgano realmente complejo de tratar. Por esta razón, es importante que la biodisponibilidad del fármaco en aquellos lugares de acción específicos de las diferentes estructuras oculares donde sea necesario un tratamiento sea adecuada.

Los avances conseguidos respecto a las tecnologías de administración de fármacos han ido evolucionando con el paso del tiempo, aunque todavía quedan grandes desafíos en lo que se refiere al tratamiento de enfermedades oculares. Estos sistemas de administración por vía ocular deben mantener las concentraciones terapéuticas del fármaco, reducir la frecuencia de dosificación y atravesar las diferentes barreras oculares.

El uso de la instilación tópico-oftálmica para el tratamiento de enfermedades que afectan al segmento anterior del ojo es la vía de administración más deseable. Estos sistemas presentan varias ventajas ya que se consideran fáciles de manejar, tienen una aceptación alta por parte del paciente y también son rentables económicamente. No obstante, también presenta ciertas limitaciones en cuanto a la baja biodisponibilidad ocular, esto es debido a la elevada tasa de renovación lacrimal lo que equivale a un aclaramiento importante a través del drenaje nasolacrimal, repercutiendo en la repetición constante de instilaciones frecuentes. Por lo tanto, es necesario que muchos de los esfuerzos estén enfocados en abordar este problema. Esta solución pasa por desarrollar sistemas que permitan aumentar la permanencia en la superficie ocular y que faciliten la entrada en los tejidos.

Por otro lado, la administración directa mediante inyecciones intravítreas de fármacos es útil en el tratamiento de patologías oculares del segmento posterior del ojo. Esta vía presenta algunas ventajas en comparación con la administración sistémica eludiendo las barreras hemato-oculares, permitiendo alcanzar niveles de fármaco más elevados en la cavidad ocular y limitando los efectos secundarios

sistémicos. Sin embargo, también presenta alguna limitación ya que se considera una ruta invasiva que suele requerir inyecciones repetidas, por este motivo, es necesario conocer la farmacocinética intravítrea de los fármacos para poder desarrollar nuevos sistemas de liberación sostenida, y así reducir el número de administraciones.

Las desventajas de la vía tópico-oftálmica y de las inyecciones intravítreas se ven reflejadas en los tratamientos de patologías inflamatorias oculares que afectan al segmento anterior y posterior del ojo como pueden ser la uveítis en su amplio espectro o la queratoconjuntivitis, patologías en las que se centra esta tesis doctoral.

La uveítis es el término utilizado para un grupo muy heterogéneo de enfermedades que causan inflamación ocular. Debido a que su origen y lugar de afectación pueden ser muy diversos, para el tratamiento de estas enfermedades es necesario emplear diferentes vías y estrategias según el lugar y el grado de afectación, por lo que un amplio abanico de fármacos y rutas de administración son necesarias para una cura eficiente de la enfermedad. A lo largo del tiempo, se han ido estudiando el uso de diferentes familias de fármacos con acción terapéutica, así como la optimización y mejora de las vías de administración para reducir los posibles efectos adversos sistémicos causados por los fármacos. La familia de corticosteroides es una de las más empleadas ya que produce efectos favorables rápidamente, sin embargo, también es frecuente encontrar efectos adversos no deseados en algunas estructuras oculares y toxicidad sistémica en tratamientos de larga duración. Por esta razón, han surgido nuevas familias de fármacos que han demostrado un efecto terapéutico frente a estas enfermedades, como son el caso de los inmunosupresores (tacrolimus) o fármacos biológicos (adalimumab), ambos fármacos estudiados en esta tesis doctoral.

El **capítulo 1** contiene una revisión bibliográfica que pretende dar una visión general de los principales aspectos que intervienen en la farmacocinética de los fármacos oculares destinados a tratar diferentes enfermedades oculares. En primer lugar, se analizaron los diferentes factores que intervienen en la administración ocular de fármacos, abarcando las diferentes rutas de entrada en el ojo. Las barreras fisiológicas y las vías de transporte de los fármacos fueron detalladamente descritas, y también se discutieron las ventajas e inconvenientes de las diferentes vías de administración al ojo.

Las vías de administración convencionales, como la tópica o la sistémica, suelen presentar importantes limitaciones, ya sea por la baja penetración ocular o por la aparición de efectos secundarios ligados a la posología, entre otros. Por lo tanto, son necesarios nuevos sistemas de administración de fármacos (DDS) que ayuden a prolongar y adecuar los intervalos de administración en las patologías

oculares. Sin embargo, el desarrollo de estos nuevos sistemas es especialmente complicado debido a varios aspectos que deben considerarse, como la farmacocinética, la inmunogenicidad, la biodegradación, la tolerabilidad y la toxicidad. En las últimas décadas se ha observado un aumento exponencial en el diseño y desarrollo de nuevos sistemas de administración de fármacos destinados al tratamiento de patologías oculares. Desgraciadamente, los conocimientos sobre la capacidad de estos sistemas para vehiculizar los fármacos en el ojo siguen siendo escasos.

Asimismo, se han producido grandes avances en cuanto a la investigación y el desarrollo de nuevas vías de administración alternativas para alcanzar concentraciones de fármaco en las diferentes partes del ojo. Todas ellas tienen características farmacocinéticas específicas que las hacen útiles para el tratamiento de patologías oculares concretas. No obstante, estas vías han mostrado diversas ventajas y limitaciones, donde la elección de una u otra depende, no sólo de la propia patología, sino de la forma farmacéutica, del fármaco utilizado y de la adherencia del paciente al tratamiento.

El **capítulo 2** engloba el estudio de elaboración y eficacia de una formulación oftálmica de tacrolimus al 0,03% *(p/v)* a partir de la presentación comercial sistémica (Prograf®) e introducida en tres tipos de vehículos: Balanced Salt Solution (BSS), solución de alcohol polivinílico (PVA, Liquifilm®) y una solución de ácido hialurónico.

Para ello, se realizaron ensayos *in vitro* (estudios de estabilidad) e *in vivo* (tiempo de permanencia en la córnea mediante Tomografía de Emisión de Positrones) de las tres posibles formulaciones. El estudio de estabilidad a diferentes condiciones de temperatura se llevó a cabo durante 90 días en congelación (-20 °C), refrigeración (2-8 °C) y a temperatura ambiente (20-25 °C). Los parámetros de osmolalidad, pH y concentración de fármaco fueron estudiados durante este período. La única formulación que se mantuvo estable durante 90 días en condiciones de refrigeración y congelación fue la del vehículo de PVA, en BSS solo aguantó en congelación y en ácido hialurónico no se preservó en ninguna de las condiciones. Las medidas de pH y de osmolalidad se mantuvieron constantes con un pH de 7,5 y unos valores de osmolalidad demasiado altos (por encima de 1000 mOsm/kg) en comparación con la osmolalidad fisiológica de la superficie ocular. En cuanto al estudio de la permanencia ocular mediante PET se hizo un seguimiento de 3 horas tras la instilación del colirio en el ojo, en el que se pudo comprobar que el colirio de tacrolimus con PVA era el que menor aclaramiento producía, aumentando el tiempo de contacto en la superficie ocular.

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A continuación, se seleccionó la mejor formulación (tacrolimus en PVA), y se evaluó su perfil toxicológico y su eficacia clínica en comparación con el colirio

comercial de ciclosporina (Restasis®). El tacrolimus con PVA mostró menor citotoxicidad que la ciclosporina siendo mejor tolerado por las células epiteliales corneales en los primeros tiempos. Por otra parte, se realizó un estudio piloto en 8 pacientes en el que se mostraron mejoras significativas en los pacientes, sin reacciones adversas apreciables. Sin embargo, 7 de los 8 pacientes mostraron un leve picor ocular después de la instilación de este colirio de tacrolimus, seguramente provocado por la alta osmolalidad de la formulación y la presencia de excipientes poco adaptados a la vía ocular presentes en Prograf®.

Basándose en los estudios de estabilidad, permanencia ocular, seguridad y eficacia clínica, es posible concluir que el colirio de tacrolimus-PVA es un candidato adecuado para su aplicación clínica en las enfermedades inflamatorias oftalmológicas.

Con la finalidad de resolver los problemas derivados de la presencia de excipientes poco adaptados en el Prograf® en el **capítulo 3** se abordó el diseño y desarrollo de dos tipos de formulaciones oftálmicas que contienen tacrolimus para el tratamiento de la uveítis. El tacrolimus es un fármaco inmunosupresor que se caracteriza por tener una muy baja solubilidad en agua y una baja estabilidad en medio acuoso, por esta razón el objetivo principal fue conseguir la solubilización del fármaco combinando diferentes proporciones de un derivado de la β-ciclodextrina, la 2-hidroxipropil-β-ciclodextrina (HPβCD). De esta manera, se logró una interacción entre el fármaco y la ciclodextrina formando la complejación del tacrolimus en la cavidad hidrofóbica de la HPβCD, generando un aumento en la solubilidad del fármaco. Toda esta información se consiguió realizando estudios de solubilidad, RMN y modelaje molecular.

Tras haber conseguido la solubilización del tacrolimus se procedió a comprobar si este complejo podía funcionar en otros solventes acuosos, como en el caso de una solución de BSS (Balanced Salt Solution), y también en Liquifilm®, una lágrima lubrificante que contiene alcohol polivinílico (PVA). Diferentes parámetros para la optimización de la formulación fueron llevados a cabo, consiguiendo fijar la composición de cada colirio y el tiempo óptimo de solubilización del tacrolimus. De estos estudios resultaron cuatro formulaciones definitivas: dos formulaciones con el 20% *(p/v)* de HPβCD y 0,01% *(p/v)* de tacrolimus utilizando como vehículo BSS en el primer caso y Liquifilm® en el segundo, y otras dos formulaciones con el 40% *(p/v)* de HPβCD y 0,02% *(p/v)* de tacrolimus, también en BSS y Liquifilm®.

Una vez que las formulaciones estaban a punto se procedió a la caracterización fisicoquímica, determinando parámetros como el pH, osmolalidad, tensión superficial de la formulación y la fuerza necesaria para dispensar una gota. Estos estudios concluyeron que las formulaciones tienen un rango de pH óptimo para

la fisiología del ojo y valores de osmolalidad de ≃320 mOsm/kg. De esta manera, se solucionó el problema de osmolalidad existente en las formulaciones clásicas preparadas en la farmacia hospitalaria que tenían valores por encima de ≃1200 mOsm/kg.

Los estudios de irritación y toxicidad ocular fueron realizados a través del test de permeabilidad y opacidad en córnea bovina y también con el ensayo en la membrana corioalantoidea de huevos fertilizados. De esta manera se comprobó que ninguna de las formulaciones generó el mínimo indicio de toxicidad o irritación. Otra característica importante que había que demostrar es que el tacrolimus permaneciese estable en solución acuosa durante un periodo razonable de tiempo. Por lo que se realizó un estudio de estabilidad en los dos colirios que más tacrolimus contenían, 0,02% *(p/v)* en BSS y Liquifilm®, con este ensayo se demostró que los colirios podían preservarse durante al menos tres meses en refrigeración (4 ± 2 °C).

El tiempo de permanencia de un colirio sobre la superficie ocular es otro parámetro sumamente importante para aumentar la biodisponibilidad del fármaco en la diana terapéutica. Por lo tanto, se realizaron estudios de mucoadhesion corneal *ex vivo* y de tiempo de permanencia en la superficie corneal *in vivo* mediante imagen PET. Tras analizar los resultados se observó que la HPβCD no solo aumenta la solubilidad y estabilidad del tacrolimus en solución, sino que también influye en las propiedades de mucoadhesividad, ya que aumentando la proporción de ciclodextrina mejora el tiempo de permanencia. La formulación con el 40% *(p/v)* de HPβCD, 0,02% *(p/v)* de tacrolimus disuelta en Liquifilm® duplicó el tiempo de permanencia en comparación con la formulación clásica de tacrolimus.

El **capítulo 4** se basa en la demostración del efecto antiinflamatorio en un modelo de uveítis inducido por endotoxina en ratas de la mejor formulación obtenida en el capítulo anterior. Para ello se dividieron 32 ratas en 4 grupos de 8 ratas cada uno: (a) un grupo de ratas sanas no tratadas, (b) un grupo de ratas con uveítis no tratadas, (c) un grupo de ratas con uveítis tratadas con el tratamiento estándar de gotas oftálmicas de dexametasona (Maxidex®) y (d) un grupo de ratas con uveítis tratadas con el colirio de TAC-HPβCD propuesto.

La uveítis fue inducida inoculando 1 mg/kg de Lipopolisacárido de *Escherichia coli* (LPS) diluido en 0,1 mL de BSS en la pata derecha del animal. Las ratas con tratamiento recibieron la instilación de los colirios 3h horas antes de la inducción de la enfermedad y cada 3h hasta cumplir las 24 h de estudio. Una vez terminado el período de estudio, las ratas fueron eutanasiadas con dióxido de carbono para proceder a la recogida de muestras, y así poder analizar cuantitativamente la presencia de leucocitos en el humor acuoso, evaluar la histología ocular y por

último realizar la cuantificación mediante PCR de la expresión de niveles de IL-6, IL-8, MIP-1α y TNFα en el ojo.

Lo primero que se comprobó fue que el modelo de uveítis se indujo de manera adecuada, que supone que tras la administración de LPS se produce una activación de los TLRs, aumentando los niveles de mediadores proinflamatorios, desarrollándose la inflamación de la úvea anterior, la coroides y la retina y produciéndose la ruptura de la barrera hematohumoral, con exudación de leucocitos al humor acuoso. Este efecto se pudo comprobar gracias a los niveles significativamente mayores de TNFα, IL-6, IL-8, MIP-1α y leucocitos, así como la evaluación histológica del grupo con uveítis sin tratar en comparación con el grupo sano.

Los niveles significativamente más bajos de TNFα, IL-6, MIP-1α y leucocitos, así como la evaluación histológica del colirio de TAC-HPβCD en comparación con el grupo enfermo confirma la eficacia de esta formulación en el tratamiento de la uveítis. En cuanto a la comparación entre la eficacia de nuestra formulación y el tratamiento estándar con dexametasona, no se encontraron diferencias estadísticamente significativas entre los niveles de citoquinas proinflamatorias del grupo de TAC-HPβCD y del grupo de dexametasona, lo que demuestra que los colirios de TAC-HPβCD podrían ser una alternativa al tratamiento tópico con corticoides en el tratamiento de este modelo de uveítis y su uso clínico traslacional debería estudiarse en futuros estudios.

Además, en lo que respecta a la expresión del ARNm del TNFα, su reducción es estadísticamente significativa sólo entre el grupo de TAC-HPβCD y el de uveítis, pero no entre el grupo de dexametasona y el de uveítis, lo cual es coherente con el mecanismo de acción del tacrolimus y podría ser especialmente beneficioso en uveítis relacionadas con el aumento de este factor, como la uveítis asociada a HLA B27, la uveítis sarcoide y la uveítis asociada a la enfermedad de Behçet, entre otras entidades de uveítis no infecciosas.

Puesto que, tal como se ha demostrado, el aumento de la expresión del TNFα juega un importante papel en las enfermedades inflamatorias oculares, en el **capítulo 5** se planteó el estudio del perfil farmacocinético ocular y del aclaramiento y distribución en tejidos de un anticuerpo monoclonal inyectado intravítreamente que actúa inhibiendo los receptores de TNFα llamado adalimumab.

El adalimumab es un fármaco anti-TNFα aprobado para el tratamiento de la uveítis por inyección subcutánea. Esta vía de administración expone a los pacientes a efectos adversos sistémicos y dificulta la obtención de

concentraciones terapéuticas del fármaco en el lugar de acción debido a las barreras anatómicas y fisiológicas del ojo.

El uso de técnicas de imagen molecular en combinación con el radiomarcaje de anticuerpos permite no sólo la observación de su patrón de distribución en el organismo, sino también su cuantificación en tiempo real. En este sentido, la Tomografía por Emisión de Positrones (PET) representa una prometedora herramienta de imagen para la evaluación no invasiva de la farmacocinética de los anticuerpos, permitiendo realizar estudios longitudinales en los que se sigue a cada animal a lo largo del tiempo. Esto representa claramente la ventaja de reducir el número de animales, según los marcos de las 3Rs. El Zirconio-89 (^{89}Zr) es un radionúclido que desempeña un papel importante en las técnicas de imagen de Inmuno-Tomografía por Emisión de Positrones (Inmuno-PET). Su larga vida media (3,3 días) es favorable para evaluar la distribución *in vivo* de los anticuerpos monoclonales (mAb), siendo de gran potencial en la monitorización de terapias basadas en anticuerpos. La técnica Inmuno-PET combina la sensibilidad del PET con la especificidad del anticuerpo, permitiendo conocer su distribución corporal y su comportamiento farmacocinético.

Para lograr el objetivo de este estudio el adalimumab se conjugó con un agente quelante llamado deferoxamina cuya función es la de atrapar los núcleos de ^{89}Zr. Una vez terminada la conjugación el anticuerpo conjugado fue radiomarcado con ^{89}Zr con una actividad específica máxima de 10 MBq/mg, consiguiendo un resultado de radiomarcaje óptimo, ya que la pureza radioquímica tras la ultrafiltración fue del 99,69%. En cuanto al diseño del estudio se emplearon dos grupos de ratas: un grupo control de ratas sanas (n=3; 6 ojos) y un grupo de ratas con uveítis inducidas de la misma forma que en el capítulo 4 (n=6, 12 ojos). Cada uno de los animales fue inyectado en el vítreo con 4 µL que contenían ≃1,74 MBq de adalimumab marcado con ^{89}Zr (en los dos ojos). La adquisición de imágenes del microPET se realizaron inmediatamente después de la inyección y en diferentes puntos temporales a lo largo de un estudio de 10 días, en esos mismos tiempos se recolectaron muestras de sangre a través de la vena de la cola.

El análisis cuantitativo fue realizado en las imágenes PET obtenidas, en ellas se dibujaron diferentes regiones de interés (ROIs) en los órganos de estudio (ojos, corazón, hígado, bazo y ganglios linfáticos cervicales) con el fin de cuantificar la actividad radioactiva emitida por el ^{89}Zr-adalimumab y así generar sus curvas cinéticas. Modelos mono- y bicompartimentales fueron empleados para ajustar los datos experimentales.

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La farmacocinética ocular del anticuerpo se ajustó a un modelo mono-compartimental, mostrando una semivida de eliminación intraocular de 15,57 horas para las ratas sanas y de 33,64 horas para las ratas con uveítis, lo que

implica que el ^{89}Zr-adalimumab permaneció alrededor de dos veces más en las ratas con la enfermedad en comparación con las sanas. Estos resultados muestran que, pese a que es esperable que el aclaramiento ocular de un fármaco en un ojo con inflamación sea más rápido debido al aumento de la permeabilidad de las barreras oculares, en este caso no ocurre esto. La capacidad del adalimumab de unirse selectivamente al factor de necrosis tumoral es bien conocida, por lo que este resultado es coherente con la sobreproducción de TNFα de los macrófagos y otras citoquinas en el proceso de uveítis, que activa las células dendríticas, iniciando la cascada inflamatoria en la que las células Th1 y Th17 migran y se infiltran en la barrera hemorretiniana causando daños.

Los resultados del análisis farmacocinético compartimental en sangre muestran un comportamiento diferente entre los animales sanos y los enfermos. La actividad frente al tiempo en los animales sanos se ajusta a un modelo bicompartimental que muestra una rápida captación del anticuerpo desde el ojo simultáneamente al proceso de distribución tisular. Sin embargo, en el caso de los animales con uveítis, la actividad frente al tiempo se ajusta mejor a un modelo de un compartimento. Esto puede deberse a que el proceso de transferencia del ojo a la sangre es mucho más lento actuando como paso limitante, por lo que no se aprecia el proceso de distribución a los órganos.

Este estudio muestra por primera vez el análisis farmacocinético ocular y sanguíneo de adalimumab en un modelo de uveítis en ratas aportando una información muy valiosa para poder desarrollar nuevos sistemas de liberación controlada de adalimumab que permitan espaciar la administración del fármaco y así mejorar la calidad de vida de los pacientes.

En **conclusión**, en esta tesis doctoral se han propuesto diferentes formulaciones de tacrolimus para la administración tópico-oftálmica con un gran potencial, respaldadas por amplios estudios preclínicos con el fin de lograr una base consistente como alternativa a otros tratamientos farmacológicos para las enfermedades inflamatorias oculares. Esto fue confirmado con amplios estudios *in vitro*, *ex vivo* e *in vivo*. Por otra parte, en lo que respecta al estudio de inyecciones intravítreas de adalimumab, esta tesis doctoral ha dilucidado la distribución y farmacocinética intravítrea del anticuerpo monoclonal que permitirá el desarrollo de nuevos sistemas de administración.

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DE COMPOSTELA

INDEX

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DE COMPOSTELA

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DE SANTIAGO
DE COMPOSTELA

INDEX

OBJECTIVES AND ORGANIZATION 1

STATEMENT OF THE PROBLEM 3

OBJECTIVES 4

ORGANIZATION 5

INTRODUCTION 7

1. UVEITIS 9

1.1 Anatomic classification 9

1.2 Clinical course classification 10

2. ETIOLOGY AND PATHOGENESIS OF UVEITIS 10

3. EPIDEMIOLOGY OF UVEITIS 13

4. RISKS FACTORS FOR UVEITIS 14

4.1 Genetic risks factors 14

4.2 Environmental risks factors 15

5. TREATMENTS 16

5.1 Corticosteroids 18

5.2 Mydriatics and cycloplegics 18

5.3 Immunosuppressive agents 18

5.3.1 T-lymphocyte inhibitors 19

5.3.2 Antimetabolites 20

5.3.3 Alkylating agents 21

5.4 Biological agents 21

6. IN VIVO ANIMAL MODELS 22

6.1 Experimental Autoimmune Uveitis model (EAU) 23

6.2 Experimental Melanin Protein-Induced Uveitis model (EMIU) 23

6.3 Experimental Autoimmune Encephalomyelitis-Associated Anterior Uveitis model (EAE/AU) 24

6.4 Endotoxin Induced Uveitis model (EIU) 24

7. IN VIVO IMAGING IN PRECLINICAL RESEARCH 24

7.1 Structural imaging techniques 25

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

7.1.1 Micro-Computed Tomography (μCT) 25
7.1.2 Micro-Magnetic Resonance Imaging (μMRI) 25
7.2 Molecular imaging techniques 26
7.2.1. Micro-Single Photon Emission Computed Tomography imaging (μSPECT) 26
7.2.2. Micro-Positron Emission Tomography imaging (μPET) 27
7.3 Radiolabeling 27
8. REFERENCES 28

CHAPTER 1 39

1. INTRODUCTION 42
2. ROUTES OF DRUG DELIVERY TO THE POSTERIOR SEGMENT OF THE EYE 44
2.1. Intravitreal Administration 44
2.1.1. Vitreous and Posterior Compartment Barriers to Drug Delivery 44
2.1.2. Advantages and Limitations 48
2.1.3. Pharmacokinetics 49
2.1.4. Drug Delivery Systems 57
2.2. Topical Administration 58
2.2.1. Ocular Barriers for the Entry of Drugs: Precorneal Factors 58
2.2.2. Corneal and Anterior Compartment Barriers 58
2.2.3. Advantages and Limitations 61
2.2.4. Pharmacokinetics 61
2.2.5. Drug Delivery Systems 64
2.3. Systemic Administration 64
2.3.1. Advantages and Limitations 65
2.3.2. Pharmacokinetics 66
2.3.3. Drug Delivery Systems 69
2.3.4. Systemic Drugs for Posterior Segment Eye Diseases 69
2.4. Periocular Administration 72
2.4.1. Advantages and Limitations 72
2.4.2. Pharmacokinetics 72
2.4.3. Subconjunctival Route 74
2.4.4. SubTenon's Route 76
2.4.5. Retrobulbar Route 77
2.4.6. Peribulbar Route 78
2.4.7. Posterior Juxtascleral Route 79

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2.4.8. Suprachoroidal Route 79
2.4.9. Subretinal Route 81
3. CONCLUSIONS 82
4. REFERENCES 83

CHAPTER 2 101

1. INTRODUCTION 104
2. MATERIALS AND METHODS 105
2.1. Preparation of formulations 105
2.2. Stability study 105
2.2.1. Quantification of tacrolimus amount 106
2.2.2. Osmolality, pH and Microbiological control growth 106
2.2.3. Statistical analysis 106
2.3. In vivo evaluation of the residence time on the ocular surface 107
2.4. Cytotoxicity assay 107
2.5. Pilot study of clinical use of TLI eye drops 108
3. RESULTS 110
3.1. Stability assay 110
3.2. In vivo evaluation of the permanence time on the ocular surface 111
3.3. Cytotoxicity assay 113
3.3. Clinical Effectiveness of TLI Eye Drops 114
4. DISCUSSION 117
5. REFERENCES 120

CHAPTER 3 129

1. INTRODUCTION 132
2. MATERIALS AND METHODS 134
2.1. Tacrolimus/HPβCD Solubilization Study 134
2.1.1. Phase Solubility Diagram 134
2.1.2. NMR studies and molecular modeling 135
2.1.3. Vehicle Solubility Study 136
2.2. Formulation Preparation Procedure 136
2.3. Optimization Procedure: Tacrolimus Solubilization Time 137
2.4. Physicochemical Characterization 138
2.4.1. pH and Osmolality Determination 138

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2.4.2. Surface Tension Determination 138
2.4.3. Squeezing Force Determination 138
2.5. Corneal Mucoadhesion 139
2.5.1. Bovine Corneal Opacity and Permeability Test (BCOP) 140
2.5.2. Hen´s Egg Test on the Chorioallantoic Membrane (HETCAM) 142
2.6. Stability Study 142
2.6.1. Quantification of Tacrolimus Amount 143
2.6.2. Osmolality, pH and Microbiological Control Growth 143
2.6.3. Statistical Analysis 143
2.7. In Vivo Evaluation of the Residence Time on the Ocular Surface 144
3. RESULTS AND DISCUSSION 145
3.1. Tacrolimus/HPβCD Solubilization Study 145
3.2. Physicochemical Characterization 150
3.2.1. pH, Osmolality and Surface Tension 150
3.2.2. Squeezing Force Determinations 151
3.3. Corneal Mucoadhesion 152
3.4. Ocular Irritancy and Toxicity 153
3.4.1. Bovine Corneal Opacity and Permeability Test (BCOP) 153
3.4.2. Hen's Egg Test on the Chorioallantoic Membrane (HETCAM) 155
3.5. Stability Study 155
3.6. In Vivo Evaluation of the Residence Time on the Ocular Surface 158
4. CONCLUSIONS 162
5. REFERENCES 162

CHAPTER 4 171

1. INTRODUCTION 174
2. MATERIALS AND METHODS 176
2.1. Formulation Elaboration Procedure 176
2.2. In Vivo Endotoxin-Induced Uveitis Model (EIU Model) 176
2.3. RNA isolation and real-time PCR analysis 178
2.4. Quantitative analysis of leucocytes in aqueous humor 178
2.5. Histological evaluation 178
2.6. Statistical analysis 179
3. RESULTS 179
3.1. Evaluation of the effect of tacrolimus on IL-6, IL-8, MIP-1α, and TNFα 179

3.2. Quantitative analysis of leucocytes in aqueous humor 181
3.3. Histological evaluation 182
4. DISCUSSION 183
5. CONCLUSIONS 185
6. REFERENCES 186

CHAPTER 5 191

1. INTRODUCTION 194
2. MATERIALS AND METHODS 195
2.1. Conjugation, radiolabeling and quality control 195
2.1.1. Adalimumab conjugation 196
2.1.2. Adalimumab radiolabeling 196
2.1.3. Quality control 196
2.2. Endotoxin-induced uveitis model (EIU) 197
2.3. Intravitreal administration of ^{89}Zr-adalimumab 197
2.4. PET acquisition and image analysis 198
2.5. Pharmacokinetic analysis 200
3. RESULTS AND DISCUSSION 202
3.1. Conjugation, radiolabeling and quality control 202
3.2. Pharmacokinetics after intravitreal administration 203
3.2.1. Ocular levels 203
3.2.2. Systemic distribution 205
4. DISCUSSION 209
5. CONCLUSIONS 212
6. REFERENCES 212

CONCLUSIONS 217

APPENDIX 229

CONFLICT OF INTEREST 231
ANIMAL EXPERIMENTS 231
CLINICAL STUDY 231
FUNDING AND ACKNOWLEDGEMENTS 232
ARTICLES DERIVED FROM THIS THESIS 232
FIGURES AUTHORSHIP 235

USC UNIVERSIDADE DE SANTIAGO

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DE COMPOSTELA

OBJECTIVES AND ORGANIZATION

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DE COMPOSTELA

OBJECTIVES AND ORGANIZATION

STATEMENT OF THE PROBLEM

Inflammatory eye diseases require effective treatment, which is related to achieving an effective drug concentration at the target site for long enough for the inflammation to subside. To accomplish this goal, special drug delivery routes or drug delivery systems that reach different parts of the eye are required. This is complicated by the fact that the eye is a singular and complex organ with a unique anatomy and physiology that contributes to its high level of protection. Consequently, access of exogenous substances to its internal structures is severely restricted.

Ocular surface diseases undergo extensive clearance, which leads to a decrease in the bioavailability of the drug in the inflamed area. For the corresponding diseases of the posterior segment of the eye, the drug has to cross numerous barriers or has to be administered by more invasive injections. These two common limitations are reflected in the difficulties in the treatment of uveitis involving different parts of the eye. Location and severity of the inflammation will influence the change from topical to systemic administration route.

The current treatment for uveitis is based on the administration of a first line of corticosteroids, both topically in the eye and systemically. Although these drugs have a highly and immediate efficacy, they also cause problems with repeated use. Long-term administration of these agents can cause serious systemic and ocular adverse effects There are more and more cases of patients with refractory problems due to treatment with corticosteroids, patients who do not respond to treatment or for whom the use of corticosteroids is contraindicated. For this reason, there is a need to use effective alternative treatments that improve the patient's life quality. The use of immunosuppressants such as cyclosporine A or tacrolimus has become the second line of therapy for uveitis. These drugs are more focused on systemic administration, causing undesirable effects. Therefore, the development of new formulations that are much more targeted to the site of action in order to minimize these contraindications is one of the most challenging tasks for pharmacists.

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On the other hand, uveitis located in the posterior ocular segment with a remarkable severity requires therapy tailored to their characteristics. Due to the

anatomical features of the eye, the access of drugs to the target site is hampered. Special route of administration, such as intravitreal injection, is needed to achieve adequate drug levels in the deeper ocular layers since in clinical practice this type of administration is not used, employing the systemic route.

The emergence of biological agents, such as anti-TNFα has opened up new routes of treatment, which have already demonstrated their efficacy. The use of these biological molecules by systemic administration has become increasingly important in recent times. However, there is still no comprehensive scientific basis for therapeutic doses or effective doses of these drugs inside the eye. Knowledge of the ocular pharmacokinetics after intravitreal injection of these anti-TNFα drugs or their distribution is another important aspect that needs to be resolved. Once the behavior of these biological molecules is clearly understood, controlled release systems can be developed to make delivery more efficient and limit invasiveness by reducing the number of injections.

OBJECTIVES

With these considerations in mind, the general objective of this thesis is to improve the pharmacological treatment of uveitis for ocular administration.

In order to achieve these purposes, specific objectives linked to this line of research were set, including:

1. To understand the main factors affecting ocular delivery for anterior and posterior eye segment treatment.
2. Characterization of classical tacrolimus eye drops reformulated from the intravenous drug in Hospital Pharmacy Departments.
   2.1. Preclinical characterization of tacrolimus eye drops.
   2.2. Clinical evaluation of tacrolimus eye drops.
3. Development of a new and safe tacrolimus ophthalmic formulation intended for the topical treatment of uveitis.
   3.1. Design, optimization, and physicochemical characterization of a hydroxypropyl-β-cyclodextrin topical ocular formulation containing tacrolimus.
   3.2. Characterize the stability of the developed formulation and translate its preparation to hospital pharmacy departments.
   3.3. Evaluate the residence time on the ocular surface of the developed eye drop.
4. Evaluation of the pharmacological effect of the tacrolimus/hydroxypropyl-β-cyclodextrin eye drop in an endotoxin-induced uveitis model.

5. Evaluation of the intravitreal route for the administration of an anti-TNFα antibody (adalimumab) in uveitis.
   5.1. Development of a radiolabeling procedure for adalimumab with Zirconium-89 (^{89}Zr).
   5.2. Evaluation of the pharmacokinetics and distribution of intravitreally injected adalimumab in healthy rat eyes.
   5.3. Evaluation of the pharmacokinetics and distribution of intravitreally injected adalimumab in uveitis rat eyes.

ORGANIZATION

Based on the formulated objectives, this doctoral thesis is presented as a compendium of four original research works and one review article. This doctoral thesis has been organized to comply with the regulation of the International Doctorate School at the University of Santiago de Compostela (Spain) regarding thesis structure, languages, and ethical and intellectual properties.

The present doctoral thesis is divided in three main sections correspondence with a general review and the two drugs studied.

The first section corresponds to a deep review of the ocular routes. It is included in the first chapter, Chapter 1.

Chapter 1 aims to provide an overview of the main aspects involved in ocular drug pharmacokinetics intended to treat eye diseases. A discussion of the different factors that are involved in the ocular drug delivery is first made, encompassing the different routes towards the posterior segment of the eye. Physiological barriers and drug transport pathways are described and the advantages and drawbacks of different administration routes to the eye are also discussed.

The second section focuses on the development, characterization and evaluation of the pharmacological effect of tacrolimus eye drops for the treatment of uveitis and other inflammatory diseases. It is divided in three chapters, from Chapter 2 to 4.

Chapter 2 comprises the characterization of three types of ocular vehicles containing tacrolimus reformulated from intravenous pharmaceutical form, the first vehicle is a Balanced Salt Solution (BSS®), the second one is a PVA solution (Liquifilm®) and the last one made from hyaluronic acid polymer. Moreover, an extensive preclinical and clinical evaluation have been performed.

Chapter 3 was based on the design, preparation, and characterization of tacrolimus/hydroxypropyl-β-cyclodextrin eye drops as topical ophthalmic formulation for uveitis treatment. A comparison has been made between the best formulation resulting from the Chapter 2 and the new tacrolimus/hydroxypropyl-β-cyclodextrin eye drop in different aspects: pH and osmolality, surface tension, squeezing force, *ex vivo* corneal mucoadhesion, ocular toxicity, stability study and *in vivo* evaluation of the residence time on the ocular surface.

Chapter 4 was aimed to investigate the pharmacological effect of tacrolimus/hydroxypropyl-β-cyclodextrin eye drops developed in Chapter 3 in an endotoxin-induced uveitis (EIU) rat model. In this work, ocular levels of several pro-inflammatory cytokines were studied, leukocytes were quantified in the aqueous humor and histological evaluation was performed in 4 different groups of rats, comparing them with each other. These groups are divided into: healthy rats, EIU untreated rats, EIU rats treated with dexamethasone and EIU rats treated with tacrolimus/hydroxypropyl-β-cyclodextrin eye drops.

The third section of the present thesis focuses on the study of the next therapeutic line for the treatment of uveitis, the use of biologic drugs such as monoclonal antibodies. It is also intended to deepen the knowledge of the intraocular pathway to help the drug reach the site of action. It consists of the last chapter, Chapter 5.

Chapter 5 is based on the PET methodology for the study of the ocular and blood pharmacokinetics following the intravitreal injection of the anti-TNFα antibody adalimumab, as well as to know the distribution of adalimumab in the whole rat body. This follow-up was carried out for 10 days. For that purpose, adalimumab was radiolabeled to the radiotracer Zirconium-89 (^{89}Zr) in order to visualize it via PET.

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INTRODUCTION

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INTRODUCTION

1. UVEITIS

Uveitis is the term used for a very heterogeneous group of diseases that cause ocular inflammation. Because their origin and site of involvement can be so diverse, a proper classification according to different parameters is necessary so that some criteria and terminology can be standardized. There are multiple causes of uveitis including infections, inflammatory diseases, trauma and idiopathic cases. The main symptoms of uveitis comprise: eye pain, redness, photophobia, lacrimation, visual disturbances (mainly blurred vision and blind spots), floating bodies in the field of vision and being the loss of vision the most serious one.

Regarding the classification of uveitis, there are several groupings depending on the type of criterion followed. Among these classification systems are according to anatomy, clinical course, cause of inflammation and histopathology. In this case the anatomical and clinical course classification will be detailed as they are the most useful.

1.1 Anatomic classification

As set forth by the International Uveitis Study Group (IUSG) and confirmed by Standardization of Uveitis Nomenclature (SUN) anatomical classification is based on the place where the inflammation predominates (1,2). It divides uveitis into anterior, intermediate, posterior and panuveitis.

Anterior uveitis are those inflammations that affect the anterior chamber of the eye (iris and ciliary body involvement). Intermediate uveitis is primarily derived from inflammation of the vitreous cavity and *pars plana*. Pars planitis is reserved only if snowballs (vitreous aggregates in the form of snowballs) are found in the vitreous. In the case of posterior uveitis, the involvement is located in the retina and choroid. Finally, the term panuveitis is reserved only when the inflammation does not have a specific location and the anterior chamber, retina and/or choroid are involved.

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Table 1. Uveitis classification according to anatomical location of inflammation (2).

Uveitis type	Initial site of inflammation	Includes
Anterior uveitis	Anterior chamber	Iritis Iridocyclitis Anterior cyclitis
Intermediate uveitis	Vitreous	Pars planitis Posterior cyclitis Hyalitis
Posterior uveitis	Retina and choroid	Choroiditis Chorioretinitis Retinochoroiditis Retinitis Neuroretinitis
Panuveitis	Anterior chamber, vitreous, retina and choroid	-

1.2 Clinical course classification

Due to the inconsistency of the terminology used in the literature, a nomenclature was established to define uveitis according to the clinical course. The onset of the uveitis, the duration of an attack and the course of this disease were agreed upon by the SUN.

Table 2. Uveitis classification according to the clinical course (2).

Category	Descriptor	Specification
Onset	Sudden	-
	Insidious	
Duration	Limited	≤ 3 months duration
	Persistent	> 3 months duration
Course	Acute	Episode characterized by sudden onset and limited duration
	Recurrent	Repeated episodes separated by periods of inactivity without treatment ≥ 3 months in duration
	Chronic	Persistent uveitis with relapse in < 3 months after discontinuing treatment

2. ETIOLOGY AND PATHOGENESIS OF UVEITIS

Inflammation can be defined as a biological reaction of the immune system that can be triggered by any etiological agent (pathogens, damaged cells and toxic compounds), whose main function is to resolve, destroy or wall off the pathogenic organism and repair the damage (3).

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The underlying cause of uveitis is usually idiopathic, meaning it is not related to any specific cause of the inflammation. This cause remains unknown in over 30-60% of patients (4,5), appreciating a higher prevalence in women (6,7). Anterior and intermediate uveitis are more often regarded as idiopathic than posterior uveitis and panuveitis (8).

Within the etiology of uveitis, it can be differentiated into infectious and non-infectious, showing a significant differentiation between developed and developing countries. In general, infectious uveitis is more common in the developing world, accounting for 30-50% of all cases of uveitis. In contrast, only a minority of cases (11-21%) occur in developed countries (9). The opposite is true for non-infectious uveitis, which is generally more common in developed countries than in developing ones (8).

Table 3. Etiological classification of uveitis.

Uveitis origin	*Developing countries*	*Developed countries*
Infectious	○ Toxoplasmosis (10,11) ○ Tuberculosis (12,13) ○ Onchocerciasis (14) ○ Cysticercosis (14) ○ Leprosy (14) ○ Herpetic uveitis (11) ○ Leptospirosis (14) ○ Other parasitic diseases	○ Toxoplasmosis (4,15,16) ○ Herpetic uveitis (15,17) ○ Herpetic retinitis (15,18,19) ○ Tuberculosis (20) ○ Syphilis (20)
Non-infectious	○ HLA-B27 associated anterior uveitis (21) ○ Fuchs uveitis syndrome (22) ○ Sarcoidosis (23) ○ Vogt-Koyanagi-Harada (24) ○ Sympathetic ophthalmia (25) ○ Birdshot chorioretinopathy (26) ○ Multifocal chorioretinitis (27) ○ Behçet disease (28)	

Table 3 shows the most important causes divided by etiology (infectious or non-infectious uveitis) and also according to the origin of the population. Prevalence of infectious uveitis in developing countries of the above etiologies varies according to the geographic region (5). Infectious causes in developed countries include toxoplasmosis (3.8-17.7%), herpetic infection (4.5-18.6%) and herpetic retinitis (0.2-3.8%) with tuberculosis and syphilis being much less frequent with an overall low prevalence (<3%) (5). Regarding non-infectious uveitis, HLA-B27 anterior uveitis has been reported as the most common type in the developed countries, prevalence of Fuchs uveitis syndrome ranges between 1.8% and 22.7% in the developed world while in the developing world only 0–5.6% (5). The

remaining non-infectious causes are highly dependent on the studied geographical area without a well-defined epidemiological profile.

When an obvious infection is not recognized, uveitis is classified as non-infectious. This type of disease is thought to be autoimmune or immune-mediated in origin. The pathogenetic mechanism of autoimmune uveitis is still poorly defined, given its remarkable heterogeneity and the many discrepancies between experimental and human uveitis. Nevertheless, the autoimmune uveitis onset is thought to be related to an aberrant T cell-mediated immune response, triggered by inflammation and directed against retinal or cross-reactive antigens (29) as shown in Figure 1.

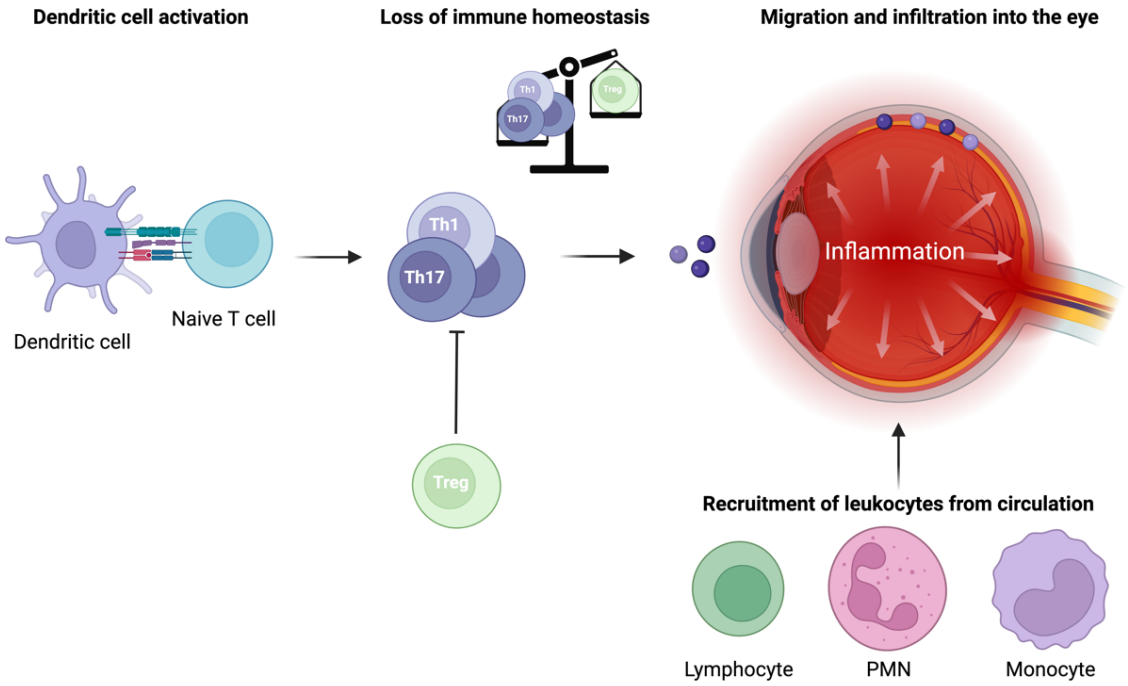


Figure 1. Uveitis mechanism of action. Created with BioRender.com.

The difference between autoimmune and immune-mediated is very slight, the former is related to an autoimmune reaction to self-proteins (autoantigens), while the latter is primarily linked to an aberrant activation of the innate inflammatory response to environmental triggers (microbial) or to autologous immune changes (tissue damage) (30).

These autoimmune inflammations may occur without any other autoimmune manifestations (idiopathic autoimmune uveitis) or may be derived from another autoimmune disease (autoimmune uveitis associated with systemic disease) such as autoimmune thyroiditis, inflammatory bowel disease, ankylosing spondyloarthritis or Behçet's disease, among others (29).

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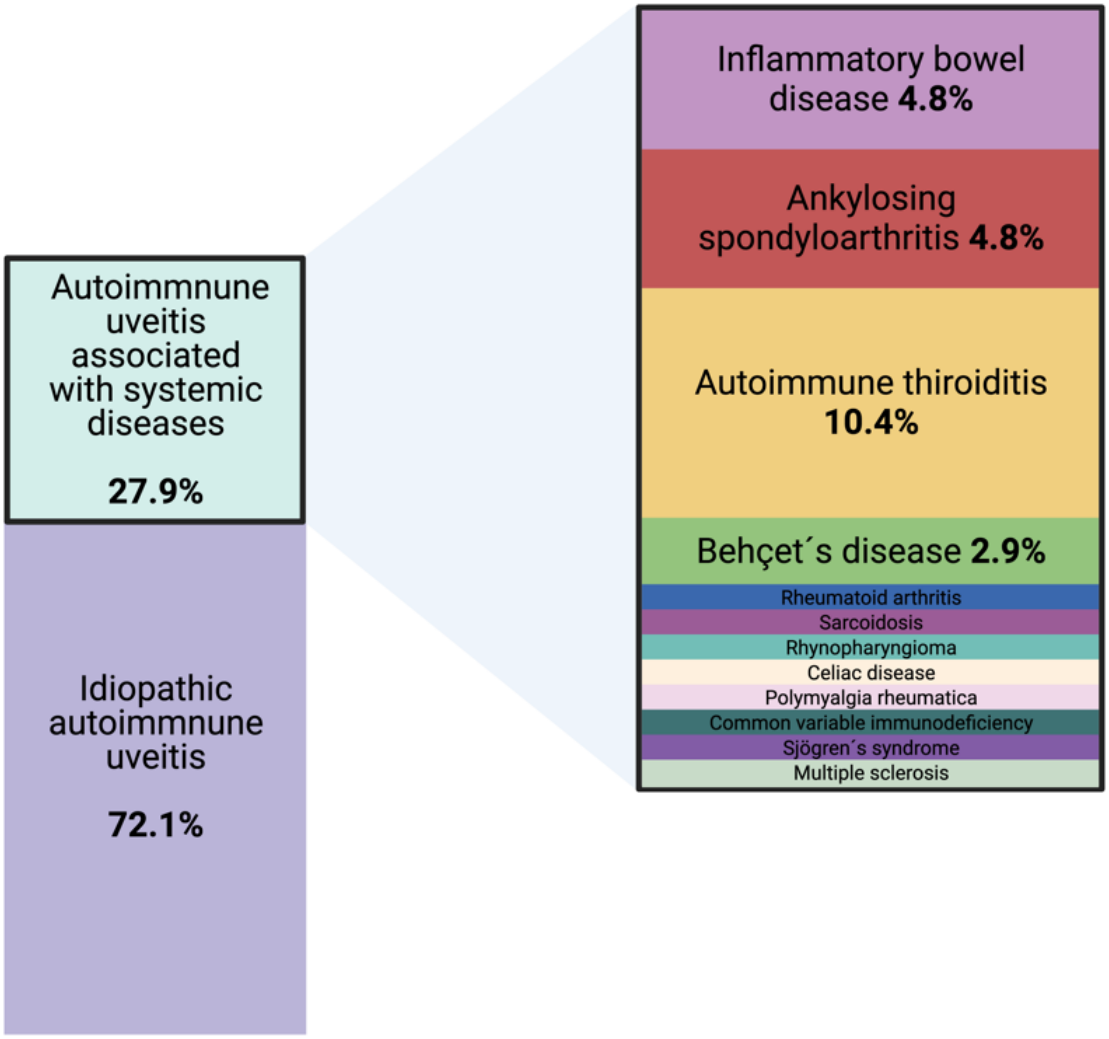


Figure 2. Schematic representation of the distribution of idiopathic autoimmune uveitis and systemic disease-associated autoimmune uveitis. Created with BioRender.com.

3. EPIDEMIOLOGY OF UVEITIS

Epidemiological studies should not only focus on their incidence and prevalence, but also on their etiology and pathogenesis, as well as on the identification of possible environmental and genetic factors that could influence their appearance and development.

Most studies dealing with the general epidemiology of uveitis have been conducted in developed countries (9). The incidence rate is a measure of the frequency of new cases of disease during a span of time and the prevalence of a disease is the total number of disease cases in a population at any point during a given period. The estimated range of incidence in developed countries was between 17 and 52 cases per 100,000 inhabitants per year and also a prevalence of 38-714 cases per 100,000 inhabitants (5,31). This pathology is even more

prevalent in developing countries where up to 25% of cases result in blindness (32,33).

The frequency of uveitis varies greatly depending on the eye anatomical site of inflammation, geographic location and the etiology. This is due to the diverse group of disorders characterized and connected by the shared presence of ocular inflammation in uveitis. Regarding epidemiology according to anatomical classification the most common form of uveitis is the anterior uveitis followed by posterior uveitis and panuveitis, while intermediate uveitis is known as the least frequent form of uveitis about 15% of all uveitis).

Anterior uveitis is about 50-90% of all cases in Western countries. In contrast, the prevalence is lower in Asian countries, this may be due to the genetic difference between the two regions, the HLA-B27 gene associated with different uveitis is much more prevalent in the Western population than in Asia (34). Intermediate uveitis is only about 15% of all uveitis and most cases are idiopathic (60-100%) (35,36).

Posterior uveitis is between 15% to 30% of the uveitis cases, the second most common form in most of studies, with the majority being of idiopathic etiology and followed by toxoplasmic retinochoroiditis (particularly high prevalence in South America) (37). Panuveitis was relatively more common in South America, Africa and Asia compared with Western populations (34).

4. RISKS FACTORS FOR UVEITIS

Although the etiology of uveitis is not fully elucidated, multiple genetic and environmental factors have been associated with the development of the disease. While the etiology of infectious uveitis is well-defined and clear, the pathogenesis of most non-infectious uveitis is not. It is believed that there are multifactorial causes among the environment, microorganisms and the patient's underlying genetics that play a joint role in increasing the susceptibility of the disease (38).

4.1 Genetic risks factors

The human leucocyte antigen (HLA) is a functional gene unit whose main function is the recognition of self and foreign antigens. A pathological HLA association means that there is a statistically significant increase in the frequency of that haplotype in patients with that disease compared to its frequency in the healthy population. Strong associations of HLA with different forms of uveitis have been identified since the early 1970s (39,40). The main associations between HLA and inflammatory eye diseases are shown in Table 4 below. However, only

a small part of the associations known so far are shown, there are many more related to uveitis and other systemic diseases or in different populations.

Table 4. Human leukocyte antigen (HLA) associations with uveitis (41).

Disease/syndrome	*HLA*	*Uveitis manifestations*
Acute anterior uveitis	B27	Anterior, unilateral, sudden onset
Behçet´s disease	B51, B52	Anterior, acute, posterior acute, or chronic
Birdshot chorioretinopathy	A29	Chronic bilateral posterior uveitis
Idiopathic intermediate uveitis/pars planitis	DR15	Chronic/recurrent, bilateral intermediate uveitis
Juvenile rheumatoid (or chronic) arthritis	DPB1*0202	Chronic bilateral anterior uveitis
Tubulointerstitial nephritis and uveitis	DRB1*0102, DQA*01	Chronic/recurrent bilateral anterior uveitis or sudden onset
Vogt-Koyanagi-Harada syndrome	DR1, DR4 (DR1*0405 in Asians)	Bilateral panuveitis of acute onset followed by chronic anterior uveitis

4.2 Environmental risks factors

Several environmental factors have been related to the development of uveitis. Age is one of the most influential factors, with the majority of cases diagnosed being young adults (29). Some studies have demonstrated that air pollution exposure increases the risk of uveitis (42). It is worth mentioning the relationship between smoking and autoimmune uveitis, smoking can increase the release of TNFα and other interleukins enhancing the function of inflammatory cells contributing the development of uveitis (43).

On the other hand, diet could be an explanation of the different risk of developing the disease depending on geographical area, food antigens mimicking retina-specific autoantigens may participate in the occurrence of uveitis. Furthermore, the gut microbiome changes the homeostasis of intestinal immune and activates T-cell specific for retina antigen in the gut to act on the course of uveitis (44). Regular physical activity helps to reduce the level of reactive oxygen species which are induced by pro-inflammatory cytokines to damage the uvea ameliorating the uveitis symptoms (43,45). Sex, race, socio-economic factors, occupation also play a decisive role in the etiopathogenesis of uveitis (46).

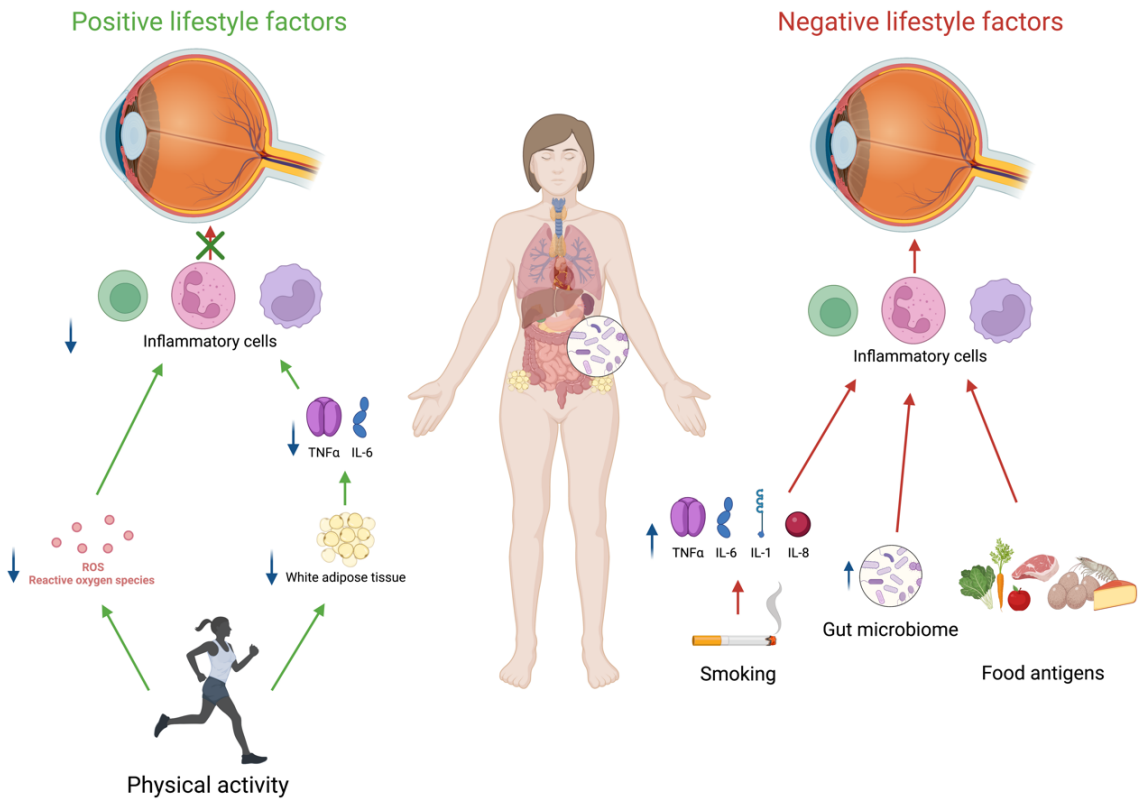


Figure 3. Role of lifestyle factors in autoimmune uveitis.

5. TREATMENTS

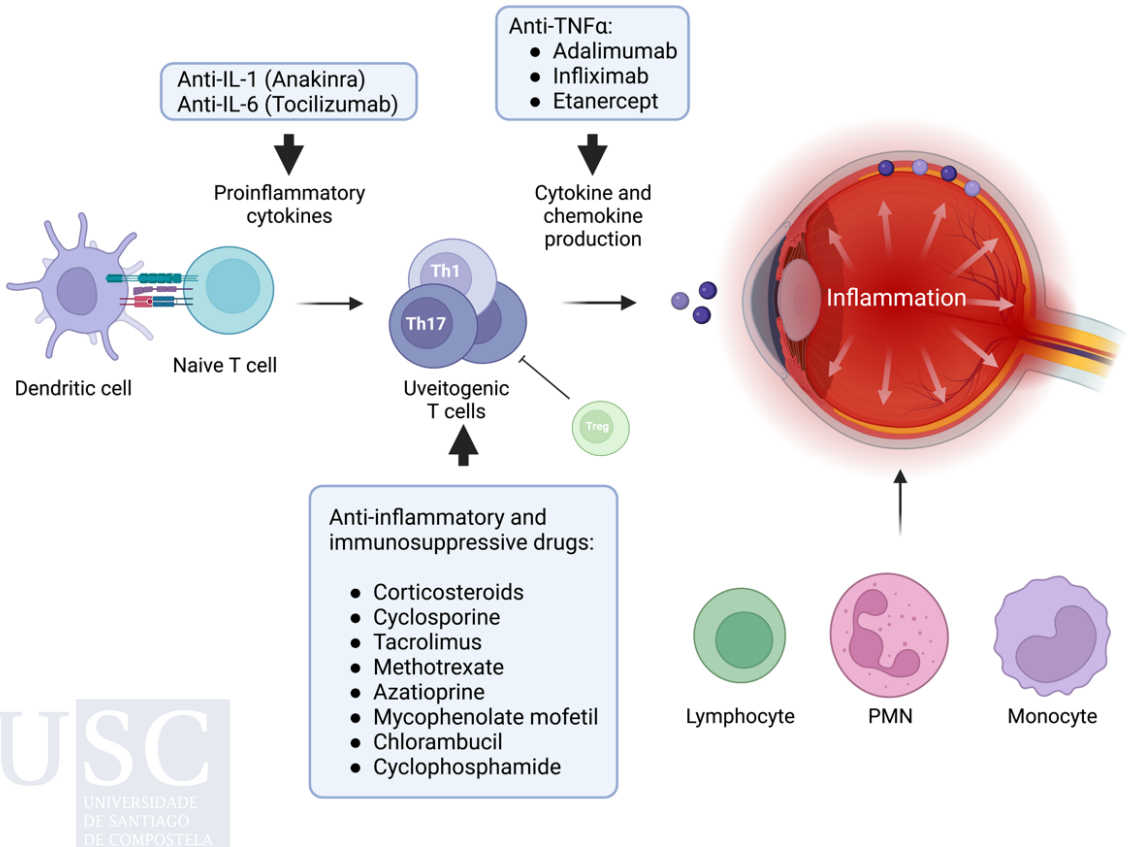
Uveitis is a disease with various phenotypes and visual prognoses, so treatment is based on the inflammation degree in the eye, the presence of risk factors and complications. Once the diagnosis has been made, treatment should be started as soon as possible following a stepwise approach, starting with the least aggressive to the most aggressive treatment until the inflammation has subsided (47). The aim of uveitis treatment is to completely eliminate the inflammation, as chronic uveitis can cause very serious repercussions. Therefore, as many drugs as necessary must be used to control the inflammation.

In the 1950s, a new era of anti-inflammatory treatment for ocular inflammatory diseases began with corticosteroids (48,49). Very favorable results were rapidly obtained with remission of the disease, however, unintended adverse effects on some ocular structures and systemic toxicity were also observed when prolonged treatments were needed (50). For this reason, alternatives with other types of drugs were sought. The emergence of immunosuppressive agents for the treatment of eye diseases in the late 1960s widened the range of therapeutic possibilities. In the beginning, the use of immunosuppressive agents was limited to cases of corticosteroid-resistant or intolerant treatment, but over time and with more clinical experience they gained recognition (51). More recently, biological agents such as anti-Tumor Necrosis Factor α (anti-TNFα) have been added to the

therapeutic spectrum. Some studies show their efficacy and usefulness in patients refractory to conventional therapy. These biological agents selectively suppress immune response by targeting specific molecules in effector mechanisms of autoimmunity and inflammation (50).

Currently, a large therapeutic arsenal is available to combat uveitis. The use of different drug families with therapeutic action has been studied, as well as the optimization and improvement of the administration routes in order to reduce the possible systemic adverse effects caused by the drugs used. Moreover, within the different delivery routes to the eye, a great deal of effort has also been devoted by pharmaceutical researchers to developing new systems and vehicles to improve bioavailability and ocular toxicity. One of the major concerns regarding the treatment of uveitis is systemic side effects, which is precisely why local administration of corticosteroids, immunosuppressive agents and biological agents represent a broad area of work to be developed (52). With this in mind, ocular administration routes of these drugs include topical application, subconjunctival and sub-tenon injections or intravitreal route (injection or implant) (53–55).

A summary of uveitis treatments and their site of action within the inflammatory process can be seen in Figure 4.



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Figure 4. Diagram of the drugs included in the therapeutic arsenal for uveitis and their site of action within the inflammatory process. Created with BioRender.com.

5.1 Corticosteroids

Corticosteroid treatment is the gold standard in clinical practice because of its rapid pharmacological effect, low cost and lack of major complications (56–58). The fact that they are very potent drugs can also cause quite a few side effects, so they should be used until the benefits outweigh the risks. Depending on the degree and site of inflammation, topical corticosteroid treatment is indicated for anterior uveitis and systemic corticosteroids for posterior uveitis or panuveitis (48).

An initial high dose of oral corticosteroids should not be prolonged for more than 1 month, at which time the dose should be gradually reduced. The major problem with these drugs is the ocular and systemic effects of long-term use. Many uveitis specialists have raised concerns about the safety of corticosteroids, and indeed "corticosteroid-free" has been one of the main goals for future developments (57). Despite this, the use of corticosteroids remains irreplaceable to this day, which is why the development of new corticosteroids that reduce adverse effects and maintain local anti-inflammatory activity remains a challenge (49,59).

5.2 Mydriatics and cycloplegics

These drugs are administered topically and are used in the treatment of acute inflammation of anterior uveitis, as they relieve pain secondary to ciliary muscle spasm and help prevent synechiae. The most commonly used drugs are: cyclopentolate, tropicamide, phenylephrine, homatropine or atropine.

5.3 Immunosuppressive agents

The therapeutic second line for uveitis is immunosuppressants. The indication for these drugs is focused on chronic uveitis that compromises or could compromise the patient's vision. The use of immunosuppressants in uveitis is indicated in corticosteroid-refractory eye disease or after systemic side effects' appearance.

These drugs are sometimes given in combination with oral corticosteroids in the acute phase of severe ocular inflammation. In this way, a larger number of patients can be controlled with low doses of corticosteroids and minimal side effects (60). In patients who are refractory to corticosteroid therapy, immunosuppressive drugs can play a greater role in controlling inflammation and can sometimes even serve as rescue therapy (61,62).

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Due to the success of immunosuppressive agents in the control of other autoimmune inflammatory disorders or in transplant rejection, they have been

introduced into clinical practice to treat uveitis (63). A large body of evidence demonstrating the advantages of immunosuppressive drugs in the treatment of uveitis has been collected, although the indications for choosing one immunosuppressant over another are still uncertain (64,65). Most of the evidence are observational case series and clinical trials comparing different drugs and different types of uveitis are needed (65). The choice of immunosuppressive agents made by uveitis specialists depends on complex factors such as individual familiarity with the drug, safety/tolerability, efficacy, patient compliance and cost.

This broad pharmacological group of immunosuppressive or immunomodulatory therapies can be divided into T-lymphocyte inhibitors, antimetabolites and alkylating agents.

5.3.1 T-lymphocyte inhibitors

Cyclosporin A was the first T-cell inhibitor used for the systemic treatment of uveitis. Multiple studies showed the satisfactory therapeutic efficacy of this drug, making it the only immunosuppressant for this disease at that time (66). Thus, it was found to be similar to oral corticosteroids in terms of efficacy (67).

However, some adverse reactions, such as renal toxicity and hypertension, required its dose reduction or discontinuation in a large proportion of patients. These results suggest that cyclosporin A is not suitable for long-term control of inflammation at high doses as a single agent (66). Close monitoring of patients on treatment with this drug is required to avoid irreversible damage (60).

Tacrolimus is a macrolide with a great immunosuppressive activity (10-100 times more potent than cyclosporine A) (68) having demonstrated in clinical studies more effectiveness at lower concentrations and a better safety profile than cyclosporine A (60,69,70).

Tacrolimus acts by inhibiting T-cell activation by blocking calcineurin, calcineurin inhibition suppresses dephosphorylation of the nuclear factor of activated T cells and its transfer into the nucleus, which results in the suppressed formation of T-cell lymphokine (IL-2) production. This inhibition is going to lead in the release inhibition of inflammatory cytokines and decreased stimulation of other inflammatory cells (71). The tacrolimus mechanism of action is shared with cyclosporine A (72).

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Systemic treatment with tacrolimus showed high efficacy in patients with refractory uveitis with excellent tolerability. Adverse effects are similar to, but always less than, those of cyclosporin A. Given their good results, attempts were

made to adapt the systemic formulations to the topical ocular route, with good results in terms of treatment efficacy. However, due to its poor water solubility and relatively high molecular weight, it has great difficulty penetrating the corneal epithelium and accumulates in the corneal stroma (73). In addition, as it is not designed as an ophthalmic drug, patients suffer discomfort because of its high osmolality, decreasing their success rate by discontinuing treatment.

The potential role of topical tacrolimus in treating patients with uveitis it should be determined yet. Further studies are needed to detect the optimum concentration and formula of topical tacrolimus (74).

5.3.2 Antimetabolites

Within the antimetabolites therapy, there are three drugs with therapeutic indications in uveitis: methotrexate, azathioprine and mycophenolate mofetil. Each of them has a different application depending on which part of the eye is affected.

Methotrexate was the first antimetabolite to be added to the therapeutic arsenal against uveitis. The effectiveness of this drug is widely reported in the literature (75), including specific uveitis such as Behçet's disease (76), sarcoidosis-related panuveitis (77) or juvenile idiopathic arthritis-associated uveitis (51). Several studies indicate that their effectiveness is moderate, but they are well tolerated by patients and rarely present adverse effects. Because of this safety profile, it is one of the drugs of choice for uveitis specialists. However, it is worth noting that most patients require 6 months to have a full effect. Remarkably, methotrexate has a higher remission success in patients with anterior uveitis and scleritis (65,75).

In the case of azathioprine, it has more recently been used for the treatment of uveitis. Its effectiveness has been demonstrated in corticosteroid-resistant uveitis, juvenile idiopathic arthritis associated iridocyclitis and intermediate uveitis but not in Behçet's disease (78,79). Although the overall efficacy of azathioprine is very close to that of methotrexate, its toxicity is a major limitation even though it may be reversible, as is the limitation due to interaction with other treatments (80).

Mycophenolate mofetil is considered a relatively new immunosuppressant and therefore experience in clinical practice is more limited. However, its acceptance has been growing due to the publications generated (64,81), showing effectiveness in the different areas of ocular inflammation. Some studies show that remission of inflammation is achieved faster with mycophenolate mofetil than with methotrexate and azathioprine (82).

5.3.3 Alkylating agents

This pharmacological group has a high percentage of disease remission after treatment. Therefore, it can be said that alkylating agents are highly effective against ocular inflammation (65,78,83). Although the success of chlorambucil (84–86) and cyclophosphamide (87,88) treatment in inflammatory eye pathology is a fact, numerous side effects are associated with the use of these agents. The risk is higher with these drugs than with alternative agents, and therefore close monitoring is very necessary in these cases (89).

With the high risk of side effects and the emergence of new immunosuppressive drugs, alkylating agents have become the last therapeutic option. Their use is only reserved in severe or recalcitrant cases of ocular inflammation (89).

5.4 Biological agents

Biological therapy has recently become increasingly important due to the discovery of new pathogenic mechanisms related to the role of pro-inflammatory cytokines and other factors causing ocular inflammation (90). TNFα is a cytokine that takes on great importance in regulating the functions of cells involved in the inflammatory process (91). TNFα, which is secreted by monocytes and macrophages, lymphocytes and mast cells, appears to play a key role in ocular inflammatory diseases (90).

Infliximab was the first anti-TNFα to be used to treat uveitis, showing good results in short-term disease remission, the effect is temporary and has a high risk of treatment failure. This is due to the development of self-antibodies against the murine component of the molecule (92,93), causing a major limitation.

Adalimumab is a humanized anti-TNFα monoclonal antibody used in the treatment of autoimmune diseases, directly blocking TNFα. Because of this, it was thought that it could also be helpful in the uveitis treatment. Adalimumab therapy efficacy has been widely demonstrated in the scientific literature.

Compared to infliximab, adalimumab has shown better long-term efficacy and more adequate safety/toxicity profiles, this may be due to the fact that in this case there is no murine component in the molecule. Some side effects can occur with anti-TNFα, limiting their use for severe ocular inflammation and/or uveitis refractory to one or more immunosuppressants. In terms of side effects, both antibodies behave similarly, the only difference between infliximab and adalimumab is the local reactions after subcutaneous injection of adalimumab. For this reason, improvement in the routes of administration of this anti-TNFα antibody to achieve a localized effect is necessary. In the case of etanercept, there

are no conclusive data clearly showing its efficacy in the treatment of inflammatory eye diseases (94).

Another biological therapy is interferons (IFNs), these molecules are low molecular weight glycoproteins that possess antiviral, antiproliferative, pro- or anti-apoptotic and immunomodulatory properties (95). IFNα is a natural cytokine that is secreted in response to viral infections, having an immunomodulatory effect and is thought to be a key mediator of "systemic" autoimmune diseases (96).

Other biological agents act at other levels of the inflammatory response, overproduction of pro-inflammatory cytokines such as IL-1 and IL-6 are involved in the immune response. Two biologic drugs act on this mechanism by counteracting this effect, anakinra as anti-IL-1 (97) and tocilizumab for IL-6 (98), both molecules have been used in the treatment of uveitis.

6. IN VIVO ANIMAL MODELS

During the last years, a large number of animal models have been developed to provide a valuable insight into the different pathways involved in uveitis pathogenesis. In each animal model the part of the eye undergoing inflammation is different, thus simulating different types of human uveitis as well. The cause of the inflammation can be caused by various retinal antigens, but other non-retinal and non-ocular antigens, such as bacterial endotoxin and cytokines, can also be used. Heterogeneity in the inflammatory response in these animal models is very pronounced, as even animals of the same species have variable responses to the same antigen. For this reason, the combination of uveitis animal models is very common. Table 5 shows some of the most representative models for the study of uveitis.

Table 5. Relationship between different uveitis animal models, the antigen/endotoxin with which it is induced and the type of uveitis it generates.

Uveitis model	Antigen/Endotoxin	Types of uveitis
EAU	S-antigen IRBP Rhodopsin Recoverin Phosducin	Posterior uveitis
EMIU	Melanin protein	Anterior uveitis Posterior uveitis Diffuse uveitis
EAE/AU	Myelin binding protein	Anterior uveitis
EIU	*E.coli* lipopolysaccharide endotoxin (LPS)	Anterior uveitis Diffuse uveitis

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

6.1 Experimental Autoimmune Uveitis model (EAU)

The first developed experimental model of uveitis was Experimental Autoimmune Uveitis (EAU), this disease model is a human endogenous uveitis that can be induced in susceptible animals by immunization with retinal antigens. This disease induction resembles human uveitis in the sense that both are mediated by T-cells (Th1) targeting the neural retina and adjacent tissues (99). The EAU clinical and pathological characteristics depend on the species and the antigen used for induction, but T-cell mediated inflammatory response is always present, mainly CD4+ cells.

EAU has been induced in a variety of species including rats, mice, rabbits and monkeys (100). No animal model in itself represents the complex spectrum of human uveitis. Although, there is no single animal model that covers the entire field of human uveitis, several models are often used to approach it. Among the above models, the rat model has the advantage of being very well characterized immunologically and immuno-genetically (101).

Within the diversity of EAU models, the most common is the Classical EAU in which the mouse is immunized by the subcutaneous injection of bovine S-antigen with the Complete Freund's adjuvant (CFA) as vehicle (102). On this basis, over the years, variations have been included in the model that have enriched the broad pathogenicity of the disease. Other antigens used to generate this inflammation are IRBP (Interphotoreceptor retinoid-binding protein) (103), rhodopsin (104), recoverin (105) and phosducin (99).

6.2 Experimental Melanin Protein-Induced Uveitis model (EMIU)

In addition to the use of retinal proteins to induce uveitis in animals, the use of non-retinal proteins such as melanin or tyrosinase-related proteins is also worth mentioning. Depending on which antigen is used for induction, uveitis of different duration and form will be produced. In the 1990s the induced EMIU model was developed by Broekhuyse et al. with non-soluble melanin proteins extracted from pigmented bovine eyes (106). Following subcutaneous injection of melanin protein in combination with Hunter's adjuvant in rats, after 10-14 days, ocular inflammation is evident in the iris, ciliary body and choroid.

Fundamentally, this model can be suited to the study of anterior uveitis (iridocyclitis), where melanin is found, as well as its spontaneous recurrence. This relapsing characteristic of the model makes it useful for the evaluation of therapies initiated during established disease and aimed at preventing recurrence.

6.3 Experimental Autoimmune Encephalomyelitis-Associated Anterior Uveitis model (EAE/AU)

In this animal model, the protein used to generate the immunization of rodents is myelin basic protein, following subcutaneous injection with CFA (107,108). This induction usually causes dilation of the iris vessels, anterior uveitis for 30 days, and is therefore ideal for the study of anterior uveitis and the inflammatory relapses associated with this disease.

6.4 Endotoxin Induced Uveitis model (EIU)

The most widely used model of autoinflammatory uveitis driven largely by innate immune mechanisms is endotoxin-induced uveitis (EIU), it was also developed in rats, and involves the systemic injection of *Escherichia coli* lipopolysaccharide endotoxin (LPS) to induce an acute anterior uveitis (30,109,110). This model is generally considered to be an inflammation of the anterior uvea, with some changes in the posterior segment (vitreous and retina), producing a severe immune response in animals. Thus, stimulation of the production of inflammatory cytokines such as tumor necrosis factor-α (TNFα) and other cytokines and inflammatory mediators are triggered.

7. IN VIVO IMAGING IN PRECLINICAL RESEARCH

Modern imaging techniques provide anatomical, functional and molecular information about what is happening in tissues through the physical phenomena that penetrate living tissue, such as magnetic resonance imaging (MRI), computed tomography (CT), single-photon emission computed tomography (SPECT) and positron emission tomography (PET), among others. In recent years, an adaptation of these imaging techniques has been carried out to be used in small animal models, by increasing the spatial resolution and the sensitivity of the scanners (111). These models are mainly rodents (mice and rats) that are too small to be managed accurately. Thus, to overcome these limitations, dedicated and miniaturized versions of clinical scanners are currently available for small animal imaging in preclinical research (112).

In a very few years, dedicated preclinical imaging scanners have reached enormous translationality by achieving high resolution and extreme sensitivity. These techniques are currently considered as an important tool in the field of preclinical and clinical research to accelerate the drug development process (113). The most promising tool of this technology focuses on non-invasive visualization of anatomical structures and physiological activity in both animal model (preclinical research) and patients (clinical practice) using exactly the same technology, which greatly maximizes its translationality. (111). It features

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

advantages over conventional study methods because it is possible to take measurements from the subject at different times without causing harm and in real-time (114). Furthermore, the reduction of the number of animals used for each study in accordance with the 3Rs principle (replacement, reduction and refinement) is an important advantage that also helps to reduce costs with the power of longitudinal studies (115,116). The combination of preclinical imaging and classical practices can achieve a considerable reduction in the expense and time spent on the development of new molecules and biologics.

These molecular imaging techniques can be differentiated into structural and functional, in the former case an animal anatomical image is obtained, while in the latter case a physiological assessment at the molecular and cellular level can be carried out.

7.1 Structural imaging techniques

7.1.1 Micro-Computed Tomography (μCT)

Computed tomography is an application of X-ray imaging that provides detailed images of organs, blood vessels, bones and soft tissue. μCT consists of a source of X-rays and a detector placed opposite each other that rotates around the subject in the center of the CT scanner. The X-rays will pass through the object to a greater or lesser extent depending on the density of the tissues, thus allowing its differentiation. The CT scan generate three-dimensional anatomical image, from which sectional or spatial images can be reconstructed (111). The use of some contrast agent is very common, as it allows the observation of soft tissue that would otherwise be difficult to differentiate.

The main application of μCT in eye-related studies is to provide an anatomical framework with high image quality for combination with functional studies, particularly in PET and SPECT imaging, as was done in several works (114,117).

7.1.2 Micro-Magnetic Resonance Imaging (μMRI)

Magnetic resonance imaging (MRI) is based on the interaction of nuclear spin with an external magnetic field. According to the properties of hydrogen nuclei (protons) contained in water or magnetically nuclei (Gadolinium, Gd), spatial maps are produced. This technique is especially useful for imaging soft tissues depicting anatomic details with high resolution in three dimensions (3D) (111).

USC UNIVERSIDADE DE SANTIAGO

This imaging technique has been used to study the structure of the eye (118,119), in studies of ophthalmic drug delivery (120,121) and some pathologies (122,123), as it allows real-time determination of the drug distribution in the eye without

damaging the tissue. Some preclinical studies have been developed to determine permanence on the ocular surface with μMRI (124).

7.2 Molecular imaging techniques

Molecular imaging technology is the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems (125,126). Molecular imaging technology can provide valuable information on the *in vivo* pharmacokinetics and distribution of drug biomarkers, to understand the permanence of a drug at its site of action, to confirm its biological activity, to show the mechanisms of a disease, or to validate the efficacy of drug treatment. Maximizing the collection of *in vivo* biological information during the preclinical phases of drug development can be valuable during the selection/optimization of promising drug candidates (111).

In vivo imaging techniques such as PET and SPECT also allow pharmacokinetic and pharmacodynamic studies in animal models without the need to sacrifice animals at every study time. "Motion frozen" PET and SPECT imaging in pharmacokinetic and pharmacodynamic studies is feasible because of the option of dynamic and static imaging. As PET and SPECT provide quantitative information, it is possible to quantify the amount of drug in the organs of interest and to monitor it over time (111).

7.2.1. Micro-Single Photon Emission Computed Tomography imaging (μSPECT)

Single-photon emission computed tomography is a cross-sectional nuclear imaging technique that uses gamma rays resulting from radionuclide decay, providing a three-dimensional spatial distribution. Some common radionuclides used in clinical practice are ^{99m}Tc, ^{67}Ga, ^{111}In or ^{123},^{125},^{131}I (127), which require to be injected into the subject. The single-photon emissions are ejected from the source multi-directionally with equal probability. Because this occurs, an aperture composed of highly attenuating material is required to identify the source path of each emission before constructing an image with a gamma camera (111).

The extended half-life of these isotopes translates to longer scan times and/or scanning periods without the need for secondary isotope administration. This means that smaller quantities of isotope are needed. These radioisotopes can also be bound to ligands to be able to follow-up the ligand according to its specific affinity for different organs and tissues. μSPECT alone is limited by low spatial resolution and it is not able to provide anatomical details, therefore this technique is usually combined with μCT scan for an adequate localization, avoiding erroneous interpretations (128). μSPECT/CT studies related to ocular distribution

(129,130) or ocular surface permanence studies (131,132) have already been carried out.

7.2.2. Micro-Positron Emission Tomography imaging (μPET)

Positron emission tomography is based on the detection of γ-photons emitted from a radiolabeled biological molecule or drug with a positron emission radioisotope, mainly isotopes with short half-lives, such as ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{68}Ga, ^{82}Rb, or with longer half-lives, such as ^{124}I or ^{89}Zr. These radiotracers are captured by tissues and emits positrons that react with electrons in the body. Because of this, photons are produced and then detected by the PET scanner, creating 3D images showing the distribution of the radiotracer in the living subject (133). Areas with higher radiotracer uptake appear more intense indicating the accumulation of the tracer in a particular organ or region of the body. As with μSPECT, μPET is usually combined with μCT or μMRI, offering an anatomical reference to the physiological data for a better identification of functional change in deep tissue against anatomical structure in a living subject.

Although the use of this modality in the ophthalmology field has a promising future (diagnosis of ocular tumors or neurophysiological studies) (134,135), it has not yet been widely used (136). However, there is a growing trend in preclinical research where PET/CT imaging is being used to study the ocular surface permanence of different formulations (117,137–140) as well as the drugs pharmacokinetics and distribution in the eye (141).

7.3 Radiolabeling

In PET and SPECT, the use of radiotracers is necessary to visualize the biological processes occurring in the living organism. The concept of radiotracer can be defined as a specific radiolabeled molecule (small molecules or biological macromolecules) that can be considered as surrogate of the *in vivo* behavior of a functional molecule (111).

Depending on the type of radiotracer, a radioisotope with a half-life compatible with the required monitoring time will have to be used. For example, one of the most commonly used radiotracers in PET imaging is ^{18}F-fluorodeoxyglucose (^{18}F-FDG). The ^{18}F half-life is 110 minutes, enough time for the fluorodeoxyglucose to be completely distributed throughout the living system. In contrast, macromolecules such as monoclonal antibodies (mAb) are much slower to distribute in the body due to the fact that their size does not facilitate the passage of physiological barriers. For these antibodies, the ^{18}F half-life is not sufficient, so radioisotopes with longer half-lives, such as ^{89}Zr (half-life of 3.3 days), are needed. For the binding of the radioisotope (^{89}Zr) and the tracer (mAb) to occur,

it is necessary to incorporate a bifunctional chelating agent that can bind to both elements, such as deferoxamine (142). In this way, the radiotracer would already be formed.

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CHAPTER 1

DRUG DELIVERY TO THE POSTERIOR SEGMENT OF THE EYE: BIOPHARMACEUTIC AND PHARMACOKINETIC CONSIDERATIONS

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CHAPTER 1

DRUG DELIVERY TO THE POSTERIOR SEGMENT OF THE EYE: BIOPHARMACEUTIC AND PHARMACOKINETIC CONSIDERATIONS

ABSTRACT

The treatment of the posterior-segment ocular diseases, such as age-related eye diseases (AMD) or diabetic retinopathy (DR), present a challenge for ophthalmologists due to the complex anatomy and physiology of the eye. This specialized organ is composed of various static and dynamic barriers that restrict drug delivery into the target site of action. Despite numerous efforts, effective intraocular drug delivery remains unresolved and, therefore, it is highly desirable to improve the current treatments of diseases affecting the posterior cavity. This review article gives an overview of pharmacokinetic and biopharmaceutics aspects for the most commonly used ocular administration routes (intravitreal, topical, systemic, and periocular), including information of the absorption, distribution, and elimination, as well as the benefits and limitations of each one. This article also encompasses different conventional and novel drug delivery systems designed and developed to improve drug pharmacokinetics intended for the posterior ocular segment treatment.

Keywords: ocular pharmacokinetics; ocular drug delivery systems; ocular routes of drug administration; intravitreal administration; topical administration

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1. INTRODUCTION

The posterior segment of the eye comprises the back two-thirds of the eye, including the vitreous humor, the retina, the choroid and the optic nerve. Posterior Segment Eye Diseases (PSEDs) are then defined as the disorders that affect these tissues with the common main outcome of varying degrees of visual impartment and blindness. The most prevalent diseases are glaucoma, age-related macular degeneration (AMD) and diabetic retinopathy (DR) (see Figure 1). Nowadays, millions of people are suffering from retinal and choroid diseases and the number is increasing every year, as the incidence significantly increases with age. Both disorders are characterized by their severity and difficulty of treating. Despite numerous efforts, effective intraocular drug delivery remains unresolved and therefore, it is highly desirable to improve the current treatments of diseases affecting the vitreous cavity.

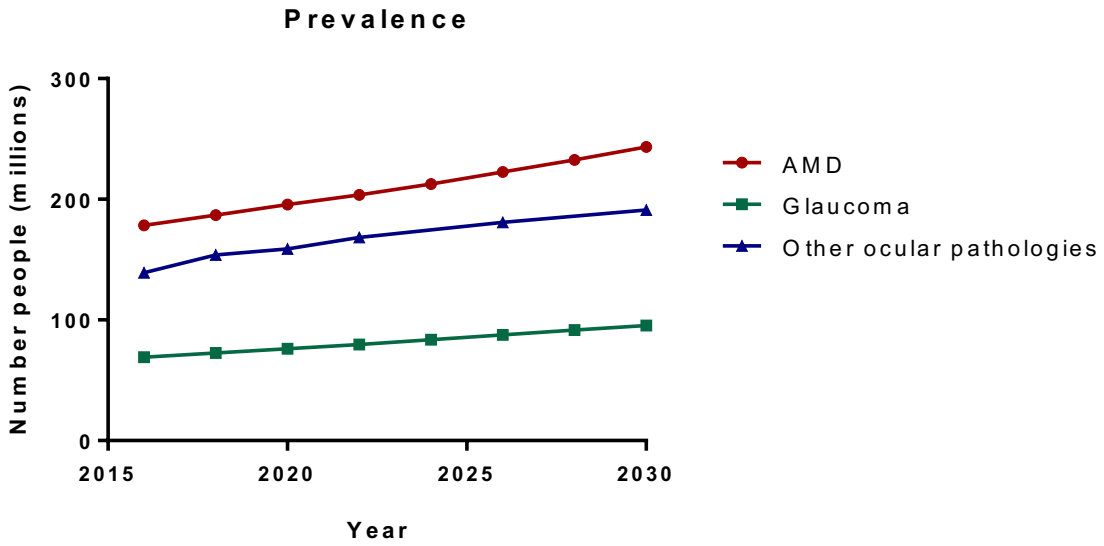


Figure 1. Prevalence of the main ocular pathologies. Data from the world report on vision published by the World Health Organization (WHO), 2019 [1].

In clinical practice, the standard procedure in treating these disorders is the intravitreal administration of injected drugs, although topical and systemic administration have also been addressed with limited results. Thus, other approaches have been developed for the treatment of posterior segment diseases such as periocular, suprachoroidal, and subretinal administration (Figure 2). All these routes of drug administration consist of the drug/system injection in the surroundings of the target site. Periocular administration includes subconjunctival, sub-Tenon's, peribulbar, retro bulbar, and posterior juxtascleral injection. However, these injections might not result in therapeutic drug levels in the target site due to the necessity of crossing several barriers to reach the

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intended site of action. This limitation could be overcome by using more effective drug delivery systems, where improved therapies may also be achieved.

One of the main drawbacks of drug delivery to the back of the eye is the narrow interval dosing because of the low effectiveness and bioavailability of the administered drug. Also, it should be taken into consideration that intravitreal and periocular routes are highly invasive, being associated to discomfort and patient compliance. Intraocularly, a prolonged drug release not only entails the patient acceptability by increasing their quality of life, but also, a notable reduction in the economic costs associated to the hospital stays due to the frequently repeated injections that are necessary to complete the treatment.

Drug delivery to the posterior segment of the eye still remains a great challenge in the pharmaceutical industry due to the complexity and particularity of the anatomy and physiology of the eye. Some advances have been made with the purpose of maintaining constant drug levels in the site of action. The anatomical ocular barriers have a great impact on drug pharmacokinetics and, subsequently, on the pharmacological effect. For this reason, understanding pharmacokinetics plays a critical role in the design of effective pharmaceutical forms.

The aim of this review is to show an overview of the main aspects involved in ocular drug pharmacokinetics intended to treat PSEDs. A discussion of the different factors that are involved in the ocular drug delivery is first made, encompassing the different routes towards the posterior segment of the eye. Physiological barriers and drug transport pathways are described and the advantages and drawbacks of different administration routes to the eye are also discussed.

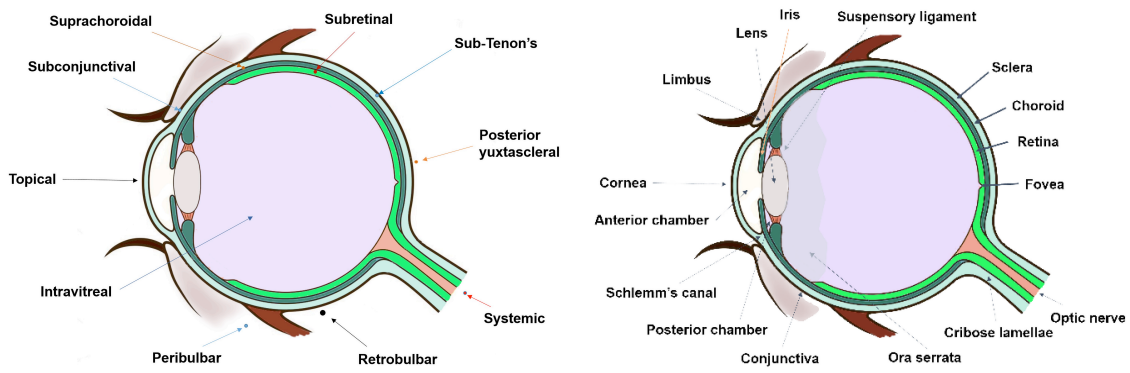


Figure 2. The first image represents a scheme of the different routes of drug administration to the posterior segment (dots symbolize the injection site of each route), while second image exemplifies the anatomy of the eye.

2. ROUTES OF DRUG DELIVERY TO THE POSTERIOR SEGMENT OF THE EYE

2.1. Intravitreal Administration

Intravitreal injection is the mainstream route of administration to treat diseases affecting the posterior segment of the eye. The drug is placed directly into the vitreous humor, though it is a highly targeted drug route. It has the inconvenience of being an invasive route. However, intravitreal drug administration is always selected to deliver to the posterior ocular segment due to the possibility of overcoming systemic exposure and obtaining high drug levels into the vitreous chamber.

2.1.1. Vitreous and Posterior Compartment Barriers to Drug Delivery

Vitreous

The vitreous body shows a transparent jelly-like structure mostly composed of types II, IX, and V/XI collagen fibers, whose spaces are filled with glycosaminoglycans, mainly hyaluronic acid. This structure acts as both a static and dynamic barrier. The static barrier is the vitreous structure by itself, although it is not a very restrictive barrier in terms of molecular mobility. The so-called dynamic barrier consists of the flow and clearance processes.

Initially, the superficial charge becomes vitally important for positively charged particles due to interactions with the components of the vitreous network such as hyaluronic acid (negatively charged), while negatively charged particles diffuse without any problem. Likewise, particle size may also impact on drug permeation, even though this parameter seems to be less clear. Certainly, several studies showed the distribution of same-sized particles (1 μm) through the vitreous is charge-dependent [2].

The convective flow from the anterior to the posterior segment of the eye should be taken into consideration for drug uptake due to the difference in pressure of the vitreous. This natural fact influences the drug distribution, having more impact on large molecules.

The drug particle size also plays a role in the type of clearance that drugs undergo, since smaller and more lipophilic drugs are mainly cleared in the posterior segment of the eye because of their ability of crossing the blood–retinal barrier (BRB), while larger and hydrophilic molecules are removed through anterior segment due to the aqueous humor outflow [2,3].

Posterior Compartment Barriers to Drug Delivery

1. Inner Limiting Membrane

The inner limiting membrane is a mechanical and electrostatic barrier located between the vitreous and the retina composed of collagen, laminin, and fibronectin. Its composition and thickness (~4 μm) may be variable along its structure, showing a 10 nm pore size.

Both drug charge and size can play an important role in the passage of molecules through this membrane, being the charge the most critical factor. Thus, positively charged nanoparticles seem to present more difficulty than neutral and negatively charged ones because of the negative charge of the membrane [3,4].

2. Neural Retina

The neural retina is the innermost layer of the eye and consists of a multi-layered structure which is responsible for transmitting the light to the brain by means of photoreceptors, neurons, and glial cells (Figure 3). These layers are: The outer limiting barrier, the outer nuclear layer, the outer plexiform layer, the inner nuclear layer, the inner plexiform layer, the ganglion cell layer, and the nerve fiber layer.

It also must be taken into account that molecules cannot freely diffuse through the retina due to Muller glial cells presence between the inner and the outer limiting membranes. Furthermore, certain components of the retina can interact with some molecules, hindering their diffusion [2,3].

3. Blood–Retinal Barrier (BRB)

The BRB is composed of two types of cells: The retinal capillary endothelial cells (which make up the inner BRB) and the retinal pigment epithelial cells (RPE) (which form the outer BRB) [5]. Both are considered an important impediment to retinal drug delivery as it restricts the drug transport between neural retina and circulating blood [4]. In the RPE, drug permeability is determined by physicochemical factors (e.g., molecular weight, lipophilicity, protein binding), as well as drug concentration gradient and their affinity with the existing transporters at that level, which can increase or decrease drug transfer through RPE [6].

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The BRB limits the drug diffusion from the vitreous humor to inner parts of the retina. It is composed of the inner BRB, which contacts with the vitreous humor and it is formed by capillary endothelial cells connected by tight junctions, and

the outer BRB, also called the RPE, which exhibits a high amount of melanin, being surrounded by choroidal capillaries [3,7,8].

Paracellular transport through retinal capillaries is limited by the tight junctions (about 2 nm), although certain larger-size molecules can transcellularly permeate by passive diffusion and/or active transport (e.g., ganciclovir or dexamethasone) [9,10]. Nevertheless, both processes are also restricted due to the absence of fenestrations and the lack of transport vesicles in the endothelial cells.

The outer BRB located between the photoreceptors and the choriocapillaris, is composed of tight, gap and adherent junctions that can be removed by molecules via passive diffusion. Also, the pump proteins that are in the layer and the invagination vesicles allow active transport (e.g., Na^+/K^+-ATPase, P-glycoprotein, Multidrug Resistance Proteins -MRP1-) [11].

Molecules permeation is restricted depending on their size, charge, and/or lipophilicity. Thus, hydrophilic compounds follow the paracellular route through tight junctions while lipophilic molecules can cross the epithelium by the transcellular route. It has been demonstrated that only small lipophilic molecules can easily diffuse from bloodstream to the vitreous and vice versa [2,3,12].

4. Choroid

The choroid is a highly vascularized barrier that lies between the RPE and the sclera (Figure 3). This layer supplies oxygen and nutrients to the retina. It has a 200 μm thickness and it is divided into five layers: The Bruch's membrane, the choriocapillaris layer, two vascular layers, and the suprachoroidal layer [2].

The Brunch´s membrane presents a 2–4 μm thickness, whose structure is composed of collagen and elastin fibers. The choriocapillaris layer is formed by highly fenestrated capillaries with a 6–12 nm pore size, allowing the passage of large molecules [13]. The suprachoroid is located between the sclera and the choroid and it is composed of collagen fibers, melanocytes, and fibroblasts.

In terms of drug delivery, it must be taken into consideration that choroid shows two different behaviors: 1) It acts as a static barrier due to suprachoroid structure and 2) it provides a dynamic barrier as a consequence of a high choriocapillaris-layer blood flow. Both actions prevent the passage of hydrophilic compounds while positively charged lipophilic drugs can stabilize bindings with the tissue, leading to slow-release depots. The molecular size of the drug also determines the diffusivity into the posterior segment [2,12].

5. Sclera

The sclera is the outer opaque layer of the eye (Figure 3), which provides support to this structure. It shows a 0.5–1 mm average thickness, although it varies along the eye. It is composed of collagen fibers, proteoglycans, and glycoproteins, creating a network in an aqueous medium [14,15]. The organization of the collagen fibers gives rise to different regions, such as: Tenon's capsule, episcleral layer, the scleral stroma tissue and the lamina fusca, melding into the underlying choroid.

Drug permeability through the sclera depends on molecular weight, molecular radius, charge and lipophilicity, although it is usually more permeable than the cornea and conjunctiva [2,12]. Large molecules, up to 150 kDa, find harder to get through the sclera and their clearance is lower than smaller molecules. Transscleral diffusion consists of the permeation through porous spaces within a collagen aqueous network. Likewise, the hydrophilic molecules (e.g., methazolamide) are able to go through the sclera [16,17]. Matrix structure of proteoglycans is negatively charged at physiological pH and, consequently, the passage of negatively charged solutes across the sclera is facilitated [18].

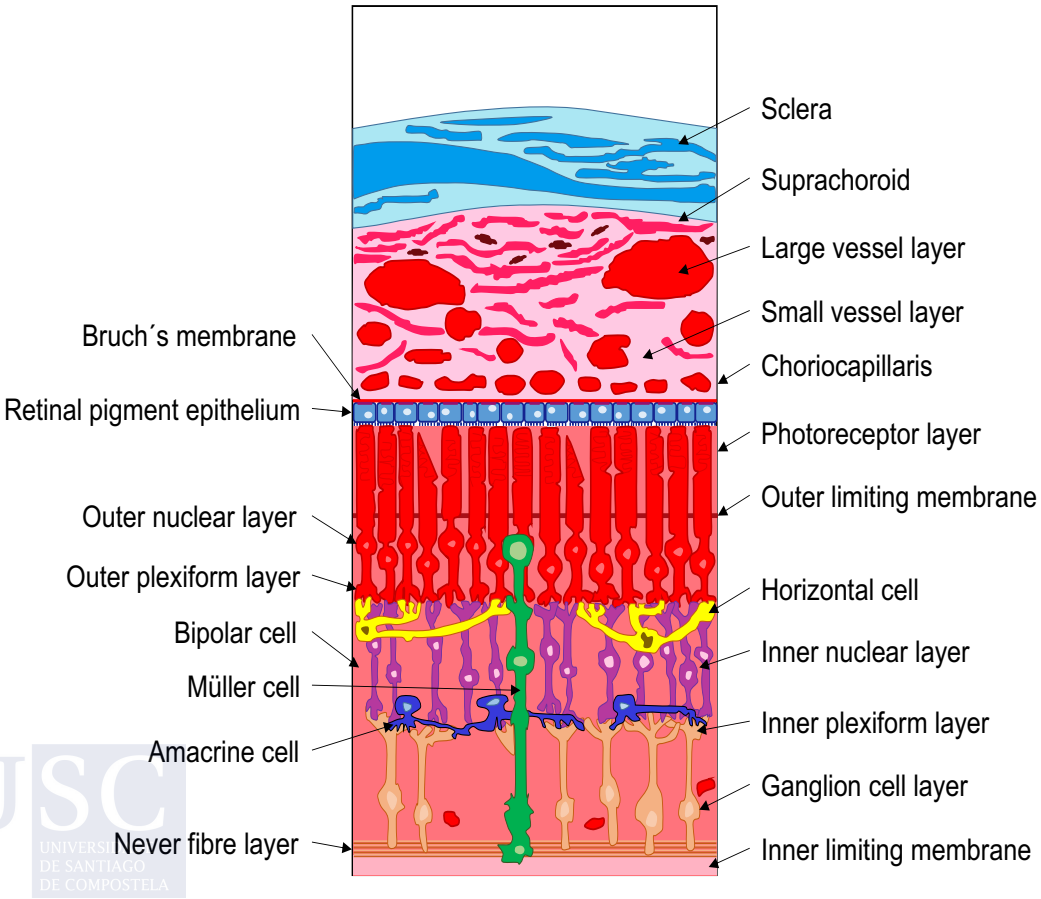


Figure 3. Schematic drawing of the sclera, choroid, and retina.

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2.1.2. Advantages and Limitations

Intravitreal administration is performed by injecting a drug solution, suspension, and even intraocular implants into the vitreous cavity. In recent years, the intravitreal injection has become the mainstream of the treatment of ocular diseases affecting the posterior segment. Direct intravitreal injection has the obvious advantage of achieving immediate therapeutic concentrations in the vitreous humor. However, sites of action are normally the retina or the choroid, so the drug is injected in the surroundings of the site of action, but it has yet to overcome some barriers in order to achieve its target site. Therefore, drug delivery into the vitreous humor does not necessarily implies that the drug will be able to exert the pharmacological effect [19]. Nevertheless, intravitreal administration has proved to be the best administration route to achieve high and effective drug concentrations in the posterior segment of the eye.

Additionally, intravitreal administration has the advantage that largely avoids the systemic exposure; hence, the systemic adverse effects are limited. However, not all side effects are completely eliminated, being the most common ones: Endophthalmitis, vitreous detachment, retinal hemorrhage, inflammation, increased intraocular pressure, conjunctival hemorrhage, retinal toxicity, and cataracts, among others [19]. As an invasive procedure, the injection itself may cause a patient's discomfort and eye pain, mainly where repeated injections are needed. The need to treat the posterior segment of the eye increases interest in getting available novel controlled release formulations to maintain the therapeutic drug concentration in the vitreous as well as minimizing the number of injections.

The vitreous humor is composed of a polysaccharide aqueous meshwork. Apparently, it would seem that it acts itself as a retention compartment against drug distribution [7]. Nevertheless, drugs are quickly removed from the vitreous via the anterior route to the aqueous humor, hampering prolonged release phenomena [20]. In addition, the relatively high volume of the vitreous chamber allows administering drug delivery systems of different size and form (implants and inserts).

In clinical practice, intravitreal injections are directly administered into the vitreous cavity and the procedure is performed by a trained specialist. As an invasive procedure, some risks may be taken into account and several precautions should be taken during the intravitreal administration, such as avoiding touching the retina or the lens [19]; the vision line must be left untouched (the vision field can be blocked); and the injection incision should be as small as possible since it can prevent the development of eye infections and the leakage of vitreous [19]. Other major risks after intravitreal injection

(although uncommon) are eye infections or vitreous hemorrhage. Depending on the disease being treated, a follow-up visit to the specialist should be scheduled for the administration of repeated intravitreal injections, always needed in chronic disorders such as DP or AMD [21].

Despite the associated complications to the injection procedure and discomfort after administration, repeated intravitreal injections are the only alternative to administer drugs as anti-VEGF, steroids, and antibiotics to treat pathologies affecting the posterior segment of the eye.

2.1.3. Pharmacokinetics

Nowadays, the pharmacokinetics and pharmacodynamics of pharmacological agents after intravitreal administration remain relatively unexplored and poorly understood. The vitreous-humor clearance mechanisms of intraocular-administered drugs limit the duration of their effect and injection's repeated administration. Nevertheless, there remains a real need to develop novel drug delivery systems intended to sustain release and more effective therapy.

Pharmacokinetics studies of intravitreal injection have demonstrated the drawbacks of an invasive administration route and the difficulty of acquiring direct samples. Most of the *in vivo* studies have been performed in rabbits as an experimental animal model [22]. Vitreous humor samples collection may require animal sacrifice, even though novel techniques such as micro-dialysis can be applied for continuous sample collections without animal detriment [23]. According to the 3Rs regulatory frameworks, the alternative is to extract aqueous humor or blood samples and then make a correlation with the drug concentration in the vitreous humor.

The drug concentration at any certain time after injection depends on the drug distribution volume, the initial injected dose and the elimination rate. Therefore, the two factors that predominantly affect the pharmacokinetics of injected drugs into the vitreous are their vitreous humor distribution and their clearance.

Drug Distribution in the Vitreous Humor

Drug distribution in the posterior segment of the eye is a crucial step in ocular pathologies treatment in order to obtain a pharmacological effect at the right target site. The factors that affect the drug distribution in the vitreous humor are predominantly its diffusion through the vitreous, the effect of the convective flow and the possible drug interactions with the vitreous humor elements. Moreover, other ocular barriers may limit the drug passage depending on the target site within the vitreous cavity [24].

Another aspect that should be taken into consideration is the effect of the injection itself, as the needle creates a channel through the vitreous humor. After the needle is being removed, it leaves behind a low resistance pathway for the passage of the drugs due to a change of the integrity of the vitreous humor [25]. The impact of both diffusion and convection flow in the drug pharmacokinetics within the vitreous humor has been studied based on different experimental data and mathematical models [7,26–29].

1. Diffusion

Once the drug is injected into the vitreous humor, it will diffuse through the vitreous humor until reaching its target tissue. The diffusion speed of this process depends on the drug physiochemical properties and the vitreous retention effect. The mesh size of the bovine vitreous humor was calculated to be approximately 500 nm [30]. Also, the different structural components presented in the vitreous constitute a barrier to the diffusion of the drugs depending of the characteristics on the drug.

Drug diffusion through the vitreous humor is created as drug concentration gradients from the injection site, which provides the moving force for the drug-particles' movement until a concentration balance is reached, where not more diffusion occurs. Regarding the drug physicochemical properties, the molecular weight and the drug net's charge are the parameters that most influence in the diffusion speed through the vitreous humor.

Generally, low molecular-weight drugs (e.g., fluorescein, glycerol, mannitol) exhibit high diffusion coefficients as they do not have the diffusion restriction through the vitreous meshwork [31,32]. In fact, the diffusivity in an aqueous solution could be a specific representation of this parameter in the vitreous humor. Diffusion of high molecular-weight molecules (e.g., FITC dextran) can be limited by the vitreous structure [33]. On the other hand, the drug net's charge could affect its diffusion since the vitreous humor is a negatively charged polymer network. Anionic and neutral molecules will not show any type of restriction in the vitreous humor considering their charge. However, cationic drugs could electrostatically interact with the negative charges of the vitreous humor [30,34]. This will lead to a decrease in the drug diffusion due to its retention in the vitreous humor.

2. Convection

Convection is the process of moving a fraction of volume of the aqueous humor produced by the ciliary processes throughout the vitreous humor towards the

retina [7,26,28,29]. This process takes place due to the differences in pressure and temperature between the anterior chamber and the retinal surface [7].

The significance of the vitreous outflow effect on drug distribution depends on drug diffusivity in the vitreous [28]. It has been seen that drugs with high diffusivity values within the vitreous are not affected by the convective flow due to the high net movement through the vitreous humor, while low molecular weight drugs do not suffer any type of restriction through the vitreous. On the other hand, the relevance of the convective flow in the movement of drugs with low diffusivity values is quite unclear, even though it is generally accepted that the convective flow has a major effect when the diffusivity is lower and the flow is increased.

Park et al. have calculated the drug diffusivity values that could be affected by the convective flow, using a mathematical model. The convection does not affect the drug distribution with high diffusivity values within the vitreous (1×10^{-5} cm^2/s) but it starts to become relevant in the case of drugs with low diffusivity values (1×10^{-5} cm^2/s), particularly if the flow is increased [26,28]. Moreover, Xu et al. predicted that convection could be responsible for the 30% of the drug movement through the vitreous humor [27]. In the light of these results, it can be concluded that the convection is not a major contributing factor in the drug distribution through the vitreous humor [3].

Since the intravitreal convective flow depends on pressure, it seems obvious that an increase in the intraocular pressure could change flow values in the vitreous humor. As an example, in some pathologies such as glaucoma and retinal detachment, where higher intraocular pressure values are noticed, an increase in the convection has been observed [26,29].

3. Drug Interaction with Vitreous Humor

Hyaluronic acid, as one of the main vitreous components (65–400 mg/mL concentration range), may establish charge interactions with intraocularly administered drugs that are positively charged [35]. This binding could importantly decrease the cationic-drug diffusion through the vitreous humor (e.g., poly-l-lysine) [3]. However, up to now, the effect of this binding on drug pharmacokinetics remains to be elucidated.

4. Drug Interaction with Proteins

Protein concentration in the vitreous humor was estimated to be 4.7 ± 1.2 μg/μL in human samples [36], being the albumin the most abundant one (60–70% of the total protein) [37]. Between 1000 and 2500 different proteins have been identified

in the vitreous cavity [36,38]. Although their concentration is very low in comparison with plasma concentration, the interaction between drugs and vitreous proteins can occur in the same way with plasma proteins. This interaction will lead to a free drug decrease to exert a pharmacological effect. Furthermore, the drug binding to a protein could slow down its diffusion through the vitreous humor increasing the residence time in the vitreous.

In some vitreoretinal pathologies, such as diabetic vitreoretinopathy, an increment in protein concentration in the vitreous humor is observed, as well as new proteins are expressed and linked to inflammation and immunity processes [36]. However, there is no available data regarding the impact of the increased protein concentration in the drug interaction with the proteins in these chronic diseases.

5. Liquefaction

One factor influencing both drug diffusion and convection processes in the vitreous humor is its own liquefaction, that is, the degeneration process of the vitreous humor associated with aging. The vitreous humor is observed in both liquid and jelly forms at the same time in the vitreous cavity [39]. Nevertheless, the proportion between these two forms gets modified with age, being observed as an increase in the liquid proportion and a decrease in the gel one [7], due to the disruption of the fibers mesh composing the vitreous humor. Vitreous liquefaction could cause an increase in the drug diffusivity, particularly in those that showed a limited diffusion, since the fibers mesh presents less movement restriction of molecules in its interior. This expanded diffusion can lead to an elimination increase, although the liquefaction itself does not directly affect the drug elimination from the posterior segment of the eye. The vitreous humor higher liquefaction, the more closely the diffusivity of the molecules in it approximates to their diffusion in water [3]. On the other hand, liquefaction and the vitreous homogeneity loss through aging are also associated with an increase in the convection flow [40].

Such data entails that the treatment of different-age-group patients with the same dosing scheme might be inappropriate, leading to overdose or insufficient dosage situations. Even though, so far it is not clear whether the vitreous liquefaction can affect the intravitreal pharmacokinetics or not [41,42].

It is also worthwhile to mention that the injection position during the administration and the injected volume may have an influence on the distribution of molecules in the vitreous humor. Different injection sites affect the drug distribution and permeability through the retina [43], whereas the injected

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volume could affect the drug elimination in different degree, depending on the injection position [43].

On the other hand, special patient situations, such as vitrectomy (procedure where the vitreous humor is removed), also determine the drug behavior into the vitreous. The motivations to perform a vitrectomy are varied, where the most common ones are: 1) Removal of infection, 2) obtention of a better access to the retina, 3) removal of scar tissue, and 4) correction of retinal detachment.

After the vitrectomy procedure, the drug elimination is greatly increased, regardless of the elimination pathway. In different animal studies, the half-life reduction of intravitreal drugs has been detected after a vitrectomy was performed, including: Amikacin [44], amphotericin B [45,46], cefazolin [47], ceftazidime [48], ciprofloxacin [49], and vancomycin [50], as well as some biologics, such as bevacizumab [51,52], ranibizumab [51,53], and aflibercept [53].

It also must be taken into account that these studies were performed in animal models and that its extrapolation to humans lacks of enough knowledge. However, it seems to be a general decrease in the half-life in humans and might affect the efficacy of the intravitreal drug, although this aspect is still under investigation [54].

Moreover, a higher risk of retinal toxicity is expected in vitrectomized eyes. The anti-infective is supposed to be placed in contact to the retinal surface on a high concentration, instead of being distributed all over the vitreous humor, consequently causing the retinal toxicity [55]. This is extremely important in drugs as amikacin since it has proven to produce retinal toxicity [56].

Surgical vitrectomy is normally followed by the filling of the vitreous chamber with silicone oil, which acts as a long-term buffer in the management of vitreous detachment. Injected silicone oil can also behave as a slow-release reservoir for some drugs [7], although it must be taken into consideration that most anti-inflammatory drugs are not soluble in the silicone oils. Several studies confirmed the drug injection into silicone oil-filled eyes led to its migration to the aqueous interface, resulting in an increase in the local concentration and the ulterior precipitation. This may cause retinal toxicity [57,58], supposedly caused by a decrease on the preretinal-space, which can also affect the drug distribution and half-life.

Therefore, the drug pharmacokinetics in silicone oil-filled eyes is still not well defined, although some authors recommend a substantial reduction of the drug dose (1/4–1/10 of the standard dose) to prevent these phenomena [59].

Drug Distribution to Surrounding Tissues

Depending on the type of disease, the target site could be located in the vitreous humor itself (e.g., infections), the retina (e.g., AMD, diabetic macular degeneration), or the choroid (e.g., serpiginous choroiditis). Therefore, the drug distribution to the surrounding tissues could be considered as part of the elimination process if the site of action is in the vitreous humor or as part of the drug distribution in order to achieve its target site. Nevertheless, studies performed in rabbit eyes have shown that the distribution volume is close to the anatomical volume of the vitreous chamber which implies that the surrounding tissues do not contribute too much to the distribution process [3].

The vitreous body is limited by the anterior blood–aqueous barrier (BAB) and posterior BRB. The inner BRB allows for the permeation of small molecules, where molecules with a size higher than 2 nm are prevented from their diffusion. Likewise, the RPE is a tight cellular layer between the photoreceptors and the choroid, which its permeability depends on the molecule's size and lipophilicity [3]. Once the drug reaches to the choroid, the drug diffusion is quite rapid, because of the higher permeability of the choroid and, subsequently, the drug is quickly removed to the blood circulation [3].

There is evidence of BRB influx-and-efflux carriers that ensure the retina is constantly supplied of nutrients and ions [7,8,60]. The evidence of efflux transporters at the BRB has been recently investigated, as the studies performed in animal models might not correlate with the results that could be obtained in humans. MDR1, BCRP, some MRP, and OATP are some of the main carrier families that have been detected in the BRB [61].

It should be noted that some drugs can be substrates of the BRB active transporters, but their contribution to the drug pharmacokinetics is still unclear. Firstly, it is needed to be addressed that the presence of active transport at the BRB could be an advantage if the drug target is in the choroid, as it will help the drug to reach the target site or even ensure that some drugs, that normally are not able to cross the BRB, can achieve the choroid. Conversely, this fact could be a disadvantage if the target site is prior to the retina or in the retina itself, the active transport will act as an elimination pathway.

Overall, the active transport contribution on the drug movement through the BRB is quite low, the effect being also reduced over the time. As the drug concentration at the vitreous humor is usually very high after administration, the transporters are prone to be saturated [61]. The drug elimination from the vitreous humor involves the drug possible metabolism in the ocular tissues and the removal from the ocular compartments to the systemic blood flow.

Drug metabolism in the vitreous humor has not been deeply investigated. Mainly, studies have aimed at the enzyme identification in the vitreous humor, but not at analyzing its impact of drug pharmacokinetics [45]. For example, the presence of enzymes such as esterases or peptidases in rabbits´ vitreous humor should be mentioned here [62]. The drug in situ metabolism have been exploited for the development of prodrugs, such as ganciclovir esters (prodrugs with no pharmacological activity) which are biotransformed into ganciclovir (drug with pharmacological activity) once injected into the vitreous humor [62].

Metabolic enzymes have been detected in other ocular tissues posterior to the elimination of the drug from the vitreous humor, such as retina, ciliary body, and iris [63]. There are two major routes of drug elimination from the vitreous: Anterior and posterior clearance (Figure 4).

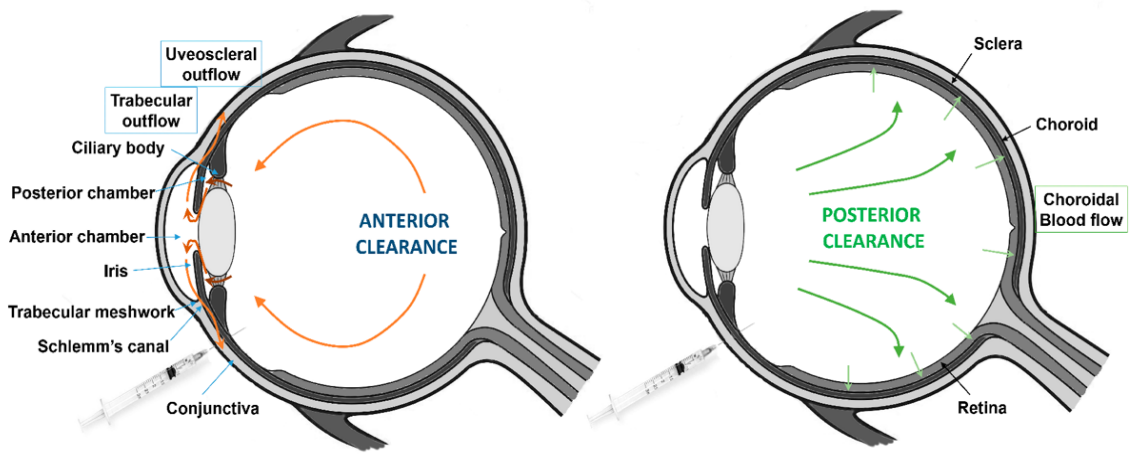


Figure 4. Schematic representation of the anterior and posterior clearance from the vitreous humor.

1. Anterior Route

After intravitreal injection, the drugs will be eliminated following the anterior route from the vitreous by a diffusion process across the lens and the ciliary body, to enter afterwards into the posterior chamber. From there, drugs are removed through the aqueous humor turnover to the anterior chamber, where they are subsequently removed along with aqueous humor by the trabecular and uveoscleral outflow [8]. The rapid turnover of the aqueous humor into the anterior chamber is the main force for the anterior clearance [7].

This elimination route is accessible for all type of drugs as they can freely move across the hyaloid membrane, avoiding the lens. However, drugs that are typically removed from this pathway are hydrophilic and large molecules that are not able to cross the retina [7]. The elimination of high molecular-weight

compounds by the anterior route has been widely studied [40,42,64] (see Table 1). In fact, there is an inverse relationship between the molecular weight and the elimination rate from the vitreous.

Experimental data have determined that drugs which are removed from the anterior pathway exhibit higher half-lives than the ones that are removed from the posterior route. The relationship between vitreous half-life and aqueous humor/vitreous humor ratio is not broadly clarified. However, it is known that the presence of the drug is higher in the aqueous humor, i.e., it is removed by the anterior route, as the half-life is higher [65].

Table 1. Vitreous half-life times for intravitreally administered drugs with different pharmacokinetic characteristics.

Pharmacologic Group	Drug	Characteristics	Half-Life Time (h)	Ref.
Corticosteroids	Dexamethasone	Low molecular weight Water insoluble	3.48	[66]
Antibiotics	Ceftizoxime	Low molecular weight Water soluble	5.70	[67]
Somatostatin analogues	Octreotide acetate	High molecular weight Water soluble	16.00	[68]
Antiviral	ISIS 2922	High molecular weight Water soluble	62.00	[69]

2. Posterior Route

In the posterior route, administered drugs permeate through the retina and subsequently are cleared by the choroidal blood flow. Drugs that are removed by the posterior route exhibit short half-lives due to the large surface area available for permeation and the presence of active transport mechanisms [65].

In posterior elimination processes, a relationship between the drug physicochemical properties and their half-lives within the vitreous humor has been identified. Durairaj et al. established that the drug molecular weight, its lipophilia, and the dose/solubility ratio at pH 7.4 are the major parameters that affect the drug half-life in the vitreous [70].

Therefore, the posterior route is the main elimination pathway for small and lipophilic molecules since they can easily cross the retina. The diffusion process could take place via the paracellular and/or transcellular route. As can be seen in Table 2, some important differences are shown between the parameters affecting the anterior and posterior elimination route of drugs from the vitreous humor.

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Table 2. Comparison of the anterior and posterior route of drug elimination from the vitreous humor [65,70].

Features	Anterior Route	Posterior Route
Tissue involved	BAB	BRB
Elimination pathway	Aqueous humor outflow	Choroidal flow
Molecule characteristics	Hydrophilic High molecular weight	Lipophilic Small molecular weight

2.1.4. Drug Delivery Systems

Several reviews about the development of drug delivery systems have been published previously [71–74]. For this reason, in this article is not going to be treated in depth.

Biodegradable implants [75], non-biodegradable implants [76], biodegradable microspheres [71], nanoparticles [77], dendrimers [78], and hydrogels [79] have been used for intravitreal drug administration. Moreover, some sophisticated systems have been developed for the treatment of chronic and refractory ocular diseases, such as a microelectromechanical system (MicroPump) [80] and a port delivery system (PDS) [81].

In addition, to the current research on new systems of intravitreal release [82], there are already commercialized formulations such as Lucentis®, Ozurdex®, Eylea®, Avastyn®, among others (see Table 3).

Table 3. Summary of the main key pharmacokinetic parameters for different intravitreally administered drugs.

Pharmacologic Group	Subgroup/Drug	Half-Life Time (h)	MRT (h)	C_{max} (μg/mL)	Ref.
Nonsteroidal anti-inflammatory drugs	Ketorolac	4.3	6.16	175	[83]
	Diclofenac	2.05	2.95	65	[83]
Antibiotics	Penicillines	10–20	5–25	1000–5000	[84–86]
	Cephalosporines	5–15	5–30	1000–2250	[48,85,87]
	Tetracyclines	10–20	NA	125–400	[86,88]
	Fluoroquinolones	3.5–5.5	0.25–5	100–500	[49,89,90]
	Monobactams	7.5	NA	1000	[91]
	Carbapenems	2.5–10	NA	50–100	[92,93]
	Macrolides	40–60	NA	100–200	[85,94,95]
Antibodies	Bevacizumab	4.32	5.92	400	[96]
	Ranibizumab	2.88	4.03	162	[97]

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2.2. Topical Administration

Ophthalmic topical administration by eye drops is commonly used for the treatment of anterior-segment diseases [98,99]. Most of the topically applied drugs are intended for the treatment of diseases that affect different layers of the cornea, the conjunctiva, iris, or the ciliary body [5]. However, topical administration for the treatment of posterior ocular diseases is considered an ineffective pharmacological strategy since therapeutic drug concentrations are not reached in the posterior segment of the eye due to low drug penetration.

2.2.1. Ocular Barriers for the Entry of Drugs: Precorneal Factors

After topical eye-drops administration, the first tissue barrier that drug molecules must overcome to access the target is the tear drainage of the excess volume through the nasolacrimal duct. In normal conditions, this drainage occurs at 1.45 $\mu L \cdot min^{-1}$ and it results in a drug loss into systemic circulation, especially related to hydrophilic molecules [100]. In fact, the loss of eye drop solution occurs until the tear volume returns to a normal range (7–9 μL).

Likewise, the thin precorneal tear film secreted by different glands and the Globet cells, which is about 8 μm thickness and with a 7 μL volume, also acts as a barrier in terms of drug absorption. It is composed of three layers: mucin, an aqueous and a lipid layer. The rate of drug elimination from the tear fluid is in the range of 0.5–1.0 $\mu L \cdot min^{-1}$ [101,102]. As a result of these facts and the systemic absorption through the conjunctiva, the ocular drug absorption is limited to less than 5% (<5%) when this delivery method is used [102].

2.2.2. Corneal and Anterior Compartment Barriers

Cornea

The cornea is the transparent portion surrounding the sixth anterior part of the eyeball with a 0.5 mm thickness and a 12 mm diameter. Tear film and aqueous humor provide nourishment and oxygen as it lacks blood vessels. The cornea consists of a collagen structure organized in six layers: Epithelium, Bowman's membrane, stroma, Dua´s layer, Descemet's membrane, and endothelium (Figure 5). The stratified, squamous and non-keratinized epithelium is the most critical barrier to penetration with a 10^{-7}–10^{-5} cm^{-1} drug permeability rate because of the fact that tight junctions impair the permeation of low lipophilic molecules [12,100,103].

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Drug absorption depends on their physicochemical characteristics. In consequence, only hydrophilic molecules can diffuse through the stroma due to the hydrophilic nature of the hydrated collagen. Only small molecules with a log D value between 2 and 3 can penetrate through all the layers. The paracellular route allows molecules with a 500 Da molecular weight or a <5.5 Å size penetrate across tight junctions. The solute charge is also an important factor, as the cornea decreases the absorption of negatively charged molecules because of the negatively charged corneal surface [12].

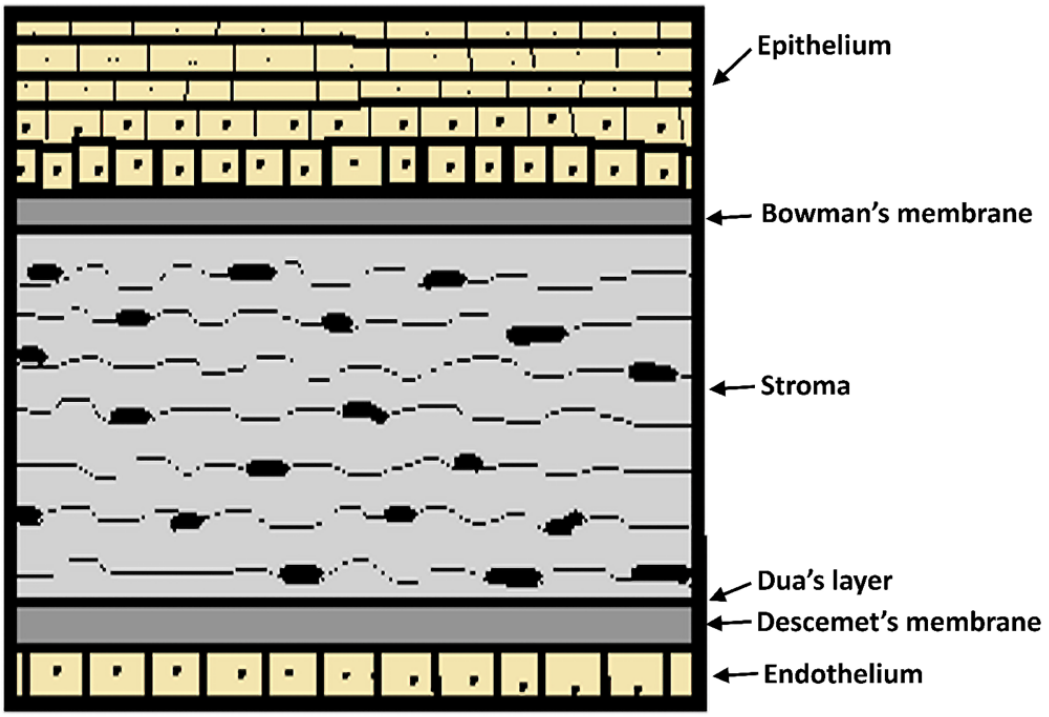


Figure 5. Schematic representation of the human corneal layers.

Conjunctiva

1. Conjunctiva

The conjunctiva is a thin vascularized stratified cylindrical epithelium with its underlying stroma covering the exposed sclera and the eyelids. This membrane shows a 17-times higher surface area than the cornea [104], although conjunctiva is less permeable for lipophilic molecules. Hydrophilic compounds absorption is also reduced because of tight junctions in the corneal epithelium. Despite this fact, the conjunctival absorption is higher than the corneal's due to the wider intercellular spacing allowing the passage of molecules up to 10 kDa. Furthermore, through the corneal route, via the conjunctiva, drugs are removed into the systemic circulation because of the presence of blood and lymphatic capillaries in the conjunctiva [12].

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2. Blood–Aqueous Barrier (BAB)

The BAB is formed by the iris and ciliary muscle vasculature endothelium and by the posterior iris and non-pigmented ciliary epithelium (Figure 6). This barrier is poorly permeable due to the tight intercellular junctions, which limit the passage of substances with high molecular weight or high hydrophilicity. This biological barrier is also responsible for the selective diffusion of ions and small solutes through space between cells. There are active transporters that alters drug permeation, depending their activity on the passive diffusion rate. However, their significance in drug absorption is still unclear and requires elucidation.

For systemically delivered drug, the tight junctions of the iris vasculature limit the passage of substances from the plasma to the iris stroma. The non-pigmented ciliary epithelium tight junctions restrict the passage of substances from ciliary body stroma to the posterior chamber [12,105]. Overall, this barrier modulates the passage of molecules between the anterior and posterior segment, as well as from plasma to aqueous humor. The drainage of aqueous humor (2.0 to 3.0 mL/min) also hinders the passage of drugs [106].

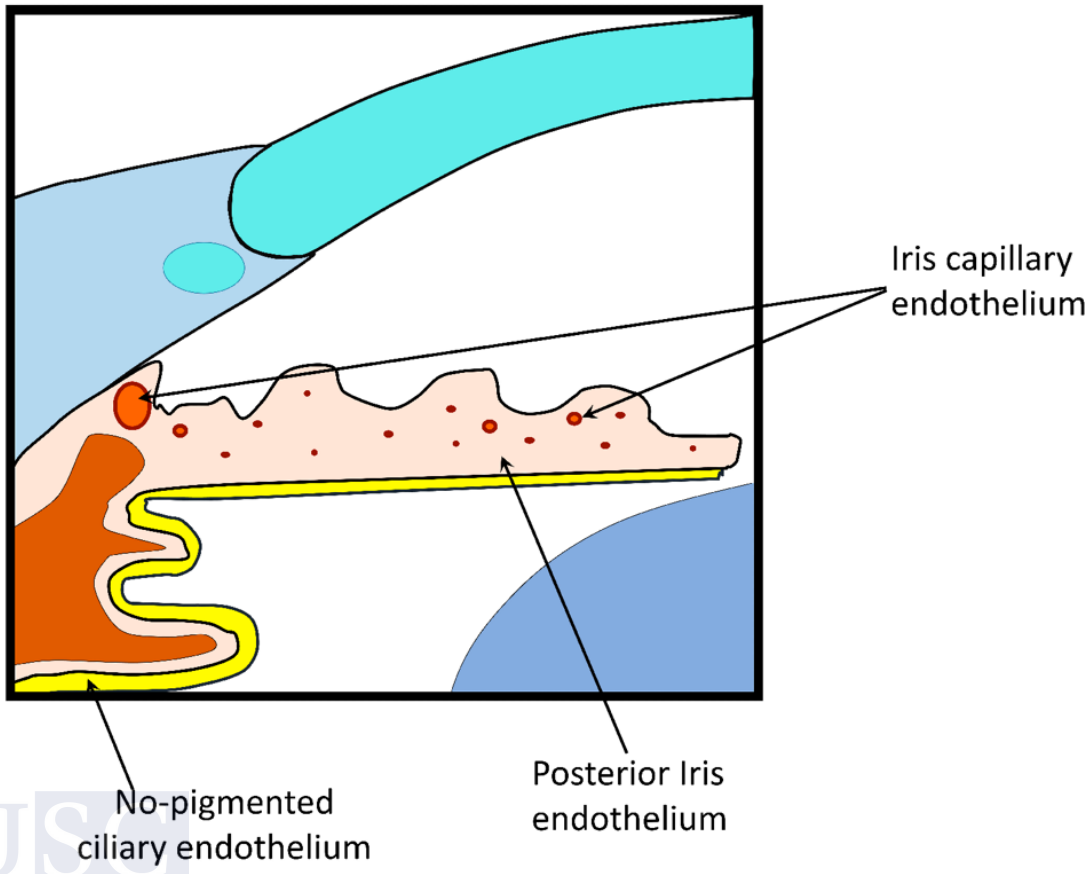


Figure 6. Blood–aqueous barrier (BAB) structure.

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2.2.3. Advantages and Limitations

Topical administration is the most challenging administration route for drugs intended to treat pathologies that are affecting the posterior segment of the eye. Once administered onto the ocular external surface, the drug absorption is restricted by the different barriers, these being: Static (cornea and the BAB), dynamic (conjunctival and lymphatic conjunctival lymphatic and lacrimal drainage), and metabolic (carriers and receptors) barriers [99].

Nevertheless, it is considered the least invasive method of ophthalmic application due to patient compliance and ease of administration. Moreover, it is not required the ophthalmologist intervention since eye drops can be instilled by the patients themselves [5].

However, significant disadvantages have been found using this administration route to the posterior segment, such as the significantly low bioavailability (1–5%) and ocular penetration (less than 0.001%) in the intraocular tissues (aqueous humor among others) [107]. The main reasons are that the drug applied topically is quickly removed by ocular protective mechanisms such as nasolacrimal drainage, blinking (6 to 15 times/minute), tear renewal (0.5–2.2 μL/min) and high lacrimal clearance [108,109].

Besides, the unproductive absorption to the systemic circulation through the conjunctiva, choroid, uveal tract and internal retina also limits the drug access to the posterior segment. Significant efforts towards the development of new drug delivery systems to the posterior segment of the eye have been carried out to overcome the obvious limitations of topical ocular administration.

2.2.4. Pharmacokinetics

Drug topical absorption into the posterior segment of the eye strictly depends on the route through which the drug is distributed (Figure 7). Thus, the drug corneal exposure will allow for its concentration to be higher in the aqueous humor, whereas higher drug concentrations in the back of the eye will be reached after direct exposure on the conjunctival surface.

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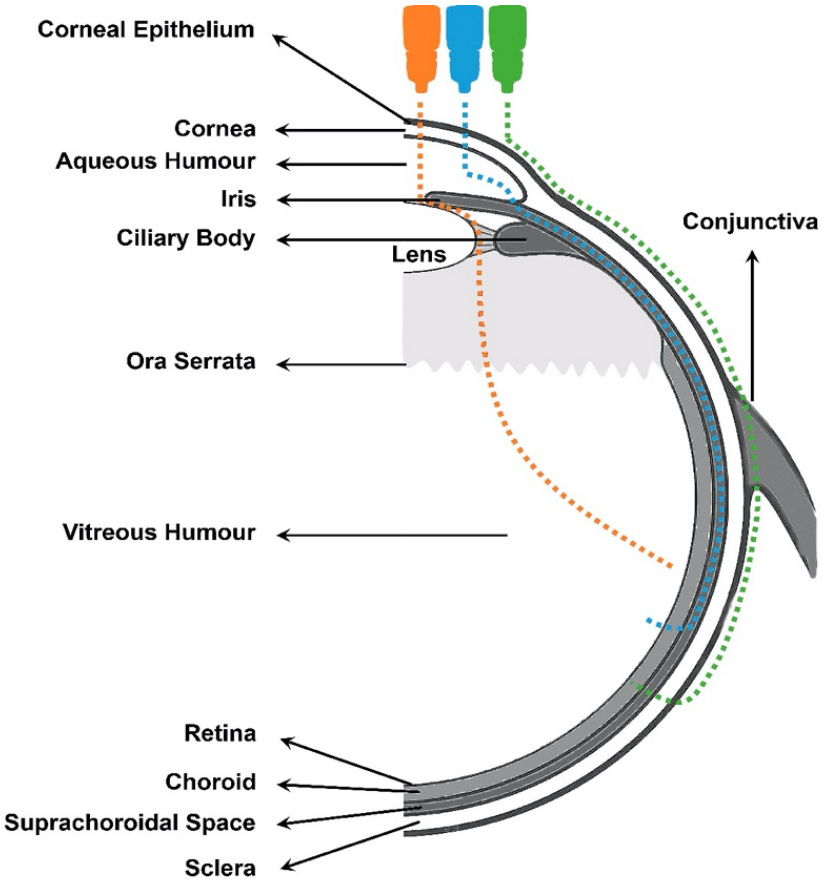


Figure 7. Different topical routes of drug absorption from the cornea/conjunctiva to the vitreous humor: Periocular route marked in green. Uvea-scleral route in blue. Transvitreal route marked in orange.

For example, the drug loss in the precorneal segment is because of the tear fluid. The administered drug will be released from the vehicle to the tear film, where tear fluid dynamics accelerate its elimination due to the complete replacement of the tear film every 2–3 min [99]. Furthermore, different enzymes and lacrimal proteins can influence the drug metabolism in the precorneal surface [107].

When administered topically, the low amount of drug that has been able to be absorbed will permeate from the cornea/conjunctiva surface to the retina across three different routes [98]: transvitreal route, uvea–scleral route and periocular route, being all of them conditioned by the nature of the drug, the pharmaceutical form and the anatomical characteristics of the eye.

The transvitreal pathway of absorption is the one followed by the drug via the transcorneal diffusion until it reaches the vitreous humor. The passage of molecules through the cornea depends on their lipophilicity, molecular weight, charge and ionization degree [110,111]. Once the drug is applied to the ocular surface by eye drops, the passage to the anterior chamber is driven by a diffusion process passing through the corneal epithelium via the paracellular or

transcellular route. The penetration through these routes always depends on the drug concentration in the tear film and the drug retention time on the ocular surface. Hydrophilic drugs cross the corneal epithelium via the paracellular route while lipophilic drugs cross the epithelial barrier along the transcellular route. Once the drug crosses the cornea and reaches the aqueous humor, it is distributed to the surrounding tissues (ciliary, iris, crystalline body) and to the vitreous humor by diffusion process [98].

The aqueous humor is mainly drained (75–85%) through the Schlemm's canal (tubular conduct with thin and porous walls that allows the absorption of large protein molecules). The rest of the elimination process is carried out through the iris and the connective tissue of the ciliary and suprachoroidal muscles, leading to the uvea towards the extraocular connective tissue. The drug removed from aqueous humor contributes to a decrease in the amount of drug that could be able to reach the posterior segment of the eye.

The iris is one of the two ocular tissues that contain melanin, a pigment responsible for its color. Melanin can interact with some drugs, causing its retention in the iris. This retentive effect is more pronounced in case of lipophilic drugs, resulting in a drug sustained action [112] but decreases its pharmacological activity [5]. Overall, the drug bioavailability in the aqueous humor through this route has been determined to be less than 5% of the applied dose [113].

The uveal-scleral route followed by the administered drug after topical administration corresponds to the transcorneal diffusion to the anterior chamber and its drainage through the aqueous humor to the uvea–scleral tissue towards the posterior tissues.

This route follows the same absorption process through the cornea until reaching the aqueous humor as the transvitreal one. Once in the aqueous humor, the drug diffuses through the suprachoroidal space to the choroid causing an unproductive absorption. To a large extent, this is due to the drug partial elimination to the systemic circulation through the BAB capillary fenestrations, giving rise to the appearance of side effects. The drug is transported towards the systemic circulation through the choriocapillaris, which can cause side effects systemically. However, some other external factors, such as prostaglandins (drug penetration improvement to the back of the eye when they are co-administered via the uveo–scleral route) can affect the process [114,115].

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The periocular drug absorption comprises the drug diffusion through the conjunctiva to the Tenon's capsule and its diffusion through the sclera, choroid, and retina.

It also must be taken into account that between 34% and 79% of the administered dose on the ocular surface is primarily removed by this way into the systemic circulation due to the high degree of conjunctival vascularization and its large surface area (16–18 cm^2) [113]. Therefore, for a drug to penetrate must have a molecular weight no greater than 70 kDa [116]. In addition, it should be considered that the drug transport through the conjunctiva, sclera, choroids and retina can occur by passive (paracellular or transcellular) or active routes (membrane carriers) [114,117]. Some lipophilic drugs can passively penetrate throughout the RPE by directly accessing the posterior segment through lateral scleral diffusion followed by penetration through the Bruch's membrane and the RPE.

2.2.5. Drug Delivery Systems

Currently, there are few commercial topical formulations specifically intended for the treatment of ocular posterior segment diseases. Even so, in recent years, new advanced topically drug release systems have been developed in order to improve the drug access to the back of the eye, such as nanoparticles [99,118], emulsions [119], nanostructured lipid carriers [120], liposomes [121], and nanosuspensions [122].

Despite the enormous efforts to develop efficient topical formulations intended to reach therapeutic drug concentrations in the posterior segment of the eye, the most relevant results have been obtained by using eye drops. In an ex vivo assays using cow eyes, the topical application of a memantine hydrochloride ring that functioned as a reservoir on the corneal surface demonstrated the efficient drug distribution of the into the choroid and retina [123].

Currently, memantine is in phase III studies in humans as a neuroprotector and neuroreparative drug in ophthalmic diseases. Another approach has been made by investigating the topical application of a brimonidine ocular solution in monkeys, rats, and rabbits. Results have shown that the formulation administered repeatedly reached an enough drug concentration to provide neuroprotection in the retina [124]. In the AMD treatment, promising results have been obtained in rabbit retina by the dexamethasone-cyclodextrin complexes topical application [125,126].

2.3. Systemic Administration

Systemic administration route constitutes an alternative route in the treatment of eye's posterior segment pathologies. It is normally used as a coadjutant treatment or as a second choice after the failure of intravitreal or subconjunctival injections treatment. It is based on drug administration by conventional routes (e.g., oral,

intravenous, or intramuscular). Once the drug reaches the bloodstream, the absorption will take place through conjunctival, episcleral and/or choroidal vessels to get the vitreous cavity, although most of the drugs do not pass the main blood–ocular barriers [6].

2.3.1. Advantages and Limitations

Systemic drug administration shows a series of advantages and disadvantages compared to other administration routes to treat posterior segment diseases. Firstly, systemic administration is very effective in the case of concomitant ocular and systemic diseases as they can both be treated with only one treatment. Although ocular effects slowly appear with the systemic administration compared to other routes, the duration of the effect is more prolonged [127].

The oral administration to treat ocular diseases presents some positive features: Non-invasive, no need to use strict sterile conditions, high patient compliance, and adherence to treatment and width availability of pharmaceutical forms that provide great drug stability [5,103,128]. Oral administration is usually combined with topical ocular administration [5].

However, the systemic route presents certain drawbacks [5,103]. The presence of physiological barriers (BAB and BRB mainly) prevents the drug passage to the eye, leading to a drug bioavailability of less than 2% [5]. This low bioavailability forces the administration of high doses of the drug to obtain therapeutic concentrations into the vitreous which may lead to systemic toxicity and severe side effects.

In addition, a lag time occurs between the drug administration and onset of pharmacological effect. An important prerequisite for a drug to be administered by oral route (for ocular applications) is to have a high drug oral bioavailability [5]. Even though, the limited access to the posterior segment of the eye implies the administration of high doses of drug or an increased administration frequency to obtain a significant therapeutic response [103,116,129]. However, these procedures can exacerbate drug toxicity as a consequence of the drug non-specific absorption to other organs [116,130,131]. Trained personnel are also required in case of intravenous or intramuscular administration.

Likewise, parenteral administration is also a systemic administration route used as an alternative pathway in posterior ocular pathologies. Ocular drugs can be administered by intravenous injection, although its use is less frequent than oral administration route [132].

2.3.2. Pharmacokinetics

Absorption to Ocular Tissues

Systemically administered drugs can easily reach the choroid due to the high vascularization of this tissue, as more than 85% of the ocular blood flow takes place in this layer, with a value of 43 mL/h [133], and choriocapillaris fenestrations [5,130].

Drug transport from blood circulation to the ocular cavity is strictly regulated by two anatomical and physiological barriers: The BAB and the BRB [8,134]. These barriers limit drug bioavailability by restricting its intercellular permeation to the anterior and posterior segment of the eye. This barrier effect is mainly due to the presence of highly complex tight junctions among epithelial and endothelial cells, which are located on the interface between blood flow and intraocular tissue. Moreover, it was observed that the drug passage improves during inflammatory conditions [135]. This is due to an increase in vascular permeability that leads to greater extravasation of components from the bloodstream to extravascular tissue [136].

Drug entry into posterior ocular tissues is mainly governed by the BRB. Thus, the relationship between drug permeability and physicochemical factors has been demonstrated, concluding that drug permeability increases with decreasing molecular weight and/or protein binding but improves with increasing lipophilicity [127]. As a result, small and lipophilic compounds can easily cross eye barriers (RPE, non-pigmented internal ciliary epithelium and retinal blood vessels), while hydrophilic and large compounds penetration is restricted [133]. However, hydrophobicity and high molecular weight tend to increase drug's half-life time in the posterior segment of the eye [137]. Therefore, RPE presents a selective permeability to highly hydrophobic drugs, whereas the penetration of hydrophilic and/or large substances being much more limited.

The type (influx or output flow) and/or location (vitreous or blood side) of the ocular transporters also condition the drug absorption and its concentration in the intraocular cavity [138].

Recently, many drug efflux pumps were identified in the ocular barriers. Among transporters with the greatest influence on the drug´s arrival to the posterior segment of the eye are the efflux transporters, these being a part of the ATP-binding cassette (ABC) protein family located in the RPE [139]. Specifically, two efflux pumps related to the development of chemoresistance were described, these being: P-glycoprotein (ABCB1) and the multidrug-resistance associated protein (MRP1) (ABCC1).

P-glycoprotein is an efflux protein located in the iris, cornea and ciliary muscle, as well as in conjunctival epithelium, non-pigmented ciliary epithelium and RPE. It is a 170 KDa membrane protein that is expressed on the apical surface of the aforementioned cells [139]. It actively promotes drug molecules' exit from retinal endothelial cells, reducing drugs accumulation within them [139].

For its part, MRP1 is a 190 KDa efflux protein encoded by the MRP1 gene on chromosome 16p13.1 and bounded to the choroidal side of the retinal barrier. It is an atypical ABC transporter with two cytosolic nucleotide-binding domains (NBD) and seventeen membrane-spanning domains (MSD) [140]. It functions as a multispecific organic anion transporter, mediating an ATP-dependent transport of drugs and other xenobiotics [141]. In any case, molecular mechanisms of these drug transporters are not completely known.

Distribution to Ocular Tissues

In general, systemically administered drugs reach the target tissues from the blood. Drug plasma transport is protein-binding dependent, giving rise to 1) free drug fraction, which is active and susceptible of excretion/metabolism and 2) protein-bound fraction that acts as a drug inactive reservoir. Only the free drug fraction can cross biological membranes and, consequently, reach the ocular tissues.

Specifically, drug distribution through blood–ocular barriers is a key factor in the achievement of an effective ocular treatment with systemically administered drugs. Before mentioned ocular barriers regulate drug transfer between blood circulation and the eye in both directions. Drugs whose transport is predominantly carried out by passive diffusion, distribution and clearance are independent of the drug transport direction, although mediated permeation by transporters could lead to a drug preferred transport direction (inward or outward) [3,128].

In any case, it must be taken into account that, although the eye barriers structure and main permeability trends have been known for some time, the distribution process from the blood circulation to the posterior segment of the eye is not yet fully elucidated. However, pharmacokinetic simulation models have been created for the drug concentrations prediction in the vitreous humor, depending on the free drug concentration and the blood flow between the general circulation and the posterior segment of the eye [133].

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Once drugs reach the vitreous humor, their distribution and elimination follow the same pattern as the followed after an intravitreal administration. The

parameters that affect the drug pharmacokinetics within vitreous humor have been discussed in the section of intravitreal administration.

1. Proteins and Biological Binding

The free drug may accumulate more than expected in any posterior ocular layers if it binds to cellular components or if it acts as a substrate for a significant active transport process. Specifically, drugs binding to ocular tissues' proteins and pigments, mainly melanin, significantly affects their transport to the posterior segment of the eye [6,130,142,143].

Melanin is a polyanionic biological pigment located in the uvea and the RPE in the ocular tissues as melanosomes or pigment granules, which are melanoprotein complexes where melanin is bound to a protein matrix [142]. Menon et al. determined the existence of 6–8 mg melanin at the ocular level [6,144]. Drug binding to melanin and proteins in ocular tissues is an important factor in drugs administration by systemic route since they can modify the drug bioavailability in the target site and, consequently, reduce its pharmacological activity [5,145].

Specifically, ocular melanin has a significant influence on drug pharmacokinetics and permeation through the retina [5,146] due to its capacity to bind (mainly reversible binding) free radicals and chemicals, especially basic (pKa values above 7) and lipophilic drugs by electrostatic, charge transfer, and van der Waals forces [5,147]. Main pharmacological consequences of the drug-melanin complexes are drug accumulation and retinal toxicity, besides the fact that larger doses are needed to obtain a therapeutic response due to the bound-to-melanin drug inactivation (e.g., betaxolol, metoprolol, and phosphodiesther oligonucleotides) [142]. Nevertheless, this drug depot may act as a reservoir over a long time, prolonging drug effects [145,146].

Drug Elimination

Regarding drug elimination process by this route, it must be taken into account that the administered drug not only faces passive barriers but also active barriers, such as clearance through the choroidal blood flow, which is extremely high, and the number and size of the choriocapillaris fenestrations that make up this system [6,148], by presenting a 70–80 nm pore size and a number between 30–50 fenestrations/μm^2 [149,150]. In general, drug elimination from the posterior segment of the eye can be carried out by two different ways, anterior and/or posterior pathways [103], following the same pattern described for the intravitreal administration route.

2.3.3. Drug Delivery Systems

The design of ophthalmic drugs systemic administration forms is aimed at achieving therapeutic concentrations in the posterior segment of the eye without causing undesired side effects. Drug targeted administration to these tissues from the systemic circulation has only been studied in preclinical animal models. Studies were carried out based on qualitative investigations performance and assessed by microscopy, immunohistochemistry, and/or angiography techniques [3].

Some of the novel systemic targeting systems studied for drug transport through the choroid to the posterior segment of the eye include [137]: 20 nm gold nanoparticles [151], polyethylene glycol (PEG) conjugated immunoliposomes [103], PLGA nanoparticles or Visudyne®. Currently, none of them is commercialized except for Visudyne®, an intravenous administration formulation used in photodynamic therapy for age-related wet macular degradation treatment [151].

Even so, several strategies related to the design of advanced delivery systems are currently under study, such as: 1) Structurally modified drugs that can effectively avoid MRP1 efflux transporter to improve ocular penetration, 2) hydrophilic drug administration through advanced delivery systems directed by transporter/receiver (superficially marked systems with an ocular-receptor specific substrate and/or vehicles attached to substrates that show a high affinity for ocular tissues) [139], and 3) drug affinity improvement to BRB transporters, such as amino acid, oligopeptides or cation and anion transporters, in order to increase drug transport to the posterior segment of the eye [152].

2.3.4. Systemic Drugs for Posterior Segment Eye Diseases

Drug systemic administration is not the preferable route in the treatment of posterior ocular segment pathologies, although it is still useful in many cases, being an administration pathway carried out by different routes (e.g., oral, intravenous, intramuscular). Thus, some drugs are administered orally (see Table 4).

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Table 4. Summary of orally administered drugs for the treatment of posterior segment ocular diseases.

Pharmacologic Group	Drug	Pathology	Administration Route	Ref.
Analgesics	Paracetamol	Ocular trauma treatment-associated pain	Oral	[153]
	NSAIDs (Flurbiprofen, Ketorolac, Diclofenac, Bromfenac and Nepafenac)	Ocular trauma treatment-associated pain	Oral	[153]
Antibiotics	Doxycycline	Neovascularization	Oral	[110,154]
	Tetracycline	Ocular rosacea	Oral	[111,155]
	Erythromycin	Orbital cellulitis	Oral	[111]
	Minomycline	Ocular rosacea	Oral	[110]
Corticosteroids	Dexamethasone	Giant cell arteritis	Oral	[153]
Immunosuppressants	Cyclosporine	Idiopathy or related-to-Behçet's-disease uveitis	Oral	[156]
Carbonic anhydrase inhibitors	Acetazolamide (Diamox sequel®)	Glaucoma	Oral	[113, 157]
	Etoxolamide	Glaucoma	Oral	[114,158]

Apart from these, orally administration formulations with antineoplastic and antiviral agents have also been studied [103]. In relation to the latter one, it must be considered that the most frequent ocular posterior segment pathologies with viral etiology are associated with immunosuppression states (e.g., AIDS or transplants). These include: Cytomegalovirus retinitis, whose treatment is based on the antivirals intravenous administration (valganciclovir, ganciclovir, foscarnet, and/or cidofovir) as well as acute retinal necrosis and progressive external retinal necrosis, whose etiology is broad (varicella-zoster, herpes simplex, cytomegalovirus, or Epstein-Bar virus) and whose treatment is based on the acyclovir (very effective), ganciclovir or foscarnet administration [159].

Similarly, parenteral administration formulations have also been developed for the same purpose, including different intramuscular and intravenous preparations. These encompass: Parenteral antibodies for the uveitis treatment [3] and hydroxocobalamin intramuscular injections (B12 vitamin, Alpha Redisol) for the treatment of B12 vitamin deficiency states, as well as certain antibiotics (e.g., penicillin, gentamicin, ceftazidime, or amikacin) used in the subsequent ocular infection's treatment (uveitis, scleritis, and/or pseudoscleritis) (see Table 5).

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DE COMPOSTELA

Likewise, the antibiotic combinations administration by continuous perfusion for the serious-eye-diseases treatment, such as endophthalmitis, is quite frequent

[127,160]. As an example, ceftazidime is the best studied cephalosporine due to its activity spectrum against Gram-negative bacilli (including *Pseudomonas aeruginosa*). It is more frequently used as a first election treatment in this pathology, in monotherapy or combined with other antibiotics, since it allows reaching ocular concentrations in the order of 35.4 mg/l after intravenous administration of a 100 mg/kg antibiotic dose [161].

Table 5. Summary of systemically administered drugs for the treatment of posterior segment ocular diseases.

Pharmacologic Group	Drug	Pathology	Target	Route	Ref.
Antibodies	Secukinumab Tocilizumab Adalimumab	Uveitis	Inflammatory cytokines	Intravenous	[162,163]
	Ustekinumab			Subcutaneous	
	Abatacept		T-cell activation		
	Rituximab		B-cell targeting	Subcutaneous	
Vitamins	B12	Vitamin B12 Deficiency Optic Neuropathy	Folate receptor	Intramuscular	[164]
Antibiotics	Penicillin Gentamicin Ceftazidime Amikacin	Uveitis Scleritis Pseudoscleritis Endophtalmitis	Bacteria	Intravenous	[165–167]

In addition to these, different intravenous drug delivery strategies have been tested for drug arrival to the posterior segment of the eye [168]. Specifically, photodynamic therapy with verteporfin has been practiced, being a choroidal neovascularization effective treatment by stopping the neovascular membrane growth. Apart from this, ocular posterior segment diagnostic intravenous techniques have also been developed, being the most prominent the fluorescein digital angiography, an exploratory technique for the retinal vasculature visualization by means of the sodium fluorescein injection (vegetable origin orange dye).

In order to point out, eyes can be also exposed to systemic drugs (as a kind of side effects) and xenobiotics not intended for ophthalmic treatment, such as bisphosphonates (whose action mechanism is based on the bone-resorption inhibition, being used in the osteoporosis prevention and treatment), which can cause ocular inflammatory reactions (uveitis, neuritis, iritis, scleritis, or pseudoscleritis) [169]. Reformulation studies have also been carried out on these drugs in order to reduce their passage from the blood to the eye, making them more selective in order to decrease associated ocular side effects [169].

2.4. Periocular Administration

The periocular route has been considered as a promising drug administration route for the posterior ocular segment diseases treatment due to the high concentrations obtained with this kind of formulations' inoculation. This route allows the drug deposition on the scleral external surface and includes the following administration routes: subconjunctival, subTenon´s, retrobulbar, peribulbar and posterior juxtascleral. These pathways differ from one another in the location and/or injection direction in the proximity of the sclera.

2.4.1. Advantages and Limitations

Periocular injections are associated with greater adherence to treatment by patients compared to intravitreal injections [139] since it is considered a less invasive administration route and capable of providing a relatively great drug bioavailability in the posterior ocular segment [150,170].

The sclera provides a relatively large area for the drug absorption (approximately 17 cm^2) [171] compared to other ocular surfaces, like the cornea. Moreover, periocular administration takes advantage of the high scleral permeability. These two factors contribute to the potential effectiveness of the periocular administration compared to other ocular routes.

On the other hand, the main drawback is that the drug needs to diffuse from the site injection to the target site, with the possibility of drug losses.

2.4.2. Pharmacokinetics

In general, although this type of administration avoids the corneal-conjunctive barrier, the drug must cross several barriers to reach the target sites in the choroid, the RPE, or the neural retina [65,150]. To do this, it must overcome several dynamic, static, and metabolic barriers that limit drug access to the posterior segment of the eye. Two different types of barriers should be mentioned: 1) Physical barriers, which include sclera, choroid (its high blood flow can remove a significant fraction of drug before it can reach neural retina), and RPE, and 2) physiological barriers, as occurs with conjunctival, episcleral, and choroidal lymphatic flow [150], whose drug elimination ability is relatively fast [3,65,103].

In any case, episcleral blood and lymphatic flows are seen as the main limiting factors in drug periocular distribution, while anatomical barriers and choroidal blood flow are less important [116,150,170].

As a result, drug is removed into the systemic circulation, decreasing ocular bioavailability thereof. However, molecules that escape from the conjunctival vasculature can penetrate through the sclera and choroid to reach the neural retina and photoreceptor cells. In addition, permeability through sclera is lipophilicity-independent (e.g., inulin, methazolamide, pilocarpine, hydrocortisone) [172], unlike corneal and conjunctival layers, being dependent on molecular radio [4,42,137,148].

Drug reflux after periocular administration is the initial loss factor and contributes to its low bioavailability in the posterior segment of the eye [116,173]. It was demonstrated that the use of an adequate injection technique, volume and/or formulation type can improve drug bioavailability and ocular penetration [116,173–175].

Absorption

Drugs administered by periocular injections can reach its target site in the posterior segment of the eye through three different routes: Transscleral or direct penetration route, systemic circulation route, and anterior route [6,176]. In the anterior route, the drug diffuses directly through the sclera and the ciliary body, or indirectly through the lacrimal fluid and the cornea because of the conjunctival circulation reflux. In the systemic circulation route, the drug goes to systemic circulation through conjunctival, episcleral and/or choroidal vessels, where it is diluted, and then it returns to the eye by the blood flow. In the direct penetration route, drug reaches the vitreous humor through the underlying tissues; it represents the most important absorption route in terms of drug penetration and distribution to the posterior chamber of the eye.

In any case, it should be taken into consideration that the scleral permeability depends on the molecular radio, scleral hydration, and intraocular pressure [176,177] instead of molecular lipophilia [17]. Although the latter improves permeability through the RPE, it also increases drug loss from the choroidal and subconjunctival space to the bloodstream [3]. Regardless of the absorption route, only a small portion of the drug reaches the posterior segment of the eye [116,178] mainly due to the drug loss through periocular space, BRB, choroidal circulation, efflux transporters, and drug binding to ocular tissue proteins [116].

Elimination

Once the drug reached its target site, small molecules are rapidly removed from the administration site, presumably through conjunctival and episcleral blood and lymphatic flow [103,139,150], whereas larger molecules have much slower

elimination kinetics, around 10 times lower [150], so that their residence time is much greater.

2.4.3. Subconjunctival Route

Subconjunctival injection has been used to administer drugs in the anterior segment of the eye, achieving higher concentrations in the anterior chamber compared to the topical administration. However, this route has also been investigated as an alternative pathway to intravitreal injection for the drug administration of retinal diseases treatment [6] due to the fact that it is considered a less invasive technique, comparing it with the intravitreal administration route [116,179]. It also minimizes the side effects, mainly endophthalmitis, cataracts, and retinal damage [116].

The drug is injected under the conjunctival membrane that covers the sclera, avoiding the conjunctival epithelial barrier. In this way, direct access to the transscleral route is achieved [116], increasing its bioavailability in aqueous humor in comparison with the topical route, which presents the corneal barrier as an impediment.

Advantages and Limitations

Subconjunctival administration shows two effective types of sustained drug delivery to the retina, both the topical and intravitreal administration. Moreover, it is an alternative route in order to allow easy and better accessibility and reduce the side effects caused by the intravitreal injection procedure (e.g., intraocular pressure and cataracts) [3].

The injection volume with the same drug concentration could be higher in this route in comparison with the intravitreal injection [3], enabling a wide dose range. In addition, the enzyme absence in the injection area is an important advantage due to the low enzymatic drug degradation.

The availability of pharmacokinetic and pharmacodynamic data about this route is limited. However, the drug delivery to the back of the eye through this route is better compared with topical and systemic administration routes [150].

This pathway also has some limitations regarding the concentration that can cross to the retina. The elimination via systemic circulation and to the tear cause a reduced bioavailability. Nevertheless, the permeability is modified by the age, according to the patient get older, the permeation through the sclera is less prevented so the amount of drug that can reach the retina is higher.

Pharmacokinetics

The permeation speed through the sclera is drug size-dependent [17], where the small molecules movement is constant. Macromolecules permeation is slower [3,150,172,180], although up to 70 kDa size molecules can easily penetrate the sclera [17,33, 116,181–183]. Once the sclera is crossed, a rapid elimination caused by the choroidal blood flow takes place due to its large choriocapillaris fenestrations. Molecules that pass through this barrier faster and better are the small lipophilic ones compared to macromolecules [3,33,172,184]. However, small molecules are also quickly removed.

Likewise, lymphatic flow plays an important role in the drug elimination since conjunctival lymphatic vessels represent 50% of the surface [185]. Once drug crosses the sclera, it must break through the choroid and the RPE to reach the vitreous humor. For all these reasons, between 80–95% of small molecules drain into the systemic circulation [150]. Approximately, 10% of the drug is available for the anterior segment of the eye and only about 0.1% reaches the retina [3]. Thus, high initial drug concentrations are needed in order to achieve effective levels in the posterior segment due to the deficient bioavailability observed.

The injection volume is an important factor in the subconjunctivally administered drug pharmacokinetics as the drug reflux from the injection site is the initial loss factor that contributes to its low bioavailability [116,173]. Adequate volumes should be used in order to achieve high drug levels in the back of the eye. If large volumes are administered (over 200 μL), the reflux of drug solution out of the injection site increases and a greater drug permeation to the aqueous humor is obtained.

Drug Delivery Systems

Different techniques have been developed in order to increase the subconjunctival administered drug penetration through the sclera. One of these is the transscleral iontophoresis, an electrodynamic technique that may improve the periocular injections efficacy in some ophthalmic drugs (see Table 6), such as corticosteroids, antibiotics, NSAIDs, and immunosuppressants [186,187].

Nano- and microparticles are interesting sustained drug delivery systems for treating posterior segment diseases. These types of systems were tested, obtaining promising results for the VEGF inhibition with corticosteroids (budesonide and dexamethasone) and selective COX2 inhibitors (celecoxib) [188–192].

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Table 6. Summary of subconjunctival administered drugs for the treatment of posterior segment ocular diseases.

Pharmacologic Group	Drug	Pathology	Ref.
Antidiabetics	Insulin	Diabetic retinopathy	[193]
Chemotherapeutics	Carboplatin	Retinoblastoma	[194]
	Topotecan		
Folic acid analogues	Methotrexate	Granulomatous panuveitis	[195]
Corticosteroids	Dexamethasone	Uveitis	[196]

Thermo-responsive hydrogels are also systems that can be used to facilitate a sustained drug delivery. This pharmaceutical form turns into a gel when contacting with the injection area due to a temperature difference. These systems are employed as an alternative to the classic intravitreal route mainly because of its minimally invasive procedure. Subconjunctival placement of insulin-impregnated hydrogels has presented advantages over topical and intravitreal injection in DR treatment [197,198].

2.4.4. SubTenon's Route

SubTenon's route is one of the most promising routes to reach the eye posterior segment due to the possibility of obtaining better pharmacokinetic profiles of the administered drugs [116,130,199]. SubTenon's injection is placed in an avascular area between Tenon´s capsule and sclera, around the upper portion of the eye and into the belly of the superior rectus muscle [179,200]. It is divided into anterior and posterior segments at the insertions of extraocular muscles and their associated fasciae [179,201].

Advantages and Limitations

As a general idea, subconjunctival injections show a better drug efficacy in the anterior segment, while subTenon's injections lead to an increased penetration to the posterior segment of the eye [202,203]. Drug passage to systemic circulation is reduced and the contact time with the sclera is prolonged as a consequence of the pharmaceutical form injection. Nevertheless, drug elimination caused by the choroidal circulation can produce a shortened duration of action [130].

Besides that, it should bear in mind that Tenon´s capsule degeneration is age-related, which leads to an easier drug diffusion towards the retrobulbar cone. As a result, better drug levels are achieved in the posterior segment of the eye [130].

USC DE COMPOSTELA

Pharmacokinetics

A 2.5 cm long blunt-tipped cannula needle is usually employed in the injection procedure into the Tenon´s capsule after a surgical incision. This is a widely used technique for local anesthesia during ocular surgery because the cannula approach avoids sharp-needle complications [103,179]. The formulation volume is up to 4 mL and is injected around the muscle belly, behind the equator [179]. The possibility to inject a large volume into the subTenon's space causes a lower and slower clearance and a more readily transscleral drug delivery [199].

Some of the most employed applications by using subTenon's administration route are: corticosteroid injections (triamcinolone acetonide (TA)) for chronic posterior uveitis [204] and cystoid macular edema treatment (see Table 7) [205]. Comparing the intravitreal effects versus subTenon's TA administration for diabetic macular edema (DMA), results showed that intravitreal TA injection had greater clinical effects than the subTenon's injection [206,207]. Nevertheless, a lower incidence of serious complications has been shown with subTenon's injections than the intravitreal ones [206,208].

Table 7. Vitreous pharmacokinetic parameters for long-acting triamcinolone acetonide subTenon's injection.

Pharmacologic Group	Drug	Half-Life Time (days)	MRT (days)	C_{max} (ng/mL)	T_{max} (h)	Ref.
Corticosteroids	Triamcinolone acetonide	17.1	23.1	22	24	[209]

2.4.5. Retrobulbar Route

Retrobulbar administration arises as an alternative route to the drug targeting the posterior ocular pathologies treatment where the optic nerve or retrobulbar spaces are involved. Injection procedure is based on drug inoculation into the space between the orbital rim and the balloon edge [176], the retrobulbar space, which lies inside the extraocular muscle cone, behind the globe, being a deeper injection and uses less volume (comparing it with the peribulbar one). The usual procedure for the retrobulbar injection implies a 3 mm insertion beyond the posterior surface of the eyeball and the maximum volumes of injections are 3–4 mL.

Advantages and Limitations

Complications associated with retrobulbar injections, although potentially severe, are uncommon. Possible complications of retrobulbar injection include retrobulbar hemorrhage, globe perforation, optic nerve damage, respiratory

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arrest, optic atrophy, retinal vascular occlusion, and extraocular muscle myopathy [210]. This route has serious risks of causing ocular damage due to the proximity of the optic, motor and sensory nerves [151].

As retrobulbar injections are performed outside the ocular globe, there is little or no influence on intraocular pressure, in contrast to the intravitreal injections [179]. Compared to peribulbar injections, retrobulbar administration is a deeper injection and uses less volume.

Moreover, an injection up to 5 mL may be made, which is a considerably higher volume compared to the other types of administration to treat the ocular posterior segment diseases. This means that higher amounts of drug can be administered.

Retrobulbar administration is mainly used for corticosteroids administration as an alternative treatment to reduce the posterior segment inflammation. It is also used for local anesthesia injection in certain intraocular surgeries, where drugs are directly administered into the muscular cone, an area located among four eye muscles in the back of the globe.

2.4.6. Peribulbar Route

Peribulbar administration enables drug injection into the muscular extraconal compartment of the eye, also called peribulbar space, which is externally localized to the rectus muscles.

Advantages and Limitations

It is frequently preferred for its ocular complications' low rate, theoretical safety and ease of application comparing it with the retrobulbar route. Although both routes showed to be useful in anesthesia or akinesia, their use is limited due to the procedure associated complications (e.g., eyeball perforation, oculomotor reflex stimulation, optic nerve trauma, and orbital hemorrhage) [160]. Moreover, the peribulbar administration is less effective for anesthetizing the eye than the retrobulbar route, although a higher volume, up to 8–10 mL, may be injected [181]. However, this technique is slower and less efficient than retrobulbar injection, besides the fact that it requires multiple injections and larger volume of the injected formulation. It also carries a higher risk of potential chemosis and an intraocular pressure increase [211].

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Currently, the peribulbar approach is a widely used method for the local anesthesia administration. The injection procedure has been briefly described for the administration of lidocaine and bupivacaine mixture as a local anesthesia pre-

treatment in modern intraocular surgery [160]. A mixture of peribulbar bupivacaine combined with TA and cefazolin was also studied as a prophylactic treatment to reduce pain and possible inflammation and/or infection associated with vitreoretinal surgery [212].

2.4.7. Posterior Juxtascleral Route

The posterior juxtascleral injection, recently developed by Alcon Laboratories®, is an alternative administration route based on the therapeutic agent deposition directly in the closest area to the macula, without puncturing the eyeball [116]. It allows achieving higher drug concentrations in the target site due to the fact that the scleral thickness decreases near the equatorial region, an aspect that promotes a greater drug penetration towards the posterior segment of the eye [3,65]. This novel technique requires inserting a blunt-curved cannula after a conjunctival incision. The curved cannula follows the scleral surface without puncturing the ocular globe and drug is injected into the juxtascleral space overlying the macula. Besides, no clinically significant safety and efficacy issues related to this administration pathway were reported [213].

In addition, this route reaches a drug therapeutic dose for up to 6 months in the macular region [174], avoiding the risk of intraocular damage and reducing side effects, such as endophthalmitis, retinal detachment and glaucoma, among others [116,174]. Besides, drug reflux, which occurs when the drug flows back out of the incision made at the insertion site, may affect drug efficacy, although Alcon Laboratories® have developed a counter pressure device to deal with this side effect. There is an increased risk of systemic drug exposure due to the drug contact with the orbital tissue when compared with intravitreal injections, though there is much less systemic exposure than occurs with either topical or systemic therapy. Currently, there are few commercial formulations which are administrated by using this administration route, such as anecortave acetate (Retaane®) [214], a cortisol synthetic analogue used for AMD treatment and prevention, or TA for DMA treatment unresponsive to laser photocoagulation [213].

2.4.8. Suprachoroidal Route

Suprachoroidal injection was introduced as a potential drug administration route to the posterior segment of the eye although it is not clinically used at present [150]. Drug is injected into the suprachoroidal space below the sclera inner surface using microneedles (micron-dimensions needles that can infuse fluid into tissue with excellent spatial targeting), up to 50 μl maximum volume.

Formulation is distributed throughout the suprachoroidal space as a consequence of the pressure exerted by the injection process itself [215].

The suprachoroidal space circumferentially goes around the eye and is located between the sclera and choroid, leading to reach the macular area [216,217]. This administration route was tested for drug delivery into the posterior segment of the eye employing surgical procedures (by introducing catheters into this space) and gave rise to a long-term therapy, where drugs could be targeted towards the choroid and retina by direct contact with the administration site [218,219].

Advantages and Limitations

Suprachoroidal delivery route theoretically presents several key advantages compared to the standard intravitreal injections [220], such as that this space does not interfere with the optical pathways and it is a good pathway to target diseases at the outer retina, photoreceptors, RPE and choroid. Moreover, its diffusional pathways and pharmacokinetics are completely different from the intravitreal one, being a possible resourceful way to target drugs to the posterior segment of the eye. In comparison to other periocular administration routes, drug diffusion from the suprachoroidal space avoids several ocular barriers (e.g., cornea, conjunctiva and sclera).

Additionally, the suprachoroidal space could act as a potential reservoir inside the eye and sustained release formulations or devices could optimize diffusional kinetics from this space. Finally, this space provides a safer way with larger biologic and/or immunogenic agents [220]. There are also disadvantages involved, such as suprachoroidal hemorrhage and choroidal detachment [221].

However, the access to the suprachoroidal space is complicated and the methods used were usually invasive and too complex to be performed as a simple office procedure [218,219]. Pre- and post-injection histology demonstrated that the potential space of the suprachoroidal region returns to a normal configuration after a brief time period [220]. Also, using die-casting methods, the suprachoroidal space is rather extensive and has the capacity to expand and accommodate a relatively large volume of material [220].

Delivery device parameters' optimization showed that microneedle length, pressure and particle size played an important role in determining a successful delivery into the suprachoroidal space. 800–1000 μm needle lengths, and 250–300 kPa applied pressures provided the most reliable delivery [215]. The usual injection volume for the suprachoroidal administration has been determined to be 25 μl [220].

Pharmacokinetics

Suprachoroidal administration leads to greater vitreous bioavailability for small lipophilic (1.5%) and hydrophilic (0.19%) molecules, while macromolecules (4.2%) show a 6–23 times improvement in terms of ocular bioavailability compared to the drug administration by subconjunctival route [150]. Compared with small molecules, macromolecules have a much longer half-life time in ocular tissues where steady state is slowly reached. Drug levels in the vitreous humor are generally reached 15 h for small lipophilic drugs, 70 h later for small hydrophilic drugs and 500 h later for larger molecules. Therefore, it was seen steady-state drug levels are quickly reached for small molecules, becoming 100–1000 times slower for macromolecules [150].

Several studies showed a significant fraction of the drug administered by the suprachoroidal route was removed through conjunctival and regional lymphatic nodes, including those of high molecular weight [6,175]. In addition, it should be taken into account that choroidal blood flow also removes a huge part of the inoculated drug (96–99%) in the case of low molecular weight compounds, whereas high molecular weight drugs will be removed from the tissue almost equally through the choriocapillaris (54%) and the subconjunctival space (41%) [150].

Drug Delivery Systems

Currently, there are no pharmaceutical forms commercialized to be administered by suprachoroidal injections. Nevertheless, this type of administration was widely studied by using fluorescein and fluorescently tagged dextrans (40 and 250 kDa), bevacizumab and polymeric particles (20 nm to 10 μm in diameter). Sulforhodamine B microneedle injection was also studied as well as nanoparticle and microparticle suspensions into the suprachoroidal space [215,222]. TA formulations were also researched as a way of DMA alternative treatment, as well as ranibizumab and bevacizumab injections, although recent studies showed these large biologic proteins are quickly removed from the suprachoroidal space [220].

2.4.9. Subretinal Route

Drug subretinal route has emerged as an alternative administration route to intravitreal administration due to its side effects and lower adherence to treatment by patients derived from the latter [223,224]. Thus, subretinal administration involves drug inoculation into the subretinal space [224], an ocular space located between RPE cells and photoreceptors [224,225].

Advantages and Limitations

Subretinal injection is an especially useful route for the posterior ocular pathologies treatment by providing a direct route with a very precise location through a minimally invasive injection. A typical volume of around 150 µl is injected, leading to a transient detachment between these two layers [2]. A lower drug dose is needed to accurately reach subretinal-space cells.

Compared to intravitreal administration, subretinal injection has a direct effect on subretinal space cells. Unlike vitreous cavity, subretinal space is an isolated closed anatomical area, which also has a greater immunological defense mechanism providing a safer route of administration in case of entry of bacteria [178,224].

Basically, three subretinal injection techniques have been studied, these being: 1) a transcorneal route through the pupil, surrounding the lens, and passing the vitreous humor and the retina [226], 2) a transscleral route through pars plana or limbus, crossing the vitreous humor and the opposite side of the retina into the subretinal space [227], and 3) a transscleral route through the choroid and Bruch's membrane without penetrating the retina [224]. All routes were equally effective regardless of the chosen one. The administration procedure is performed under direct visualization by using a surgical microscope and where blister formation should be observed as a sign of the procedure success.

Drug Delivery Systems

Subretinal administration has been considered as one of the best strategies for gene therapy using viral vectors, a carried-out treatment effectively achieved for pigmentary retinitis (PR) and Leber's congenital amaurosis (LCA) [225,227]. Gene expression is however limited to the injection site, suggesting that the primary barrier for efficient therapy following subretinal injection is the retina itself [2]. In addition, it was reported macrophages subretinal injection leads to pathological fibrosis, which could be used for the advanced AMD evaluation [224]. Subretinal delivery can also be used for stem cell transplantation in ocular degenerative diseases, which was reported *in vivo* studies and aimed at clinical applications [224].

3. CONCLUSIONS

The eye is one of the most inaccessible organs in terms of obtaining therapeutic drug concentrations, especially in the treatment of posterior segment ocular pathologies. Conventional administration pathways, such as topical or systemic

routes, usually show important limitations, either by low ocular penetration or by the appearance of side effects linked to the posology, among others.

New drug delivery systems (DDS) are needed to prolong the administration intervals for posterior segment ocular pathologies, even though the development of novel DDS is particularly complicated due to several aspects must be considered, such as pharmacokinetics, immunogenicity, biodegradation, tolerability and toxicity, among others. Apart from these, different technological requirements must be taken into account, including reproducible manufacturing, clinical performance and sterility.

In the last few decades, an exponential increase in the design and development of novel DDS intended for the treatment of posterior segment ocular pathologies was observed. Biodegradable and non-biodegradable implants, microparticles, nanoparticles, microneedles, and microelectromechanical systems are the most innovative ones. Unfortunately, knowledge about drug targeting to the posterior segment of the eye is still sparse and, thus, there is not many DDS in clinical trials. Even so, memantine ophthalmic formulations as neuroprotective are in III phase studies at the moment, inter alia.

Likewise, huge advances were made in terms of research and development of new alternative administration routes to the posterior segment of the eye, including intravitreal and periocular pathways as the most relevant ones, as well as their subtypes. All of them, including conventional administration routes (topical and systemic) show a series of specific pharmacokinetic characteristics, making them useful for the treatment of certain posterior segment ocular pathologies. However, these pathways have shown several advantages and limitations, where the election of one or another is dependent on, not just the pathology itself, but the pharmaceutical form, the drug used and the patient´s adherence to treatment, among others.

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CHAPTER 2

PRECLINICAL CHARACTERIZATION AND CLINICAL EVALUATION OF TACROLIMUS EYE DROPS

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CHAPTER 2

PRECLINICAL CHARACTERIZATION AND CLINICAL EVALUATION OF TACROLIMUS EYE DROPS

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 (BSS, PVA and Hyaluronic Acid). For this, we have performed *in vitro* (stability studies) and *in vivo* assays (corneal permanence time measured directly and 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 ocular permanence 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, ocular permanence, safety and clinical effectiveness studies, we concluded that tacrolimus-PVA eye drops are a suitable candidate for its clinical application in inflammatory ophthalmology diseases.

Keywords: corneal residence time; cytotoxicity; effectiveness; eye drops; stability; tacrolimus

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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 [1,2]. 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 [3], or other complex ocular diseases as uveitis [4]. 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 [5].

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 [6]. 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 [7]. The second line of treatment is based on topical immunosuppressive drugs as cyclosporine and tacrolimus. Cyclosporine and tacrolimus are not structurally related; however, their mechanisms of action are similar. These drugs bind to 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 TNFα, all of which cause the reduction of T-cell activation [8]. Its mechanism of action has promoted its use in pathologies such as corneal graft rejection [9,10], inflammatory conjunctival and corneal diseases [11,12], uveitis [13,14] or graft-versus-host disease [15].

The use of ophthalmic cyclosporine has been described for several decades, yet only a few products have been successful in reaching the pharmaceutical marketplace. 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 [16,17]. 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 [18,19]. Several types of tacrolimus formulations as ointments [20,21], emulsions [22], liposomes [23], dextran conjugates [24] or cyclodextrins complexation [25] 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 [26]. The increase of patient's compliance and the establishment of appropriate dosing schedules are key factors for improving the treatment of many ophthalmic pathologies [27]. 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 and 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 milliliters 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 x106 - 2.0x106 Da) dissolved in BSS® to obtain a mucoadhesive hyaluronic acid hydrogel at 0.4%.

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 of 100 μL from each formulation and from each preservation condition was withdrawn with a micropipette 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 (4,6x50 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 minutes.

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 minutes before quantification. Each sample was assayed in triplicate. The analytical method was validated according to International Conference on Harmonization guideline recommendations [28].

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 48h, 15 days and 10 days respectively. At the end of each incubation period, microbiological growth was observed and determined.

2.2.3. Statistical analysis

The margins set as indicated in Pharmaceutical Codex [29] 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±1ºC) and humidity (60±5%) and with day-night cycles regulated by artificial light (12/12 hours). The animals were treated according to the guidelines for laboratory animals [30,31]. 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 [32]. 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. 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 mono-exponential decay equation using a single compartmental model was performed using pKSolver [33]. 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

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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 [34,35]. Briefly, Cell index (CI) [36] 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 hours**.** 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) [37]**.** NCI is defined by the following equation: $NCI = CI_{(t)} / CI_{(t \text{ of the dose})}$; in which $CI_{(t)}$ is CI at time 't', and $CI_{(t \text{ of the dose})}$ 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 [38].

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 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 [39]. 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%) [40]. 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 [41]. 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.

Table 1. Grading scales for objective clinical signs clinical signs. *Elevated papillae in ½ or more of the upper palpebral conjunctiva.

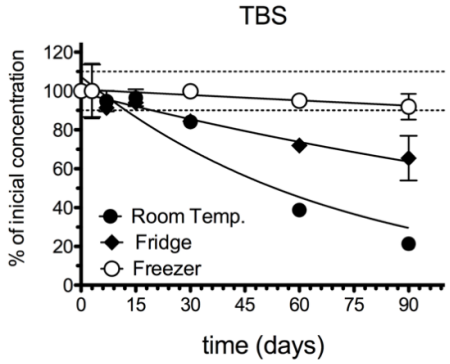
Signs	Score	Definition
Conjunctiva	3	Important dilatation of vessels
	2	Moderate dilatation of vessels
	1	Mild dilatation vessels
	0	None
Papillae	3	Giant (>1 mm and elevated*)
	2	Moderate (0.5-0.9 mm and flats
	1	Mild (0.1-0.4 mm and flats)
	0	None
Limbus Trantas´dots	3	9 or more dots
	2	5-8 dots
	1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

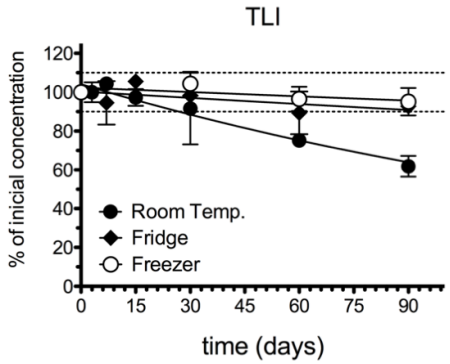
Safety was assessed from ocular findings, visual acuity, adverse events and laboratory test results. All adverse events observed by the ophthalmologist or reported by patients during the six months of treatment were recorded. For laboratory tests, blood samples were obtained from a cubital vein after 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 [42].

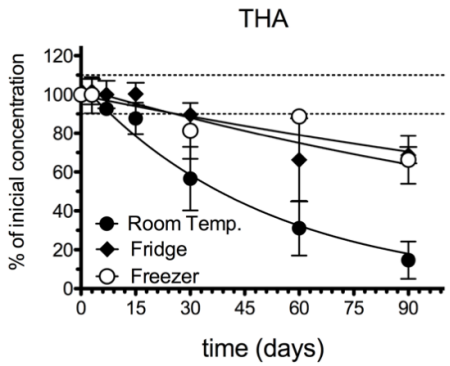
3. RESULTS

3.1. Stability assay

Figure 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.







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Figure 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

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 permanence time on the ocular surface

The ocular residence time of the tacrolimus formulations was calculated by using small-animal PET imaging in rats. Figure 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.

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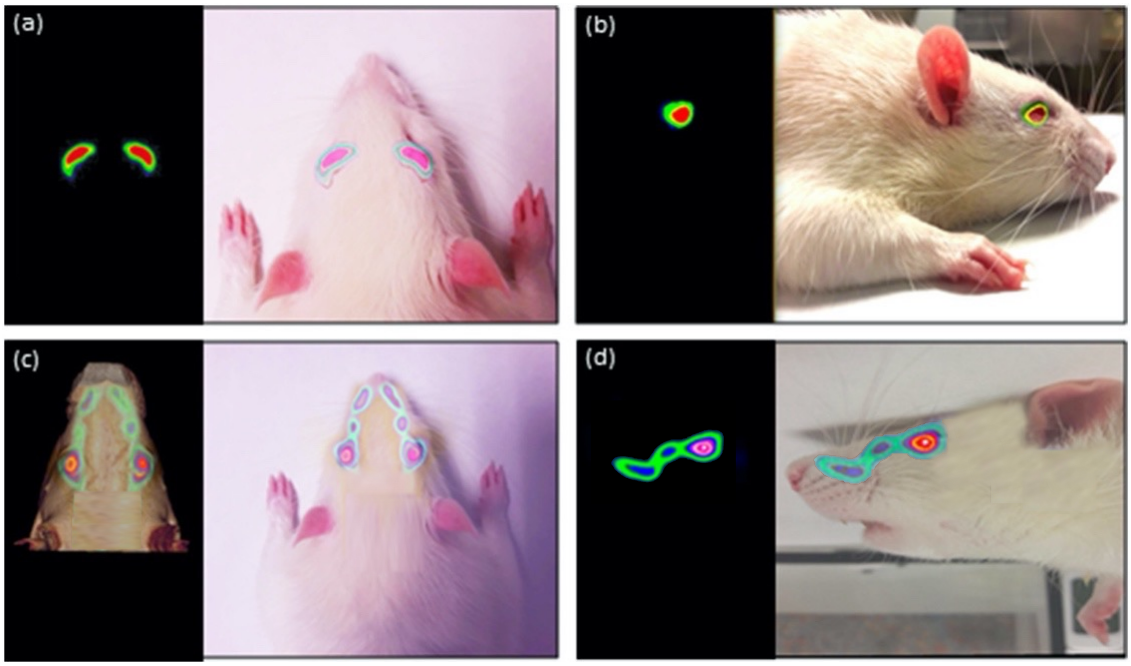


Figure 2. Fusion PET-CT-Real images of the rat's head in which the formulation remains on the ocular surface after instillation [(a) and (b)] subsequently observed as it is eliminated by the nasolacrimal ducts [(c) and (d)]. Sagittal (a) and axial (b) PET image and fusion PET-Real images of the rat 10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

In Figure 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 α <0.05). Data were fitted to a mono exponential model dependent on time and the pharmacokinetic parameters obtained by the fitting to the model are shown in Table 3. We obtained an average half-life time and a mean residence time of 77.1 and 111.3 minutes in the case of the TLI. The values for THA are 51.6 / 74.7 minutes and 20.7 / 29.9 minutes for TBS. One-way ANOVA for AUC and MRT gives significant differences between TLI and TBS (α<0.05).

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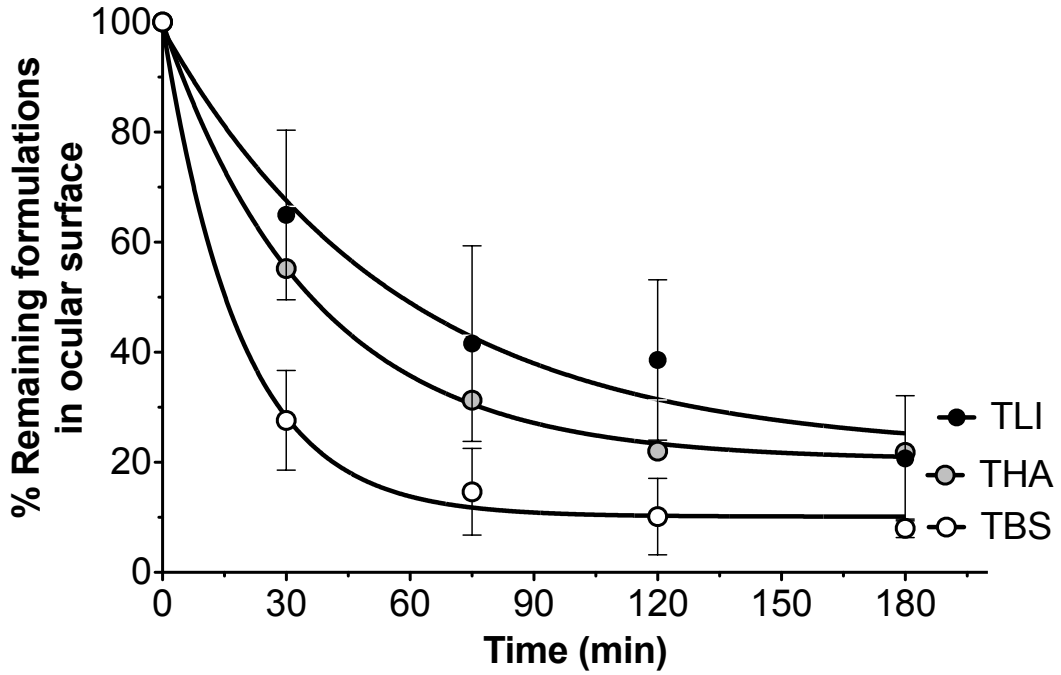


Figure 3. Clearance rate of the formulations (TLI, THA, TBS) from the ocular surface determined by PET.

Table 3. Parameters obtained by the fitting of the % formulation remaining on ocular surface obtained by PET imaging. *Determination coefficient of the fitting to mono-exponential decay equation.

Formulation	$t_{1/2}$ (min)	R^{2*}	AUC_0^{∞} (mg/L·min)*	MRT (min)*
TLI	77.1 ± 31.4	0.9782	10720.7 ± 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

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 (Figure 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 hours and 1.19 μg/ml (OCT exceeds 252 times this value) at 15 hours. 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

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values of 6.44 µg/ml from 4 hours (OCT exceed 77 times this value) and 6.67 µg/ml from 15 hours (OCT exceed 74 times this value).

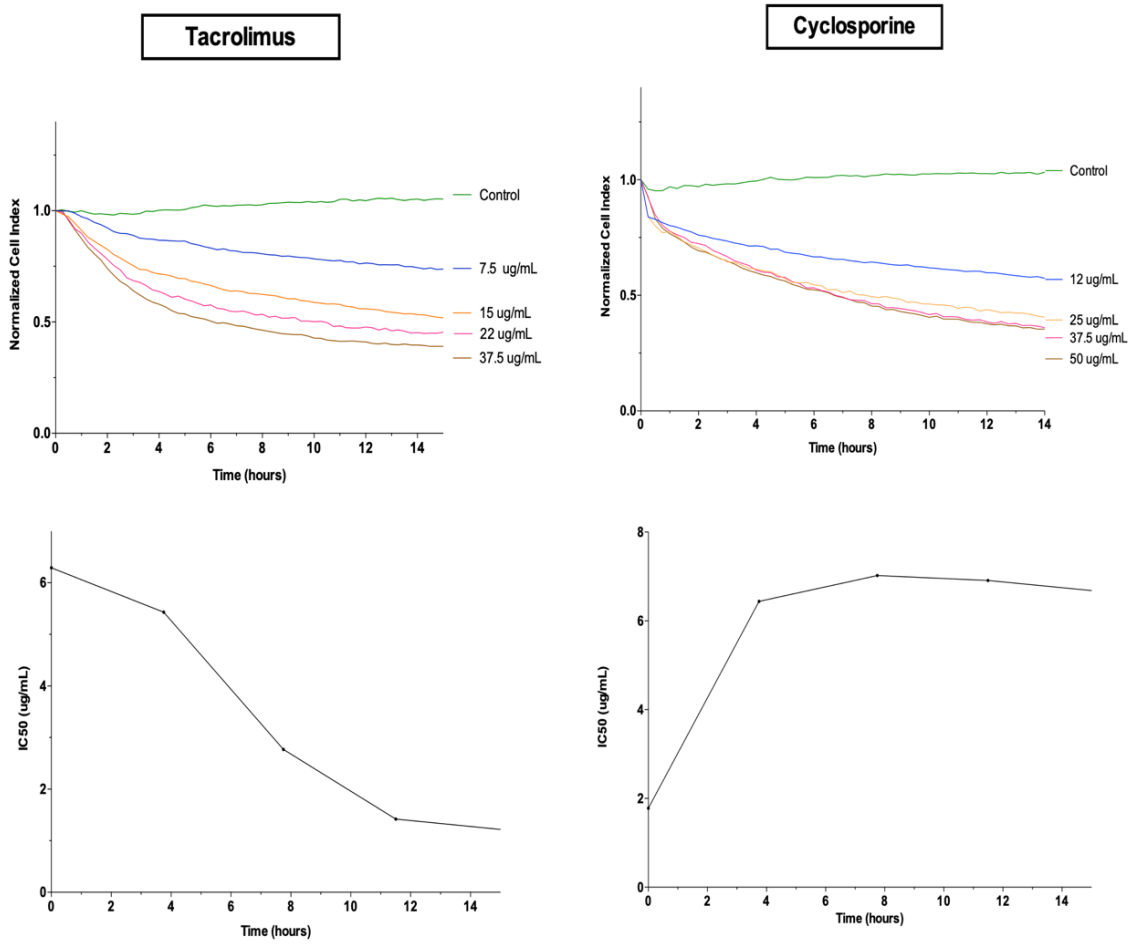


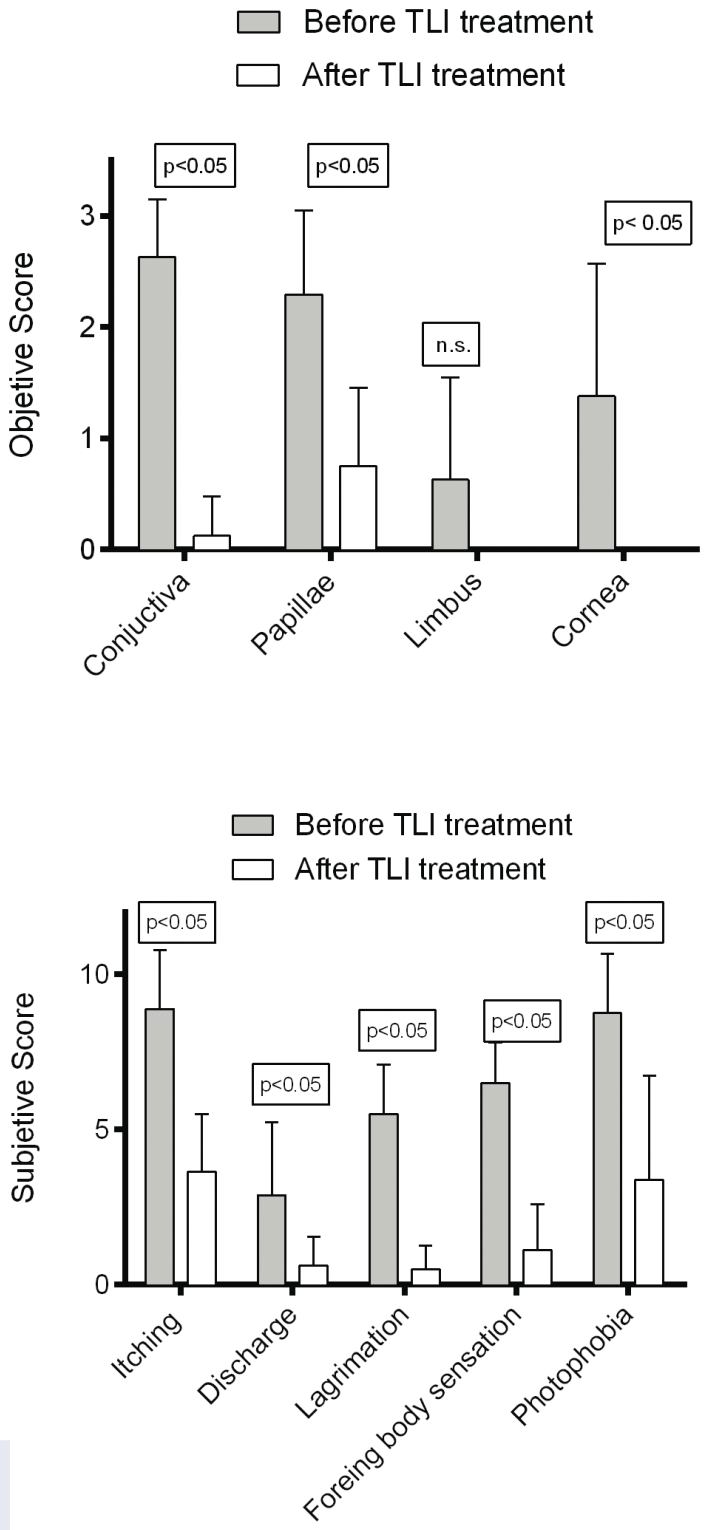
Figure 4. Cytotoxicity kinetics and evolution of the CI50 over time of tacrolimus, TLI formulation (left) and cyclosporine eye drops, Restasis® (right).

These results indicate that at initial and intermediate times (< 4 hours), 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.

3.3. 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. Figure 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 Figure 5 it is possible to observe the changes in each one of the parameters studied.



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Figure 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.

Figure 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 shows mild eye itch after the instillation of TLI with a mean score of 3 ± 1.8.

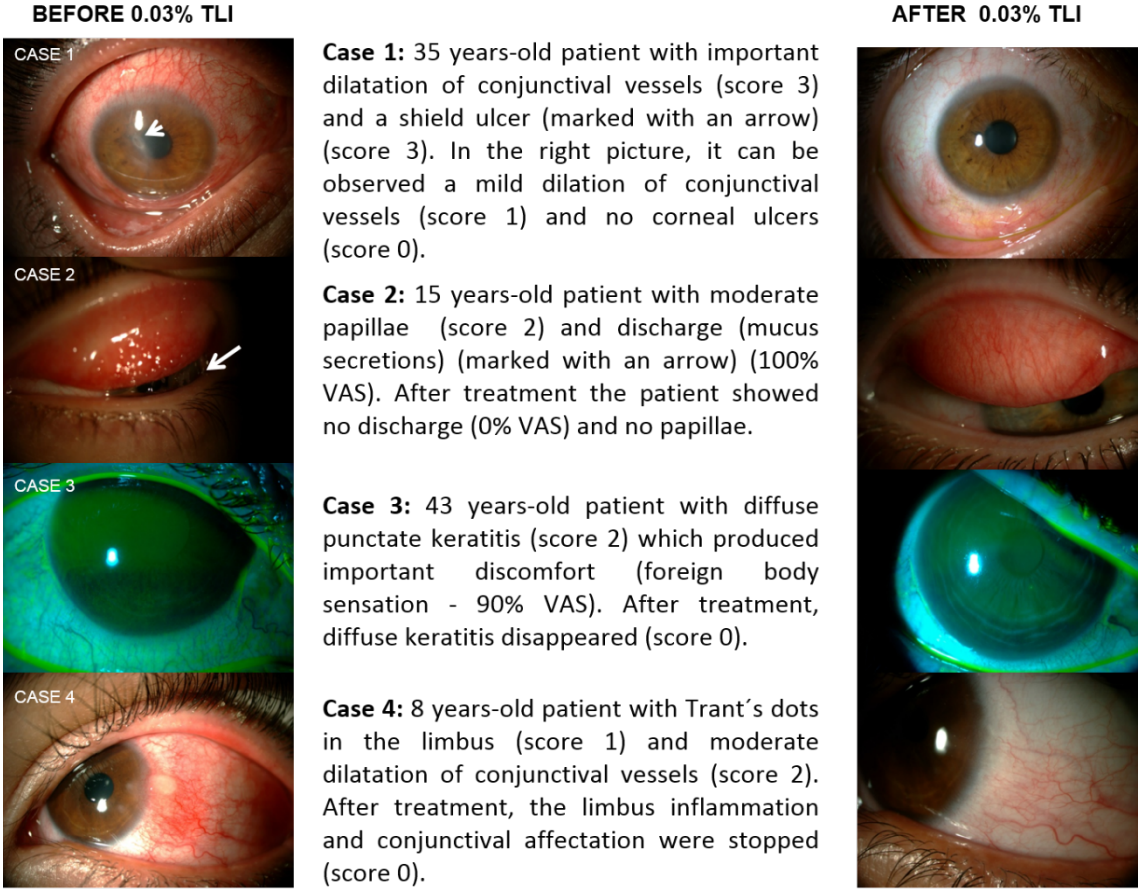


Figure 6. Representative images of some patients before and after treatment with TLI.

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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

4. DISCUSSION

The development of new ophthalmic topical vehicles for increasing the permanence of drugs on the ocular surface is important to improve adherence [43]. Therefore, new methodologies are required to provide precise information on the residence time of a formulation on the ocular surface [44]. 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 [45,46]. We have used a novel methodology based on PET technology to determine the permanence 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 permanence assays show that both vehicles are mucoadhesive 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) [47].

Hyaluronic acid has been shown to be a useful excipient to increase the permanence of many eye drops and artificial tears [48], however, in our case the permanence is greater with polyvinyl alcohol. This fact has already been observed by other authors, where the combination of tacrolimus with hyaluronic decreases permanence [49]. 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 [50,51][51]. In order to determine the concentration of active principle over time and in different preservation conditions we have adapted an HPLC method previously described by other authors [52]. 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 similar to those described by other authors [53]. Once determined that TLI is the formulation with greater permanence and more stable, its innocuity and clinical effectiveness were assessed.

One of the advantages of RTCA is the determination of IC50 in real-time [54]. These measurements allow understanding the dynamic toxicity behavior and the range of exposure time [55]. As we have observed from the ocular permanence 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 [56]. We have observed that TLI in contact with the cells for less than 4 hours 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 [57], benzalkonium chloride [58] and ethanol [59]; and Restasis®, contains cytotoxic excipients as castor oil [60] or polysorbate [61]. 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 [62–64].

Nowadays, tacrolimus concentrations used in clinical practice are very variable (range from 0.01% to 0.1%) [21,39,64–66] and usually the concentration used is lower than in cyclosporine eye drops [16,67,68]. Probably the lower concentration of tacrolimus used is because this drug is 50-100 times more potent than cyclosporine [69–71]. 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 [72], 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 signaling, tacrolimus appears to affect dendritic cell function by interfering with MHC class II-restricted antigen presentation after binding to another FKBP [8]. Concretely, tacrolimus blocks the early phase of T-cell activation, thus inhibiting both T-lymphocyte signal transduction and IL-2 transcription. It is also inhibits the release of histamine from mast cells and affects prostaglandin synthesis [73,74][74].

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 [75–79].

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, permanence, safety studies and clinical effectiveness, TLI is a suitable candidate for clinical application in inflammatory ophthalmology diseases.

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CHAPTER 3

DEVELOPMENT AND CHARACTERIZATION OF A TACROLIMUS/HYDROXYPROPYL-β-CYCLODEXTRIN EYE DROP

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CHAPTER 3

DEVELOPMENT AND CHARACTERIZATION OF A TACROLIMUS/HYDROXYPROPYL-β-CYCLODEXTRIN EYE DROP

ABSTRACT

Uveitis is a vision inflammatory disorder with a high prevalence in developing countries. Currently, marketed treatments remain limited and reformulation is usually performed to obtain a tacrolimus eye drop as a therapeutic alternative in corticosteroid-refractory eye disease. The aim of this work was to develop a mucoadhesive, non-toxic and stable topical ophthalmic formulation that can be safely prepared in hospital pharmacy departments. Four different ophthalmic formulations were prepared based on the tacrolimus/hydroxypropyl-β-cyclodextrin (HPβCD) inclusion complexes' formation. Phase solubility diagrams, Nuclear Magnetic Resonance (NMR) and molecular modeling studies showed the formation of 1:1 and 1:2 tacrolimus/HPβCD inclusion complexes, being possible to obtain a 0.02% (*w*/*v*) tacrolimus concentration by using 40% (*w*/*v*) HPβCD aqueous solutions. Formulations also showed good ophthalmic properties in terms of pH, osmolality and safety. Stability studies proved these formulations to be stable for at least 3 months in refrigeration. *Ex vivo* bioadhesion and *in vivo* ocular permanence showed good mucoadhesive properties with higher ocular permanence compared to the reference pharmacy compounding used in clinical settings ($t_{1/2}$ of 86.2 min for the eyedrop elaborated with 40% (*w*/*v*) HPβCD and Liquifilm® versus 46.3 min for the reference formulation). Thus, these novel eye drops present high potential as a safe alternative for uveitis treatment, as well as a versatile composition to include new drugs intended for topical ophthalmic administration.

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Keywords: tacrolimus; hydroxypropyl-β-cyclodextrin; topical ophthalmic administration; eye drops; uveitis; PET/CT imaging

1. INTRODUCTION

Uveitis is a sight-threatening inflammatory disorder that affects a wide range of ages in the world population, being the main cause of 5–20% of blindness in Europe and United States and over 25% in developing countries [1,2]. This affection can lead to other types of complications, including cataract, increased intraocular pressure (IOP), macular edema (ME) or glaucoma, compromising visual loss [3–5]. Therefore, it causes a great clinical and socioeconomic impact on the life quality of patients [6–8] and early diagnosis and treatment are important to prevent complications.

Uveitis is a recurrent and common ophthalmic disease that has an idiopathic etiology, caused by a complication of an infection or associated with a systemic disease (infectious or autoimmune disorder) [3,9]. The pathogenesis of uveitis is often caused by an autoimmune response. Inflammatory cytokines promote the activation of T cells and trigger recruitment of large numbers of circulation inflammatory leukocytes into the eye. This process may cause irreversible tissue damage and visual impairment. Topical corticosteroids constitute the first therapeutic line to treat the disease, but remarkable adverse effects can appear due to continuous treatment with these drugs. The use of immunosuppressants in uveitis is indicated in corticosteroid-refractory eye disease or after systemic side effects' appearance.

Tacrolimus is a macrolide with a high molecular weight (804.02 g/mol) isolated from *Streptomyces tsukubaensis*, with a great immunosuppressive activity (100 times more potent than cyclosporine A) [10,11] that inhibits T cell proliferation and suppresses the release of inflammatory cytokines; it can theoretically be used to reduce inflammatory activity in uveitis patients [3]. This drug has been used in different ocular diseases including corneal graft rejection [12–14], vernal keratoconjunctivitis (VKC) [15–17], dry eye [11,18], uveitis [5,19,20], scleritis [21,22] or graft-versus-host disease [23–25], among others. Additionally, clinical studies have shown tacrolimus' high effectivity compared to other immunosuppressants such as cyclosporine, at lower concentrations [26].

Nowadays, tacrolimus eye drops are not marketed and all its use rests in the elaboration in hospital pharmacy departments (HPDs). The preparation of ophthalmic tacrolimus formulations is limited by its poor water solubility (1–12 μg/mL) [27,28] due to the hydrolytic mechanism [29]. For this reason, 0.03% (*w*/*v*) tacrolimus eye drops are being obtained by reformulation from intravenous drug presentation (Prograf®) [15]. However, in this formulation, tacrolimus is solubilized in ethanol, containing irritating compounds that usually cause discomfort and unpleasantness to the patient. Based on these statements, it would be interesting to design new topical ophthalmic formulations with the

lowest possible toxic potential and better tolerability. Several types of tacrolimus formulations such as niosomes [30], nanoemulsions [5], microspheres [31], nanocapsules [32], micelles [33], emulsions [34] or liposomes [35] have also been described by other authors. Nonetheless, if these are not synthesized to be marketed, their elaboration in HPDs will be complicated.

The need to increase the solubility and stability of the drug becomes a task of extreme necessity. There are several types of β-cyclodextrin (βCD) derivatives, although most of them have not been authorized or there are not enough preclinical studies to support their use at the topical ophthalmic level [36]. Some β-cyclodextrins have been used by other authors as complexing agents with several drugs [27,37,38]. The oligosaccharide 2-hydroxypropyl-β-cyclodextrin (HPβCD) is a cyclic oligosaccharide formed by seven units of α-1,4-linked glucose and a hydroxypropylated group, with a lipophilic central cavity and a hydrophilic outer surface. The rigid doughnut-shaped structure makes it a natural complexing agent, being able to form inclusion complexes with several drugs, where their structure (or part of it) may fit in the cyclodextrin cavity [39]. The complexation between drugs and cyclodextrin affects many drugs' physicochemical properties, including their chemical stability and aqueous solubility [39]. Therefore, this hydrophilic cyclodextrin derivative can form highly water-soluble complexes with lipophilic drugs, as it happens with tacrolimus. There are few reports on the use of cyclodextrins to improve the pharmaceutical characteristics of tacrolimus [37]. However, HPβCD has been chosen in this work due to the fact that, according to the European Medicines Agency (EMA), it is the safest and most appropriated cyclodextrin for topical ophthalmic administration, proving that it was not toxic [40]; this fact becomes important when transferring research to the clinic. Hence, there is already a commercialized formulation (Indocollyre® 0.1% ophthalmic solution) complexing HPβCD and indomethacin [41]. The improvement of aqueous tacrolimus eye drops is still a challenge because of its low chemical stability and solubility in particular [27].

The aim of this work was based on the design and development of different topical ophthalmic formulations containing tacrolimus as an alternative to the reformulated Prograf® intravenous solution (REF). The characterization of these tacrolimus-loaded ophthalmic formulations incorporating the improvements that cyclodextrin (HPβCD) properties can provide in terms of tacrolimus solubility and stability in aqueous solution was also carried out. Cyclodextrin properties may also improve the mucoadhesive characteristics of the formulations, leading to a retention time increase on the ocular surface. In addition, the use of new molecular imaging techniques such as positron emission tomography/computed tomography imaging (PET/CT imaging) was incorporated into the present study in order to better understand the *in vivo*

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formulations' permanence on the corneal surface. On the other hand, the evaluation of the eye drops' safety was necessary to verify that there were no corneal surface alterations. Based on this background, the novelty of this work relies on the obtention of a consistent preclinical base for a new safe, stable and bioadhesive tacrolimus eye drop designed for appropriate preparation by HPDs. Besides, additional goals were also pre-established such as ease of preparation, scalability from the laboratory scale to HPDs and patient comfort improvement.

2. MATERIALS AND METHODS

Tacrolimus powder was acquired from Guinama® S.L.U. (La Pobla de Vallbona, Spain), 2-hydroxypropyl-β-cyclodextrin Kleptose® HPB (HPβCD; M_W = 1399 Da, substitution degree = 0.65 molar ratio) was provided from Roquette Laisa S.A.® (Valencia, Spain), Liquifilm® was purchased from Allergan® Pharmaceuticals Ireland (Mayo, Ireland), Balanced Salt Solution (BSS®) was acquired from Alcon® laboratories (Fort Worth , TX, USA) and Prograf® (5 mg/mL, ampoules) was purchased from Astellas Pharma S.A.® (Madrid, Spain). Ultrapure water MilliQ® (Millipore Iberica; Madrid, Spain) was used throughout the whole work. All other chemicals and reagents were of the highest purity grade commercially available.

2.1. Tacrolimus/HPβCD Solubilization Study

2.1.1. Phase Solubility Diagram

A phase solubility diagram was used to estimate the stability constant (*K*) of the tacrolimus/HPβCD inclusion complex. The solubility measurements were carried out following the previous work of our research group, described by Anguiano-Igea et al. [42]. This protocol was adjusted to the Higuchi and Connors phase solubility model [43]. A solubility study was carried out by adding an excess amount of the tacrolimus powder to MilliQ® aqueous solutions containing increasing concentrations of HPβCD (from 0 to 400 mg/mL). The vials containing these suspensions were then shaken in a VWR incubated mini-shaker (VWR International, Radnor, PA, USA) at 25 °C and 250 rpm for 7 days until reaching an equilibrium. Subsequently, each aliquot was centrifuged for 30 min at 15,300 rcf (Centrifuge 5804 R; Eppendorf®, Hamburg, Germany) and 100 µL of the supernatant was diluted with 400 µL of MilliQ® water (1:5 dilution) prior to measurement. Each HPβCD concentration was assayed in quintuplicate.

Tacrolimus concentration was determined for each sample using an HPLC system (Agilent 1260 series; Agilent Technologies®, Santa Clara, CA, USA) equipped with a Diode Array Detector HS, a solvent delivery quaternary pump system, 400 bar maximum pressure and an autosampler. The software model

OpenLAB CDS 3D UV (PDA) was used for the data processing. The analysis was performed under an isocratic method. The column used was a Poroshell 120, EC-C18 (4.6 x 100 mm, 4 μm) and at a temperature of 60 °C. The mobile phase was water-acetonitrile (35:65 (*v*/*v*)) using a 1.5 mL.min^{-1} flow rate. A 210-nm wavelength was employed for the tacrolimus quantification. The volume of the injected sample was 10 μL and the retention time was 3 min. The analytical method was validated according to International Conference on Harmonization (ICH) guideline recommendations [44] and the mathematical adjustments were made in GraphPad Prism® 8 v.8.2.1 software (GraphPad Software, San Diego, CA, USA, 2019.).

A_P phase solubility types are usually observed when a drug molecule forms a complex with more than one CD molecule, assuming a consecutive complexation. A quadratic model equation allows the estimation of both stability constants ($K_{1:1}$ and $K_{1:2}$). The value of $K_{1:2}$ is often lower than that of $K_{1:1}$. These constants values were calculated using the following Equation 1:

$$S = S_0 + (K_{1:1} \cdot S_0 + K_{1:1} \cdot K_{1:2} \cdot S_0 \cdot [HP\beta CD]) \cdot [HP\beta CD], \quad (1)$$

where S is the total solubility, S_0 is the free drug solubility and $K_{1:1}$ and $K_{1:2}$ are the stability constants of the complex tacrolimus/HPβCD. $K_{1:1}$ and $K_{1:2}$ values were calculated by non-linear regression using GraphPad Prism® 8 v.8.2.1 software.

2.1.2. NMR studies and molecular modeling

NMR experiments were conducted at two different temperature conditions, 278 and 298 K, on a Bruker NEO 17.6 T spectrometer (proton resonance 750 MHz) (Billerica, MA, USA), equipped with a $^{1}H/^{13}C/^{15}N$ triple resonance PA-TXI probe with a deuterium lock channel and a shielded Pulse Field Gradient (PFG) z-gradient. The spectrometer control software was TopSpin 4.0 (Billerica, MA, USA, 2020.). The reported chemical shifts are referenced to the lock deuterium solvent. Spectra were processed and analyzed using Mestrenova v14.0 software (Mestrelab Research S.L., Santiago de Compostela, Spain, 2019).

One-dimensional saturation transfer difference ^{1}H spectra (STD) [45] were measured. The experiment consisted of a selective saturation pulse train, a WET selective solvent suppression module and a 90-degree hard-pulse followed by *fid (free induction decay)* acquisition. The selective saturation consisted of a train of soft Gaussian-shaped pulses of 50-ms duration with a 1-ms interpulse delay. The selective saturation was applied during 2 s at a specific frequency of the ^{1}H spectrum, covering a region of the spectrum of ± 125 Hz around the chosen frequency (i.e., ± 0.17 ppm in a 750 spectrometer). The STD^{off} saturation was applied at 20 ppm. The STD^{on} saturation was applied at the frequency of one

specific signal of tacrolimus separated by more than 300 Hz from any signals of the HPβCD. The STD^{on} and STD^{off} scans were measured in alternate scans and subtracted by the phase cycling, providing the subtracted STD^{off-on} spectra. Three STD spectra were obtained by STD^{on} saturation of the tacrolimus signals at 6.30, 6.12 and 2.12 ppm, respectively. Each spectrum was acquired in 15 min with 128 scans and a 6.75-s total scan duration consisting of a 2-s pre-scan d_1, a 2-s STD saturation time and a 2.75-s *fid* acquisition.

Molecular modeling was also performed to have an orientation of which is the most likely interaction between tacrolimus and HPβCD molecules using an MM+ force field in HyperChem®. The HPβCD used in this work (Kleptose® HPB) was a mixture of hydroxypropylated-β-CDs with a substitution mean degree of 0.65 (0.58–0.68; a mean of 4.2 hydroxypropyl groups per cyclodextrin molecule). An HPβCD molecule type with an average of four hydroxypropyl groups per native CD unit was used to obtain the molecular model.

2.1.3. Vehicle Solubility Study

In addition to conducting a MilliQ® water solubility study, it was studied whether the tacrolimus solubility varied in different media in which the final tacrolimus eye drops could be formulated. The tacrolimus solubility was tested in two different vehicles besides MilliQ® water, these being BSS® and Liquifilm®. Three HPβCD proportions (20%, 30% and 40% (*w*/*v*)) were used sixfold with each vehicle and a two-way ANOVA was subsequently applied to compare the formulations' solubility and to check for significant differences among them. This assay was carried out as a way to select the formulations to be tested for further analysis.

2.2. Formulation Preparation Procedure

The 2-hydroxypropyl-β-cyclodextrin (HPβCD) solutions were prepared by dissolving them in the two vehicles studied in the previous assay (BSS® and Liquifilm®); these were selected because they are ophthalmic vehicles that are frequently used in HPDs for eye drop formulation. BSS® is an isotonic salt solution for use in irrigating eye tissues, with pH 7.5 and a ≈ 300 mOsm/kg osmolality [46]. Furthermore, Liquifilm® is an artificial eye tear that contains 1.4% polyvinyl alcohol (PVA) and a benzalkonium chloride preservative as well as different salts [47].

Based on the vehicle solubility study results (see Section 2.1. Tacrolimus solubilization with HPβCD results), the HPβCD and tacrolimus concentrations were chosen. The formulations were labeled as TBS 20 (20% (*w*/*v*) HPβCD and 0.01% (*w*/*v*) of tacrolimus in BSS®), TBS 40 (40% (*w*/*v*) HPβCD and 0.02% (*w*/*v*) of

tacrolimus in BSS®), TLI 20 (20% (*w*/*v*) HPβCD and 0.01% (*w*/*v*) of tacrolimus in Liquifilm®) and TLI 40 (40% (*w*/*v*) HPβCD and 0.02% (*w*/*v*) of tacrolimus in Liquifilm®) (see Table 1). Once the cyclodextrin was dissolved, tacrolimus powder was added under magnetic stirring (>750 rpm) until all the tacrolimus powder was dissolved.

The reference formulation was prepared just as it is formulated in an HPD; a mixture of 0.03% (*w*/*v*) tacrolimus in Liquifilm® was prepared by a Prograf® (5 mg/mL) intravenous ampoule dilution as it was done in previous work [15], instead of using the tacrolimus powder. All formulations were kept under refrigeration conditions (4 ± 2 °C) to avoid degradation processes of the drug when they were prepared. Tacrolimus concentrations used in clinical practice are very variable (0.005% to 0.1% range) [11,17,48,49]; it should be noted that the selection of the designated tacrolimus concentration has been carried out on the basis of previous experimental studies (see Section 2.1.3. Vehicle solubility study).

Table 1. Tacrolimus eye drops composition.

Formulation	Composition		
	HPβCD (*w*/*v*, %)	**Tacrolimus (*w*/*v*, %)**	**Vehicles**
TBS 20	20	0.01	BSS®
TLI 20	20	0.01	Liquifilm®
TBS 40	40	0.02	BSS®
TLI 40	40	0.02	Liquifilm®
REF	-	0.03	Liquifilm®

2.3. Optimization Procedure: Tacrolimus Solubilization Time

The purpose of this assay was to know the required tacrolimus solubilization time in the eye drops so that when the formulation has to be replicated in an HPD, the drug will not be removed when performing the sterilizing filtration, ensuring quality, final product efficacy and patient safety.

This study was designed to establish the minimum stirring time of the tacrolimus powder to achieve the desired concentration in the solution media. HPβCD dissolution was carried out in triplicate (10 mL per formulation), and the amount of tacrolimus powder was added once all the cyclodextrin was dissolved. The solutions were shaken in a Cimarec I multipoint magnetic stirrer (ThermoFisher® Scientific; Waltham, MA, USA) at 900 rpm and 30 °C during the whole study period.

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At predetermined times (24, 40, 48, 67, 72, 90 and 96 h), samples were collected without stopping the stirring, thus achieving the dissolution homogeneity.

Samples were centrifuged (Centrifuge 5804 R; Eppendorf®, Germany) at 15,300 rcf and 25 °C for 30 minutes to remove any undissolved particles. Subsequently, 400 µL of the supernatant was taken to measure the tacrolimus concentration by HPLC (Agilent 1260 series; Agilent Technologies®, Santa Clara, CA, USA) with the method described above (see Section 2.1.1. Phase solubility diagram). The tacrolimus concentration was corrected in each measure according to the volume that was left (0.5 mL less in each sample). Resulting data were compared and a statistical analysis was performed using a two-way ANOVA to analyze the influence of the solubilization time and the composition of the formulations. The analysis was carried out using GraphPad Prism® 8 v.8.2.1 software.

2.4. Physicochemical Characterization

2.4.1. pH and Osmolality Determination

The osmolality measurements were performed with an OsmoSpecial 1 osmometer (Astori Tecnica®; Poncarale, Italy), while the pH was measured using a pH meter (HI5221 HANNA®, Italy) at 25 ± 0.5 °C. Each determination was carried out in triplicate.

2.4.2. Surface Tension Determination

Surface tension at the surface-to-air interface was measured using the du Nöuy ring method [50]. A Lauda TD1 tensiometer (LAUDA Scientific GmbH®, Lauda-Königshofen, Germany) fitted with a platinum ring (2-cm diameter) was used to measure the surface tension of the tacrolimus formulations. A platinum du Nöuy ring was immersed into the liquid and then lifted to obtain tension values. The measurements were made at room temperature.

All glassware used for the surface tension measurements was washed with MilliQ® water and then dried in a clean oven before use. The platinum du Nöuy ring was washed with alcoholic KOH, rinsed in MilliQ® water and flamed until red-hot before each measurement. The determinations were carried out in triplicate.

2.4.3. Squeezing Force Determination

The squeezing force test evaluates the force needed to dispense a drop from 5-mL polypropylene eyedropper bottles that are commonly used in HPDs. A comparison of the cyclodextrin formulations with each other and between the reference one was made in order to assess the presence or absence of significant differences depending on their composition.

This method was performed following the method established by Charles H. Cox, with minor modifications [51], in a Shimadzu® texturometer (Kyoto, Japan). Figure 1 describes in detail the protocol followed to obtain the squeeze force measurements. The eyedropper bottle containing 5 mL of formulation was placed on a plate with a 45° inclination just below the load cell (1000 N maximum force).

Test parameters were set after the method optimization; the upper probe moved down at a speed of 0.5 mm/s and an assessment of the required force to spill one drop of the formulation was performed. Five eyedropper bottles of each formulation were tested in quintuplicate (25 measurements per formulation). A one-way ANOVA was carried out to find out if there were significant differences. Tukey´s multiple comparisons test was subsequently performed in order to compare all formulations.

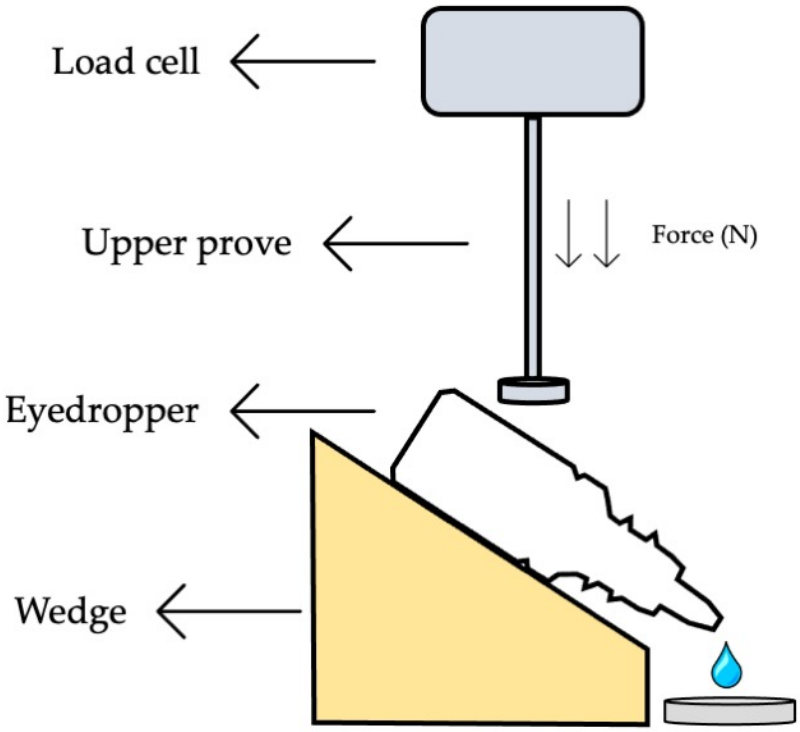


Figure 1. Scheme of the squeezing force determination.

2.5. Corneal Mucoadhesion

Tacrolimus formulations' mucoadhesion was measured following a method designed and developed by our research group using a Shimadzu® texturometer [52]. Corneal bioadhesion was used to quantitatively determine the interaction between the formulation and the corneal bovine surface. As presented in Figure 2, fresh bovine corneas were adjusted to a cornea mold made of clay and

subsequently fixed to a texturometer load cell (20 N maximum force). Formulations were dropped in a glass bottle (40 mm diameter, 20 mm height) and placed in the lower part of the analyzer.

Corneas were immersed 2 mm deep into the formulation at a 1 mm/s speed and force data (N) were obtained. Then, corneas were kept in touch with the formulation for 30 s, and just after returning to the starting point at a 1 mm/s speed, work (mJ) was measured. Mucoadhesion work was calculated from the area under the curve (AUC) of the force–displacement curve. All method parameters were previously studied and subsequently applied. Each measurement was carried out in triplicate.

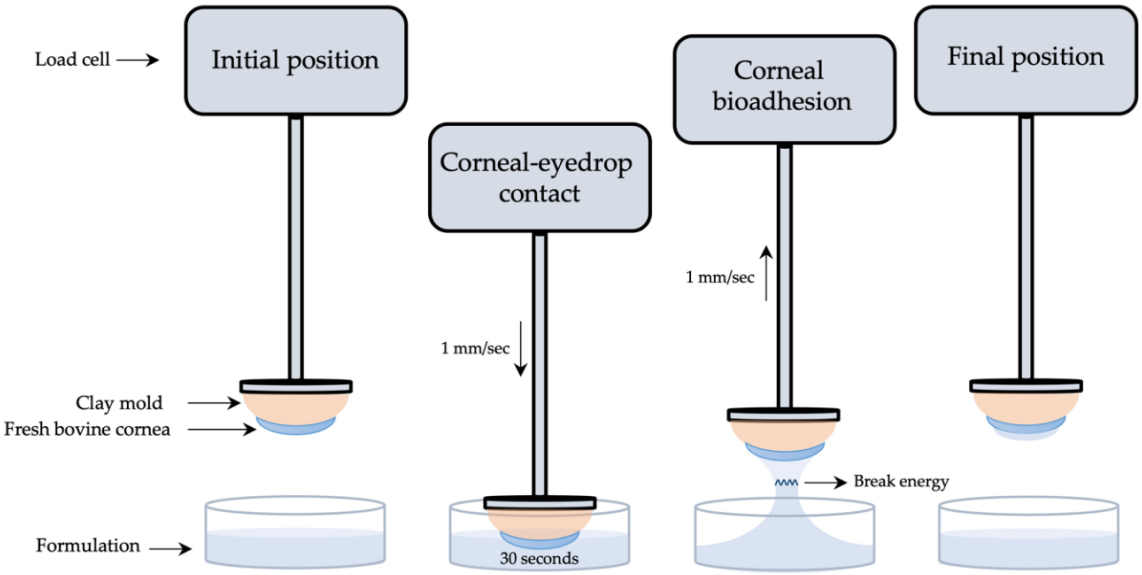


Figure 2. Scheme of the corneal mucoadhesion method.

2.5.1. Bovine Corneal Opacity and Permeability Test (BCOP)

The Bovine Corneal Opacity and Permeability test (BCOP) is an organotypic assay used to identify potential ocular corrosives and severe irritants. This method was carried out using slaughterhouse materials (fresh bovine corneas), allowing the animal replacement compliance and avoiding the use of laboratory animals. This assay was founded on the method established by Tchao et al. [53] and adapted by Gautheron et al. [54], with minor modifications.

Freshly excised eyes were obtained from a local slaughterhouse and transported to the laboratory in optimal conditions. A macroscopic inspection of the eyes was performed, and only free-of-defects corneas were dissected and used for the test. Fresh corneas were mounted in Franz diffusion cells with the corneal epithelial surface facing upwards, dividing the two different chambers of the diffusion cell

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(donor and receptor). A thermostatic bath with a controlled temperature (37 ± 2 °C) and under stirring (100 rpm) during the whole assay was used to incubate all Franz diffusion cells.

Corneal opacity changes were measured by two different techniques, these being photometry and UV–Vis spectrophotometry. Photometry was assessed using a luxmeter (Gossen® Mavolux 5032C USB; Nürnberg, Germany), where corneas were placed between two cylindrical supporting black holders (fabricated with polylactic acid filaments using a 3D printer, Witbox® BQ, Madrid, Spain) and illuminated with a pipe light (Olympus® Highlight 200, Tokyo, Japan) with fixed brightness values [55].

Corneal transparency was measured in transmittance values by UV–Vis spectrophotometry (from 200 to 800 nm), allowing the light to pass through the corneas, from the source to the receiver of the spectrophotometer (Cary 60 UV-Vis, Agilent Technologies®; Santa Clara, CA, USA). Each formulation was tested in triplicate.

The following protocol was performed for formulations' addition and opacity measurements: (I) determination of the initial opacity values for freshly excised corneas by photometry and UV–Vis spectrophotometry; a corneal blank was made before photometry determination in order to remove basal light, while the cornea itself was used as a blank (800-nm wavelength) before spectrophotometry determination; (II) addition of 1 mL Phosphate-Buffered Saline (PBS) into the donor chamber of the vertical diffusion cells and cornea incubation were performed for 10 minutes, followed by opacity determination; (III) an amount of 1 mL of the formulation was then added to the upper chamber for 10 minutes, followed by its subtraction and further addition of PBS for 120 minutes; after this time, the opacity determination was repeated.

Once the opacity was measured, the same corneas were used to evaluate the corneal permeability changes, so 1 mL of 0.4% (*w*/*v*) fluorescein aqueous solution was added in the donor compartment, keeping in touch with the corneal epithelium side. After 90 min, the corneal permeability determination was evaluated by measuring the optical density (OD) in the medium (PBS) of the lower compartment at a 490-nm wavelength.

An *in vitro* irritation score (IVIS) can be calculated with the measurements of corneal opacity and permeability. The IVIS was determined by the following Equation 2:

$$IVIS = \text{mean opacity value} + (15 \times \text{mean permeability } OD_{490} \text{ value}), \quad (2)$$

2.5.2. Hen´s Egg Test on the Chorioallantoic Membrane (HETCAM)

The hen's egg test on the chorioallantoic membrane (HETCAM) assay is one of the new organotypic models that allows the identification of irritative reactions, and it has become the international standard assay for acute eye irritation and corrosion (OECD TG 405, 2002; EC B.5). This assay was performed to evaluate possible acute ocular irritation caused by the present tacrolimus formulations.

Fertilized Broiler eggs (50–60 g weight) were obtained from the regional hatchery technology center and incubated for nine days in specific conditions (37 ± 0.5 °C and 65% ± 5% RH). The eggs were automatically rotated in an automatic rotational incubator every 2 h until the eighth day, where rotation was stopped, and the eggs were kept in the axial position during 24 h for the proper placement of the chorioallantoic membrane (CAM). The protocol used was adapted from the procedure described by Spielmann and Liebsch [56].

At the ninth day of incubation, embryonated eggs were opened by their upper part with a tiny drill (Dremel®, Madrid, Spain) and the inner membrane was moistened with 0.9% (*w*/*v*) NaCl aqueous solution so that it could be removed later. Briefly, 300 µL of 0.9% (*w*/*v*) NaCl solution (negative control), 0.1% (*w*/*v*) NaOH solution (positive control) and tacrolimus formulations (TBS 20, TLI 20, TBS 40, TLI 40 and REF) were directly instilled onto the CAM (2 eggs per compound). The membrane was observed over a 5-min period using an Olympus® SZ61TR Stereomicroscope and Olympus® CellSens Entry software.

Hemorrhage, lysis and coagulation of the CAM were measured; these reactions can be quantified through an irritation score (IS) following the Kalweit et al. criteria [57]. Based on this, they can be classified as follows: 0–0.9, no irritation; 1–4.9, slight irritation; 5–8.9, moderate irritation; and 9–21, severe irritation.

2.6. Stability Study

The stability study was only carried out with the formulations containing the greatest amount of drugs due to the fact that all the formulations have the same composition, the formulations with 40% (w/v) HPβCD being the most representative, as they have a greater amount of the components with activity and based on the clinical common usability and the drug concentration similarity at present.

Based on this, both formulations (TBS 40 and TLI 40) were aseptically prepared under laminar air flow. Briefly, 2 mL of each gel was conditioned into a 5-mL polypropylene eyedropper, previously sterilized, and the dropper was closed

with a polypropylene cap. Three batches of each formulation were prepared and subjected to a double filtration with 0.22-µm PES (polyethersulfone) filters.

The stability of each eyedrop was studied in unopened multidose eyedroppers for 4 months at three different temperature conditions: in refrigeration (4 ± 2 °C), at room temperature (25 ± 2 °C) and at oven temperature (40 ± 2 °C), protected from light exposure in all cases. Three units per formulation were subjected to tacrolimus quantification, osmolality determination, pH measurements and microbiological control growth at predetermined times (0, 15, 30, 45, 78 and 120 days). All samples were also visually inspected for any macroscopic changes (e.g., color, turbidity and precipitation).

2.6.1. Quantification of Tacrolimus Amount

The determination of tacrolimus was performed as previously mentioned (see Section 2.1.1. Phase solubility diagrams). Refrigerated and accelerated samples were kept at room temperature for 20 minutes before quantification. Each sample was assayed in triplicate. The analytical method was validated according to International Conference on Harmonization (ICH) guideline recommendations [44].

2.6.2. Osmolality, pH and Microbiological Control Growth

The pH and osmolality measurements were performed following the same experimental procedure previously described (see Section 2.4.1. pH and osmolality determination). In order to study the microbiological stability, 1 mL of each formulation was added in blood agar plates, Sabouraud/Chloranphenicol agar plates and liquid thioglycate medium plates. These samples were grown at 37 °C for predetermined periods (48 hours, 15 days and 10 days, respectively). Once each incubation period ended, microbiological growth was observed and determined.

2.6.3. Statistical Analysis

Pharmaceutical Codex [58] has established the margins of the expiration period of the formulations as being once the concentration of active ingredient is reduced by 10% concerning the initial concentration. The percentage of unaltered drug versus time was fitted to a first order kinetics using GraphPad Prism® 8 v.8.2.1 software and the degradation constant (K), expiration time (t_{90}) and determination coefficient (R^2) were calculated. pH and osmolality monitoring were performed to observe the presence of any changes. Microbiological stability was considered adequate when no microbial growth was provided in the

cultured samples. The presence of any abnormal macroscopic particles in the formulations was not considered acceptable.

2.7. 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 Compostela (Spain). The animals were kept in individual cages under controlled temperature (22 ± 1 °C) and humidity (60 ± 5%), with day–night cycles regulated by artificial light (12/12 hours) and feeding *ad libitum*. The animals were treated according to the ARVO statement for the use of animals in ophthalmic and vision research as well as the approved guidelines for laboratory animals [59,60]. Experiments (idis12072017) were approved by the Institutional Committee of the Health Research Institute of Santiago de Compostela (IDIS) following the Galician Network Committee for Ethics Research, 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 computed tomography (CT) procedures for conducting the radiolabeling of the formulations and the quantitative ocular permanence study were described in our previous works [15,61,62]. Briefly, PET and CT images were acquired using the Albira PET/CT Preclinical Imaging System (Bruker Biospin®; Woodbridge, Connecticut, USA). Anesthetized animals (2.5% (v/v) isoflurane/oxygen) were positioned into the imaging bed, monitoring the respiration frequency. Afterward, 7.5 μL of each tacrolimus formulation, previously radiolabeled with ^{18}F-fluorodeoxyglucose (^{18}F-FDG), was instilled into the conjunctival sac of both eyes using a micropipette. The administered radioactivity was 0.20–0.25 MBq per eye. After the instillation, static PET frames at different times (0, 30, 75, 120, 240 and 300 min) were acquired as a way to evaluate the pharmacokinetics behavior. To prevent the rats from scratching their eyes by removing the instilled formulations, an Elizabethan collar was placed between PET studies. Two animals (4 eyes) were tested for each formulation in order to accomplish the 3Rs regulatory frameworks [63].

Image analysis was performed using the Amide´s Medical Image Data Analysis Tool [64]. Regions of interest (ROIs) were manually drawn for the different frames by delimiting the total radiotracer uptake of each eye using a spherical volume of 1767.1 m^3 (15 × 15 × 15 mm). The radiotracer uptake over time was corrected by the radioisotope decay (^{18}F half-life: 109.7 min). Subsequently, a clearance rate for each formulation was obtained in terms of the ocular remaining radioactivity uptake over time after instillation. The data were fitted using a non-compartmental analysis in order to calculate the elimination constant (K), the

half-life ($t_{1/2}$) and the zero and first moment pharmacokinetic parameters, area under curve ($AUC_{0-\infty}$) and mean residence time (MRT) using GraphPad Prism® 8 v.8.2.1 software.

3. RESULTS AND DISCUSSION

3.1. Tacrolimus/HPβCD Solubilization Study

The phase solubility diagram of tacrolimus in aqueous HPβCD solutions at 25 °C is shown in Figure 3. The resulting profile indicates an A_P-type diagram that shows a positive deviation from characteristic linearity, this being supported by previous studies [27,37]. Thus, the formation of a soluble complex in the aqueous media with the formation of high-order drug/CD complexes at high cyclodextrin concentrations is assumed. It must be taken into account that the formation of 1:1 and 1:2 drug/CD complexes and both $K_{1:1}$ and $K_{1:2}$ stability constants were calculated. Tacrolimus aqueous solubility in the absence of cyclodextrins was 4 ± 0.67 μg/mL, and $K_{1:1}$ and $K_{1:2}$ values were 143.1 ± 10.3 and 2.1 ± 0.6 M^{-1}, respectively.

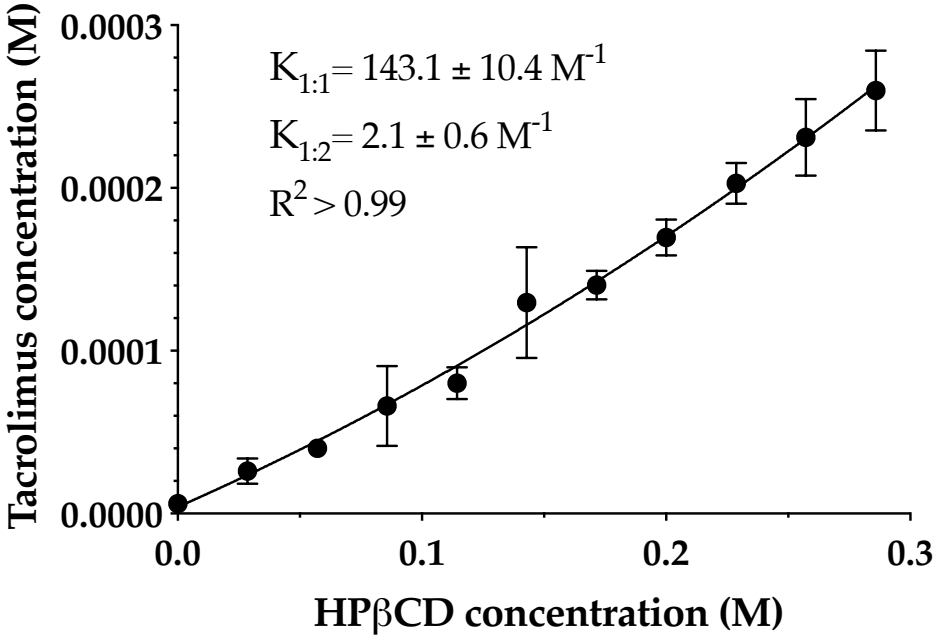


Figure 3. The phase solubility diagram of tacrolimus/hydroxypropyl-β-cyclodextrin (HPβCD) at 25 °C in purified water. Stability constants values represent the mean ± SD (n = 5).

The low values of stability constant indicate that tacrolimus and HPβCD interactions were weak, especially in the 1:2 complex. The main reason for this weak interaction between the drug and cyclodextrin may be the high molecular weight and the complex molecular structure of tacrolimus that hampered an

adequate adaptation to the cyclodextrin cavity. This study was made only with HPβCD because according to the EMA, it is the safest cyclodextrin and most appropriated for topical ophthalmic administration together with sulfobutylether-β-cyclodextrin (SBEβCD), proving that it is not toxic [40]. This fact becomes important when transferring research to the clinic. Thus, NMR studies were performed in order to study the part of the drug and cyclodextrin structure involved in the complex formation.

The intermolecular interaction between tacrolimus and HPβCD in aqueous solution was studied by NMR in the tacrolimus/HPβCD mixture prepared in D_2O at a 200 mM HPβCD concentration and tacrolimus saturation (0.2 mM). The ^1H spectrum of the mixture at 278 K (Figure 4a) shows resonances at the chemical shifts expected for tacrolimus signals (a reference spectrum of pure tacrolimus in MeOD is given in Figure 4d) with a considerable broadening, a possible indication that the tacrolimus is forming a large aggregate or self-aggregate in the water solution due to its very low polar characteristics.

To test for binding between the tacrolimus and HPβCD in the mixtures, a one-dimensional saturation transfer difference spectrum (STD) was used, being a well-known NMR technique for screening ligands binding to protein receptors [45,65]. STD spectra were measured with auto-subtraction of alternate scans acquired with off- and on-irradiation providing the so-called STD^{on-off} spectra [45].

In these STD spectra, the on-irradiation was placed in a certain proton signal of tacrolimus that is sufficiently separated in frequency from any HPβCD signal to prevent them being directly saturated. Three STD^{on-off} spectra were measured at 278 and 298 K under otherwise identical conditions. Interestingly, only the STD^{on-off} spectra measured at the lower temperature (278 K, but not at 298 K) provided STD signal responses for the HPβCD species, as can be seen in Figure 4b,c,d. This is due to the partial transfer of saturation from the tacrolimus signal being irradiated to the HPβCD resonances, which reflects that there is a binding equilibrium between these two molecules (Figure 4).

The fact that the STD responses are only obtained in the spectra measured at 278 K and not at 298 K could indicate that there is low affinity between the two molecules at 298 K (i.e., binding equilibrium with Kd > 10 mM [66]), while the affinity is notably enhanced at 278 K (i.e., Kd < 1 mM [66]). Overall, these NMR results strongly suggest that tacrolimus is forming a large self-aggregate in the water solution and it is partially solubilized by forming a complex with HPβCD.

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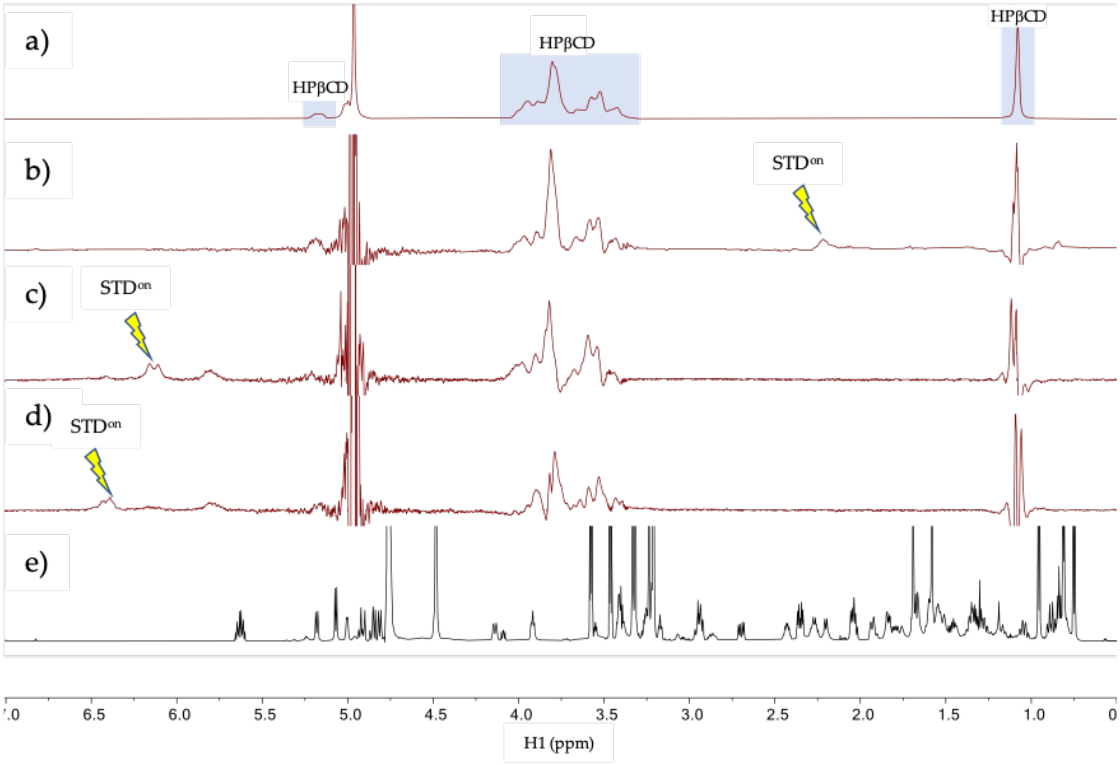


Figure 4. NMR spectra of tacrolimus/HPβCD interaction. **(a–d)** NMR spectra of the mixture tacrolimus/HPβCD in D_2O at 278 K. **(a)** HPβCD ^1H spectrum; **(b)** $STD^{on\text{-}off}$ spectrum with on-irradiation at 2.12 ppm; **(c)** $STD^{on\text{-}off}$ spectrum with on-irradiation at 6.12 ppm; **(d)** $STD^{on\text{-}off}$ spectrum with on-irradiation at 6.30 ppm and **(e)** Reference ^1H spectrum of pure tacrolimus in MeOD. The arrows indicate the approximate position of the on-saturation applied. Signals of HPβCD are indicated in spectrum **a**.

The molecular modeling of the tacrolimus/HPβCD interaction of 1:1 and 1:2 stoichiometry inclusion complexes is shown in Figure 5. The molecular docking studies indicated the higher probability of tacrolimus inclusion by its 4-hydroxy-3-methoxycyclohexyl group.

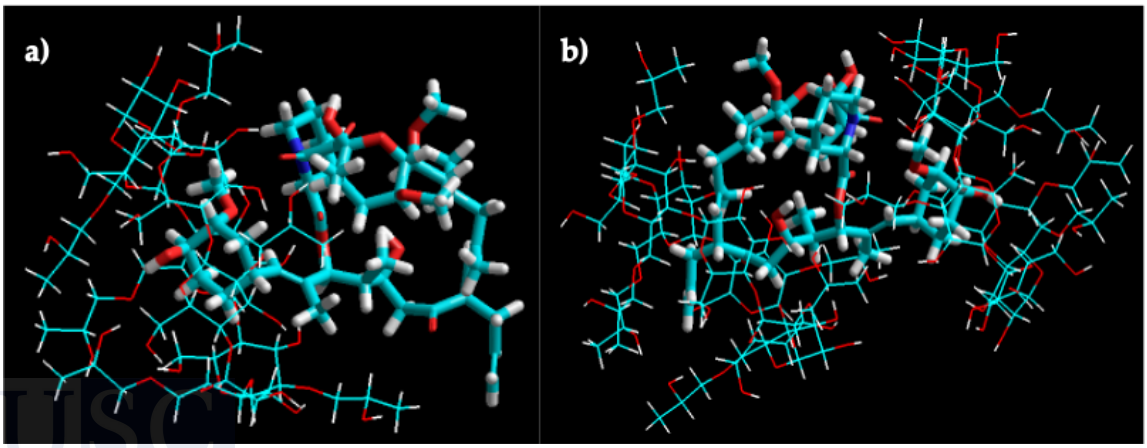


Figure 5. Molecular modeling of tacrolimus/HPβCD interaction. Molecular modeling of the 1:1 **(a)** and 1:2 **(b)** complex structures for tacrolimus and HPβCD obtained by manual docking and energy minimization using an MM+ force field in HyperChem®.

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The effect of BSS® and Liquifilm® (as ophthalmic vehicles) on the complexation was also studied (Figure 6). Hence, a vehicle solubility study was experimentally carried out to determine the tacrolimus behavior with increasing HPβCD concentration media. As presented in Figure 6, a proportional increase in the tacrolimus solubility was observed by increasing the amount of cyclodextrin included into the vehicle.

A two-way ANOVA was subsequently performed to compare all the media and assess the inclusion complexes' formation. The resulting data showed no significant differences between the 20% (*w*/*v*) and 30% (*w*/*v*) HPβCD in all vehicles; nonetheless, statistically significant differences ($\alpha < 0.05$) were found between 20% (*w*/*v*) and 30% (*w*/*v*) HPβCD solutions compared to the 40% (*w*/*v*) HPβCD solutions in all the vehicles. According to these results, the 30% (*w*/*v*) HPβCD formulations were discarded and only 20% (*w*/*v*) and 40% (*w*/*v*) HPβCD solutions were used in further studies.

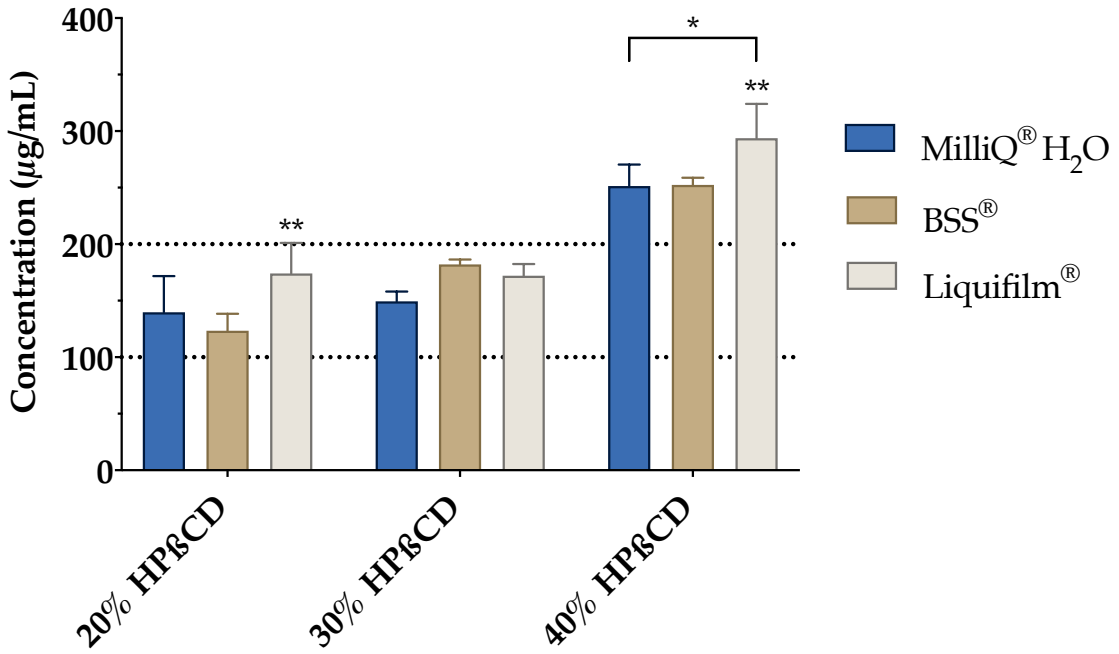


Figure 6. Tacrolimus solubility with 20%, 30% and 40% (*w*/*v*) of HPβCD in Balanced Salt Solution (BSS®) and Liquifilm® vehicles. Statistical analysis: two-way ANOVA followed by Tukey´s multiple comparison test (* $\alpha < 0.05$ compared with 20% (*w*/*v*) and 30% (*w*/*v*) HPβCD in all vehicles); one-way ANOVA followed by Tukey´s multiple comparison test (** $\alpha < 0.05$ compared with the other two vehicles (MilliQ® water and BSS®).

The resulting data also showed significant differences ($\alpha < 0.05$) among all the formulations for both HPβCD concentrations. Tukey´s multiple comparison test was then performed, and significant differences were also observed between Liquifilm® and the other two vehicles (MilliQ® water and BSS®) but there were none found between MilliQ® water and BSS®. The tacrolimus solubility in

Liquifilm®-diluted formulations may be attributed to the ternary complex formation (tacrolimus/HPβCD/PVA), increasing tacrolimus solubility by a synergistic solubilization effect [67].

Formulations were successfully prepared by an inclusion complex/dissolution technique. Tacrolimus was incorporated into the HPβCD hydrophobic cavity, leading to an increase in its water solubility. The solubilization time was determined in order to know the time necessary to completely dissolve the required dose (Figure 7).

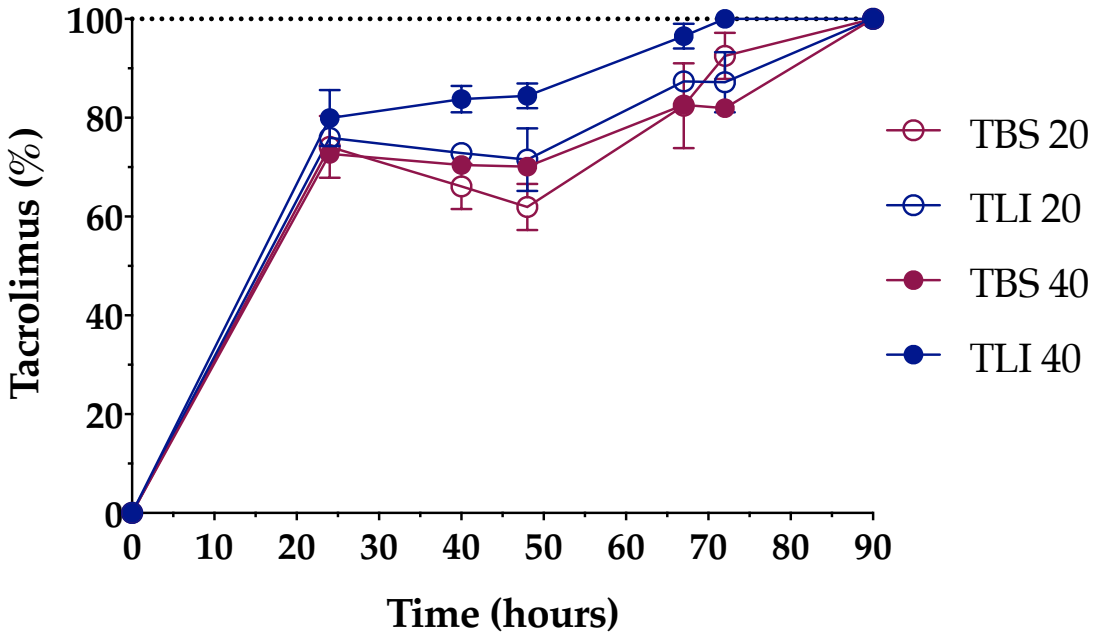


Figure 7. Dissolution time comparison among the different tacrolimus formulations. The 100% tacrolimus concentration corresponds to 0.01% (*w*/*v*) tacrolimus for TBS 20 and TLI 20 and 0.02% (*w*/*v*) tacrolimus for TBS 40 and TLI 40.

The time needed to prepare the aforementioned tacrolimus topical ophthalmic formulations will be a key preparation parameter in HPDs. As presented in Figure 7, 20% (*w*/*v*) HPβCD formulations containing tacrolimus reached 100% solubilization in a 90-h period, while 40% (*w*/*v*) HPβCD formulations containing tacrolimus showed different solubilization time, being quicker for the TLI 40 (72-h period, against the 90-h period for TBS 40).

A two-way ANOVA was further carried out to study differences in tacrolimus solubilization time among the formulations. The resulting data showed statistically significant differences between TLI and TBS formulations ($\alpha < 0.05$) from 40 h onwards. Nevertheless, no differences were found between TLI 20 and TLI 40, nor between TBS 20 and TBS 40.

3.2. Physicochemical Characterization

3.2.1. pH, Osmolality and Surface Tension

pH and osmolality determinations were carried out in order to ensure that the formulations show values within the ocular physiological range. As presented in Table 2, all formulations met the pH and osmolality specifications described for topical ophthalmic administration. These results support the idea that these new formulations could be used without generating adverse effects in the ocular surface, compared to the control formulation (REF) [15] where, despite showing a pH value within the physiological range (pH 4 to 8) [68], the osmolality values (1220.5 mOsm/kg) were four times higher than the limit value for topical ophthalmic administration. Likewise, hypertonic topical ophthalmic formulations may alter the tear osmolarity and, thus, induce ocular inflammation, as described by Dutescu et al. [69].

Table 2. pH, osmolality and surface tension results of tacrolimus/HPβCD formulations.

Formulations	pH		Osmolality (mOsm/kg)		Surface Tension (mN/m)	
	Mean	SD	Mean	SD	Mean	SD
TBS 20	7.036	0.021	359.3	5.86	54.63	0.93
TLI 20	6.986	0.005	283.6	2.52	58.46	1.10
TBS 40	7.203	0.005	628	6.93	51.5	0.61
TLI 40	6.933	0.005	383	18.08	58.43	0.21
REF	7.3	0.014	1220.5	18.57	47.5	0.2

Surface tension determination constitutes a key assay for a topical ophthalmic formulation. Appropriate surface tension values guarantee that a formulation may be spread evenly over the entire corneal surface, ensuring the drug optimal ocular penetration and also enhancing the comfort of the user when applying eye drops [70]. The ideal eye drop will have a surface tension similar to the tear film fluid (42–46 mN/m) [71]. Nonetheless, Han et al. previously studied the surface tension of commercialized ophthalmic formulations and the results showed that these topical ophthalmic solutions had surface tensions greater than that of tears (34.3–70.9 mN/m), most likely increasing the tear film stability and allowing for a greater lubrication of the eye [72].

Therefore, in the case of the designed formulations (TBS 20, TLI 20, TBS 40 and TLI 40), the surface tension values were slightly higher than physiological parameters, but they were still at an optimal value (see Table 2). These surface tension values can be given by HPβCD, which shows values of 54.8–57.5 mN/m in solution, as it was previously mentioned by Saokham et al. [67]. However, the

reference formulation (REF) showed lower surface tension values than the others, being associated to the presence of ethanol in its composition.

A one-way ANOVA was applied to determine the surface tension of each formulation, and statistically significant differences were observed ($\alpha < 0.05$). Tukey's multiple comparison test was also performed, and no statistically significant differences were found between the TLI 20 and TLI 40 formulations, but significant differences were observed between the rest of the formulations ($\alpha < 0.05$).

3.2.2. Squeezing Force Determinations

Figure 8 show the squeezing force test results for the studied formulations. Based on the research of Conner et al. [73], a large percentage of patients (>50%) receiving local ophthalmic treatments reported that self-management is difficult due to the need to apply force to the eyedropper in order to get the preparation out. Besides, high doses may cause the patient to suffer from side effects, while underdosing may cause damage or prolonged drug therapy. The volume and structure uniformity of the formulation drops are also important properties to ensure accurate drug dosage and avoid treatment variability.

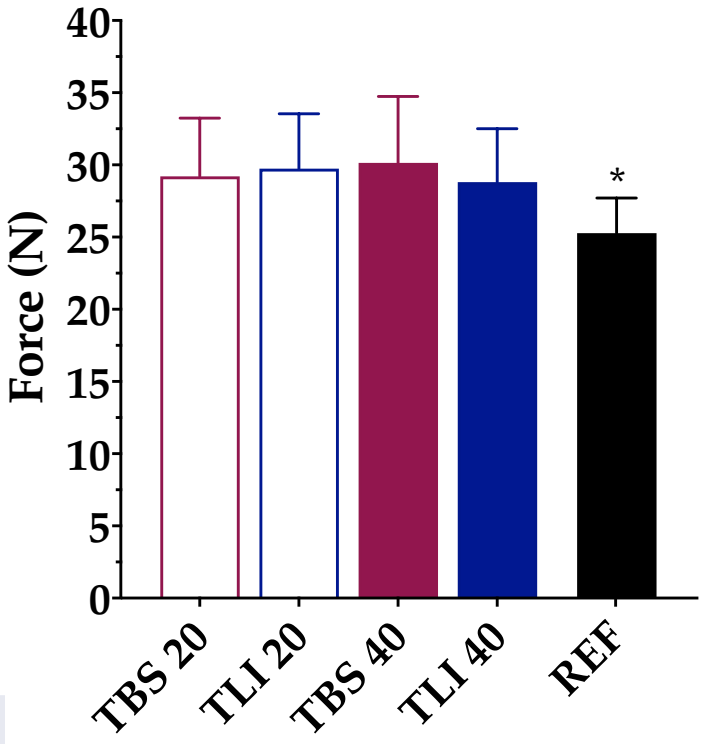


Figure 8. Comparison of squeeze force (N) values among different tacrolimus formulations (TBS 20, TLI 20, TBS 40, TLI 40 and REF). Statistical analysis: one-way ANOVA followed by Tukey´s multiple comparison test (* $\alpha < 0.05$ compared with prepared formulations).

The squeezing force test may be affected by different factors such as the formulation viscosity, surface tension or dropper tip design [74]. Therefore, the same type of packaging for testing was used. A one-way ANOVA was applied to determine the required force to dispense a drop of each formulation, and statistically significant differences were observed ($\alpha < 0.05$). Tukey's multiple comparison test was also applied, and no statistically significant differences were found between the tacrolimus/HPβCD formulations, but significant differences were observed between the reference formulation (REF) and the prepared formulations ($\alpha < 0.05$).

3.3. Corneal Mucoadhesion

Knowing that the mucoadhesion properties of the topical ophthalmic formulations will give an approximate idea of the permanence time on the ocular surface, bioadhesion work measurements were performed with the studied topical ophthalmic formulations. All data were assessed through breaking strength (N) and bioadhesion work (mJ) measurements (Figure 9).

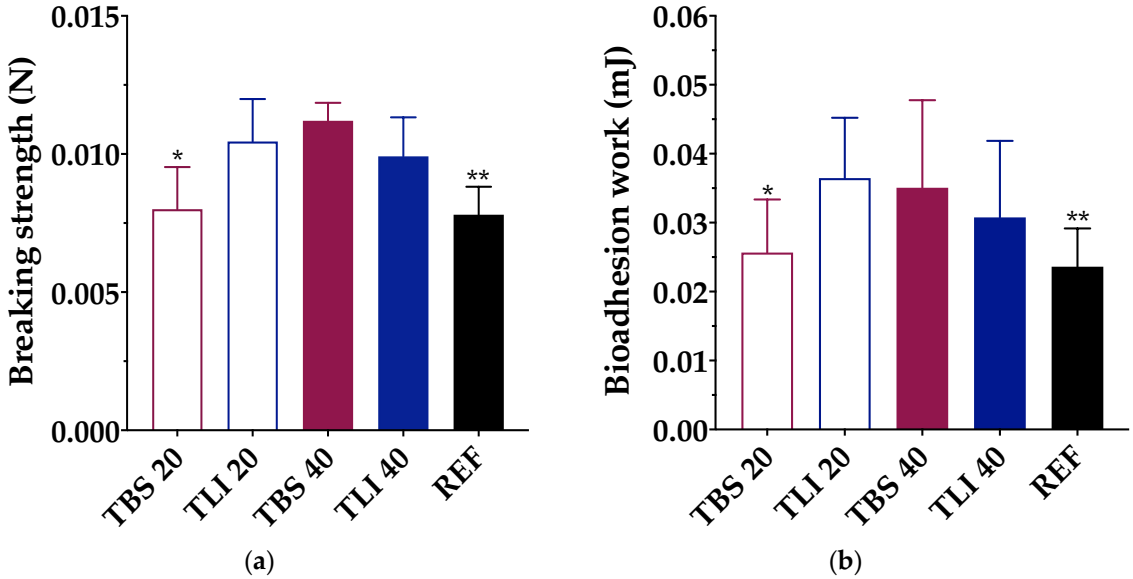


Figure 9. (a) Maximum breaking strength (N) and **(b)** bioadhesion work (mJ) obtained for each formulation using bovine cornea as a substrate. Statistical analysis: (**a**) one-way ANOVA followed by Tukey´s multiple comparison test (* $\alpha < 0.05$ compared with TLI 20, TBS 40 and TLI 40 and ** $\alpha < 0.05$ compared with TLI 20, TBS 40 and TLI 40); (**b**) one-way ANOVA followed by Tukey´s multiple comparison test (* $\alpha < 0.05$ compared with TLI 20 and ** $\alpha < 0.05$ compared with TLI 20 and TBS 40).

A one-way ANOVA was performed to define the bioadhesion work of the studied formulations, and statistically significant differences were observed ($\alpha < 0.05$). Tukey's multiple comparison test was also applied, and statistically significant differences were found between the TBS 20 and TLI 20 formulations

and between the REF and TLI 20 and TBS 40, but no significant differences were observed between the rest of the formulations ($\alpha > 0.05$).

The highest bioadhesion work values were obtained for the TLI 20 (0.036 ± 0.009 mJ) and TBS 40 (0.035 ± 0.013 mJ) formulations, and the lowest values were obtained for TBS 20 (0.026 ± 0.008 mJ) and REF (0.023 ± 0.005 mJ). In the case of TLI 40 (0.031 ± 0.011 mJ), no statistically significant differences were observed compared with TLI 20 and TBS 40. It was observed that during the separation stage of the cornea from the formulation, a formulation film remained adhered to the cornea when the load cell was lifted. It can be assumed, then, that the formulation interacts with the corneal surface with more intensity than the own cohesive forces of the formulation. Therefore, the adhesive bond fails due to the formulation fracture, making the viscosity and consistency play a fundamental role.

An *in vivo* ocular permanence study was subsequently performed using a PET/CT imaging technique to confirm the bioadhesive behavior of the formulations.

3.4. Ocular Irritancy and Toxicity

Some eye irritation and toxicity assessment tests have shown considerable potential to eliminate procedures that were historically performed by animal experimentation, such as the Draize rabbit eye irritation test which presents one of the most criticized and contested animal tests [75]. The efficacy of these *in vitro* and *ex vivo* procedures has been well studied by pharmaceutical industries and some national regulatory agencies [76]. The replacement of the Draize rabbit eye test with alternative models includes physicochemical tests, cell and tissue culture systems or organotypic models as eye components or isolated eyes [76–78]. The strategy to replace the Draize test by combining several animal-free methods has raised expectations [75]. The combination of two different methods has been proposed in this work. The Bovine Corneal Opacity and Permeability test (BCOP), which allows to detect whether the tested compounds cause a moderate, severe or very severe irritation, combined with the hen's egg test on the chorioallantoic membrane assay (HETCAM) covers the whole spectrum of irritation, since mild or very mild irritation signs can be detected.

3.4.1. Bovine Corneal Opacity and Permeability Test (BCOP)

Transmittance values of opacity and permeability assays were studied to assess whether formulations induce corrosivity or irritation (see Figures 10 and 11). No significant structural changes were observed in terms of corneal opacity and fluorescein permeability when comparing all formulations to the negative

control solution (PBS) but were observed with regard to the positive control (ethanol) ($\alpha < 0.05$).

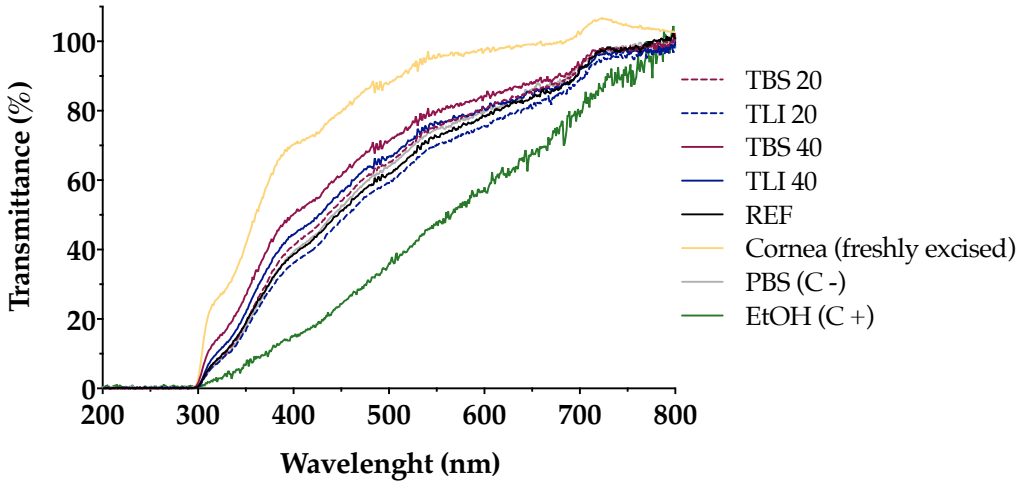
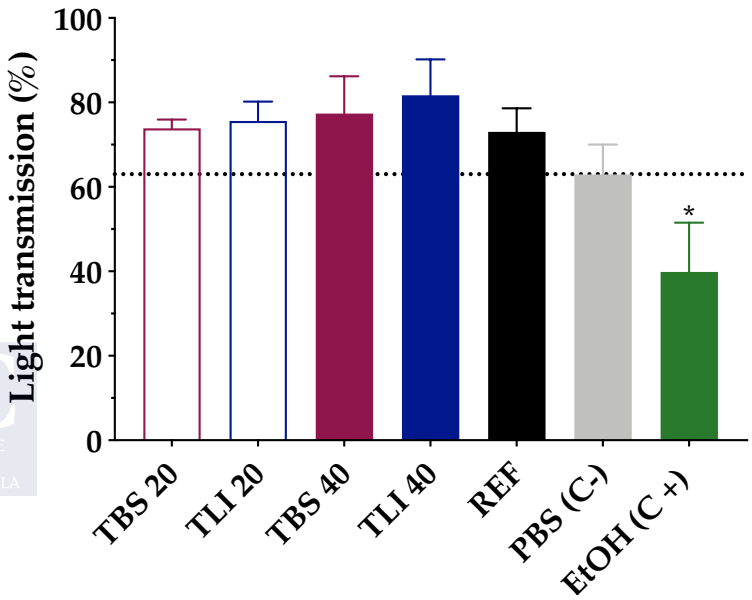


Figure 10. Ultraviolet–visible (UV–Vis) scan (from 200 to 800 nm) of corneal transmittance (%) values of bovine corneas treated with TBS 20, TLI 20, TBS 40, TLI 40, REF, PBS (negative control) and ethanol (positive control) after 10 min tacrolimus formulation treatment and 120 min PBS treatment.

Similarly, opacity and permeability data were corrected for background or control values prior to further statistical determinations being estimated. The IVIS score was then calculated, and all formulations resulted in an *in vitro* irritation score of 0 (IVIS = 0), showing no toxic effects compared to control formulations. Likewise, the fluorescein permeability test was further applied on the corneas previously treated with the tested formulations. The resulting data showed no statistically significant differences between all the formulations; however, significant differences ($\alpha < 0.05$) were observed between the studied formulation and the positive control (ethanol).



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Figure 11. Opacity values of bovine corneas treated with TBS 20, TLI 20, TBS 40, TLI 40, REF, PBS (negative control) and ethanol (positive control) after 10 min tacrolimus formulation treatment and 120 min PBS treatment. Here, 63% light transmission corresponds to the total light transmitted through bovine corneas incubated in PBS. Statistical analysis: one-way ANOVA followed by Tukey´s multiple comparison test (* $\alpha < 0.05$ compared with prepared formulations, reference formulation and negative control).

3.4.2. Hen's Egg Test on the Chorioallantoic Membrane (HETCAM)

All tacrolimus eyedrops were tested on the egg's CAM as well as two controls, NaCl as a negative control (C-) and NaOH as a positive control (C+). All formulations were evaluated, and data were compared to the NaCl and NaOH solutions' results. All formulations showed no toxic effects (irritation score = 0) compared them with the positive control formulation (see Figure 12). This agrees with previously described BCOP test results.

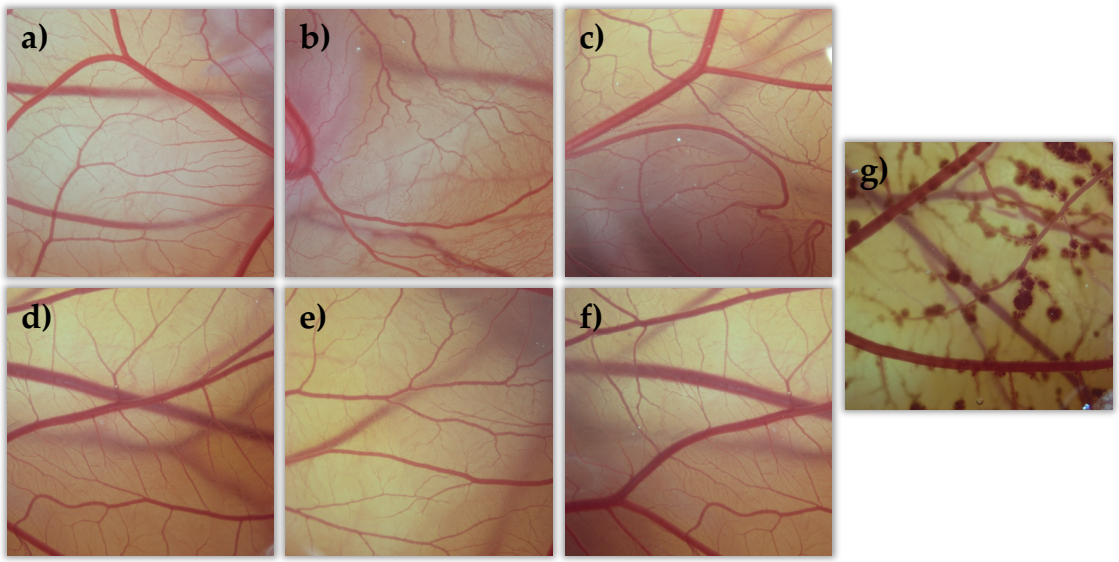


Figure 12. Hen's egg test on the chorioallantoic membrane (HETCAM) images 5 minutes post-instillation for the different formulations. **a)** NaCl (C-); **b)** TBS 20; **c)** TLI 20; **d)** TBS 40; **e)** TLI 40; **f)**: REF; **g)** NaOH (C+).

3.5. Stability Study

Stability studies of pharmaceutical compounds are essential to ensure drug efficacy [79,80] as well as to know if degradation products can cause toxic side effects and other undesired effects [27]. In this assay, only TBS 40 and TLI 40 were studied due to all formulations containing the exact same qualitative composition; thus, the formulations with the highest tacrolimus concentrations were tested as representative; these formulations were chosen based on the clinical common usability and the drug concentration similarity at present.

As presented in Figure 13, it can be seen that temperature was an important factor in the stability under storage of tacrolimus/HPβCD ophthalmic formulations. TBS 40 and TLI 40 showed the same tacrolimus degradation pattern for each storage condition and no statistically significant differences were found between them ($\alpha > 0.05$).

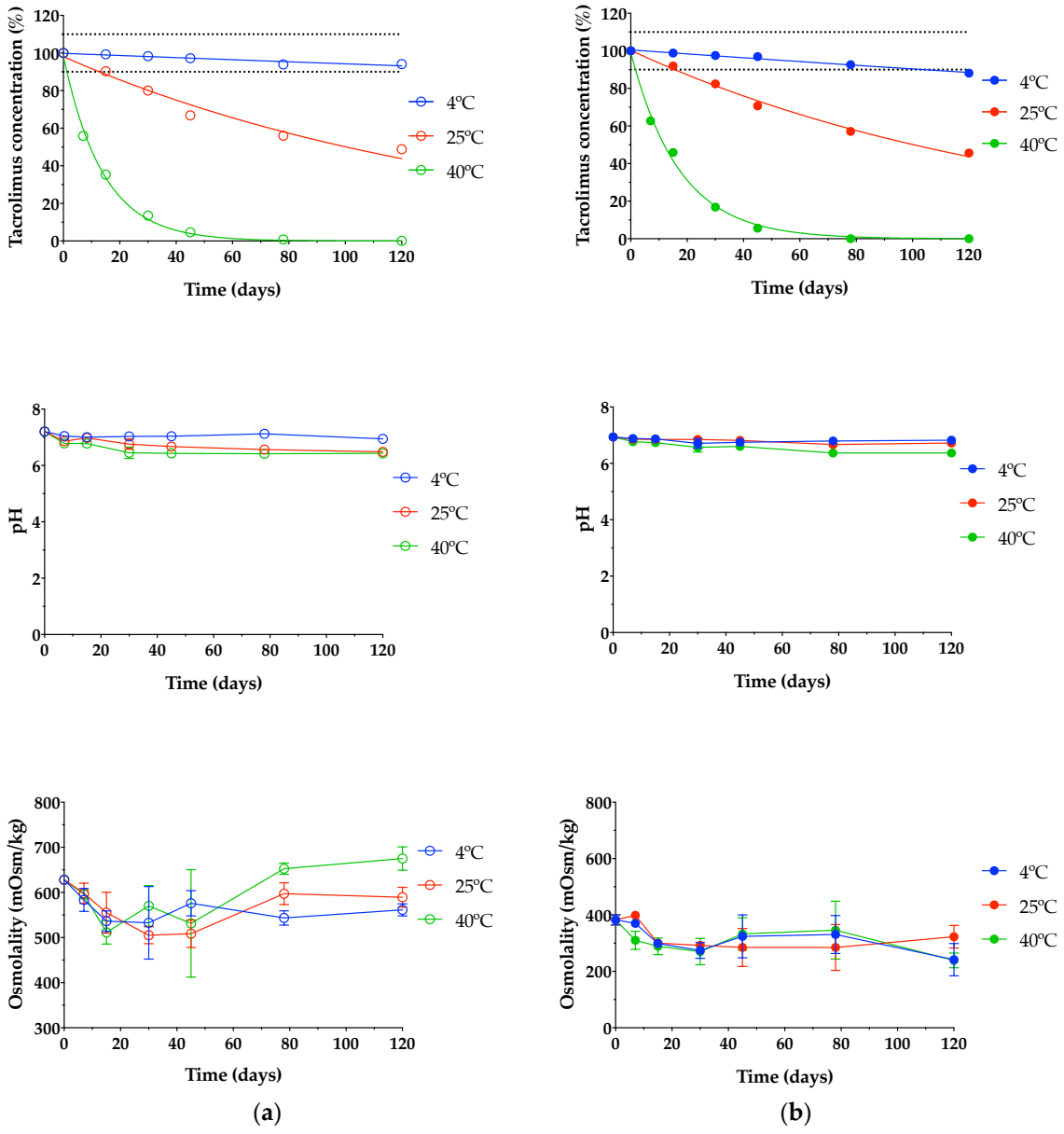


Figure 13. Tacrolimus concentration, pH and osmolality stability of **(a)** TBS 40 and **(b)** TLI 40 stored under three different temperature conditions: in refrigeration (4 ± 2 °C), at room temperature (25 ± 2 °C) and at oven temperature (40 ± 2 °C) during a 4-month stability test.

Table 3 shows the degradation constant (K), expiration time (t_{90}) and determination coefficient (R^2) values for both tacrolimus eye drops. It must be taken into account that TBS 40 showed a higher degradation rate (1.23 times) than

TLI 40 for the oven temperature condition, though significant degradation rate variations were not observed for the other two storage conditions.

Eye drops stored at oven temperature (40 ± 2 °C) were not stable in the first 7 days of the study, observing a rapid tacrolimus degradation process. In the case of eye drops kept at room temperature (25 ± 2 °C), the tacrolimus degradation was not so abrupt, but from day 15 of the study, the tacrolimus concentration was reduced to below 90% of the initial concentration, so the formulation was no longer stable. Nonetheless, both eye drops preserved in refrigeration (4 ± 2 °C) were stable beyond the 3-month period; specifically, the initial concentration of tacrolimus previously fell below 90% in the TLI 40 formulation compared to the TBS 40 formulation. Therefore, the optimal storage condition for tacrolimus topical ophthalmic formulations was at 4 °C.

Table 3. The degradation constant *K*, t_{90} and R^2 of two tacrolimus eye drops (TBS 40 and TLI 40) obtained by interpolation of the calculated regression line (% of remaining tacrolimus concentration vs time).

Formulation	Storage Condition	*K* (days^{-1})		t_{90} (days)		R^2	
		Mean	SD	Mean	SD	Mean	SD
TBS 40	Refrigeration (4°C)	0.0006	4.3×10^{-5}	184.32	13.5	0.8890	0.0179
	Room temperature (25°C)	0.0067	0.0002	15.72	0.53	0.9645	0.0057
	Oven temperature (40°C)	0.0713	0.0016	1.48	0.03	0.9966	0.0012
TLI 40	Refrigeration (4°C)	0.0011	5.8×10^{-5}	98.50	5.2	0.9410	0.040
	Room temperature (25°C)	0.0067	0.0002	15.66	0.40	0.9867	0.0035
	Oven temperature (40°C)	0.0577	0.0004	1.82	0.01	0.9959	0.0007

In this way, it was determined that eye drops kept in refrigeration condition have an available period of at least 3 months. In the case of TLI 40, the presence of benzalkonium chloride may be beneficial to the stability of tacrolimus once opened by minimizing microbial contamination.

Prajapati et al. studied tacrolimus degradation in aqueous HPβCD solutions [27]. Their results showed an inversely proportional correlation between tacrolimus degradation rate and HPβCD concentration values, with a maximum degradation value where no HPβCD was included into the formulations. The presence of 40% (*w*/*v*) HPβCD in the formulations used in the present study suggested that the increased stability of tacrolimus was due to the inclusion of complex formation between HPβCD and tacrolimus.

Likewise, pH and osmolality measurements showed no significant changes over the course of the study regardless of storage condition ($\alpha > 0.05$). The results guaranteed that all tested formulations were also in the appropriate range for

topical ophthalmic administration, ensuring suitability for an accurate tacrolimus ocular penetration.

Furthermore, microbiological control growth is a test to take into account the quality control of the prepared formulations since it ensures the sterility of the preparations and, therefore, the conditions of asepsis in the production process. Sterility is one of the most important requirements when preparing ophthalmic formulations as it reduces the risk of eye infections.

In this study, an adequate conservation of the eye drops was observed for each condition since no presence of microorganisms was found in any of the studied samples. In addition, no macroscopic changes (e.g., color, turbidity and precipitation) were observed during the whole study period. Thus, the absence of microorganisms and suspended particles was in good agreement with the preparation of this type of topical ophthalmic formulations in HPDs, by a simple technique with non-strict equipment requirements.

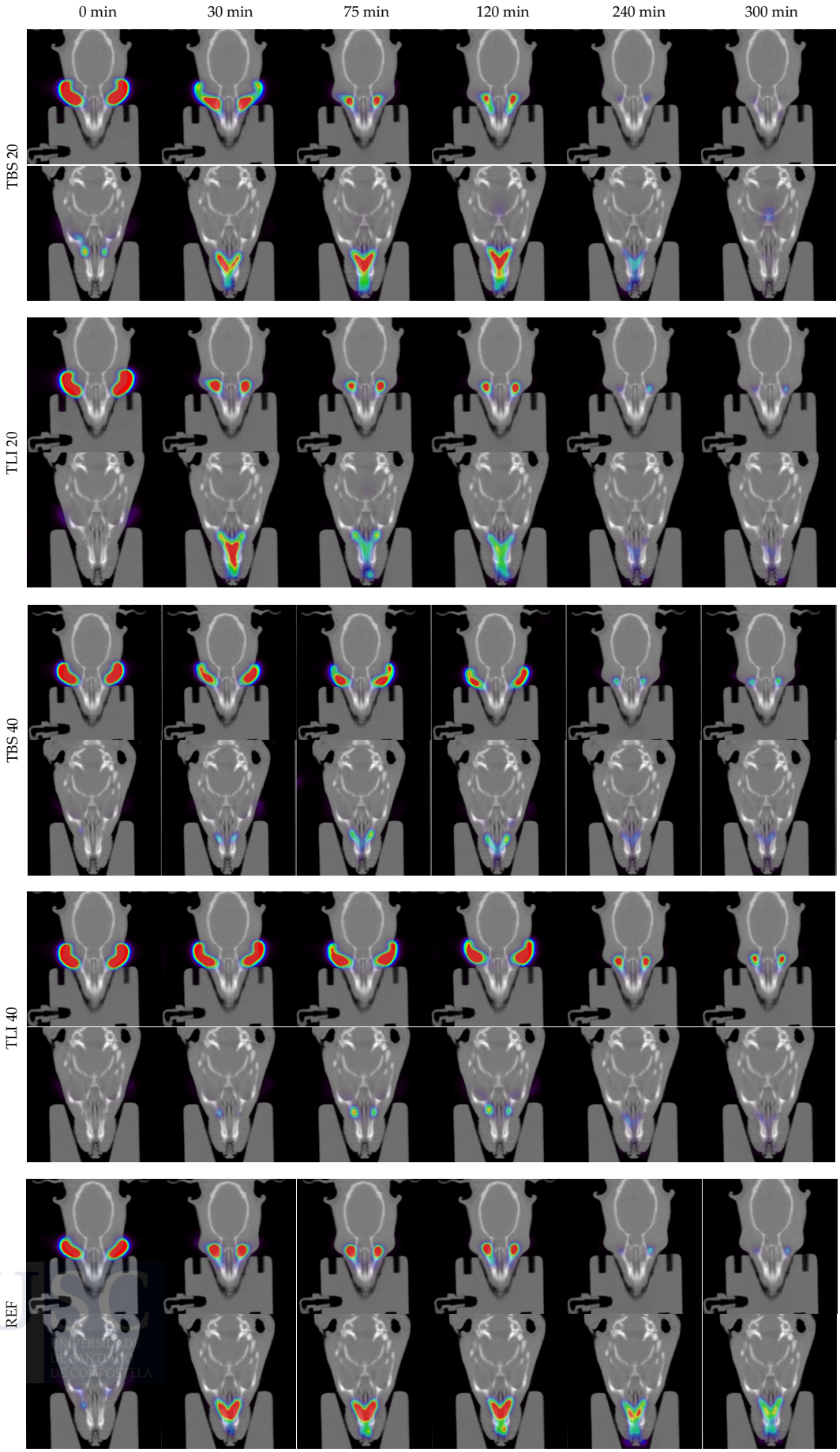
3.6. In Vivo Evaluation of the Residence Time on the Ocular Surface

The development of new ophthalmic topical vehicles for increasing drugs' permanence on the ocular surface is important to improve drug bioavailability in the eye as well as the treatment adherence by patients [81]. PET/CT imaging is a relatively new imaging modality that provides a quantifiable signal on the pharmacokinetic profile of the topically administered radiopharmaceutical. In this way, it allows to obtain a signal in the eye after an eye drop instillation and its subsequent follow-up at different study times, and to be able to follow the distribution of the formulation in the nasolacrimal duct and nasal cavity [62].

In this study, the PET/CT imaging technique was used to determine the residence times of the proposed tacrolimus topical ophthalmic formulations on the ocular surface, and they were compared with the tacrolimus eye drop (REF) elaborated by HPDs.

In Figure 14, the permanence of the studied eye drops at different times can be seen more visually, with PET/CT images showing both coronal planes (rat eyes and nasolacrimal duct).

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Figure 14. Fused positron emission tomography (PET)/computed tomography (CT) images displayed in coronal plane representing rat eyes (above) and nasolacrimal duct (below) with TBS 20, TLI 20, TBS 40, TLI 40 and REF at 0, 30, 75, 120, 240 and 300 minutes post-administration. The formulation signal on the eye surface is coded on a color scale: blue areas show low radioactive activity whereas red areas show high radioactive activity.

Figure 15 shows the clearance rate for each tested formulation compared to the tacrolimus eye drop (REF) elaborated by HPDs. The clearance rate was represented in terms of the ocular remaining radioactivity uptake over time after instillation and the corresponding fits in order to estimate all pharmacokinetic parameters (K, $t_{1/2}$, AUC_{0}^{∞} and MRT) and the remaining formulation at 75 min (%), which are represented in Table 4. All the parameters indicated a significant increase in the ocular retention time for the TBS 40 and TLI 40 formulations compared to TBS 20 and TLI 20; the best formulation result was obtained for TLI 40. The results from the ocular permanence assays showed that formulations containing higher concentrations of HPβCD had a longer eye residence time, regardless of the vehicle used. Adding HPβCD to formulations, apart from increasing the solubility and stability of tacrolimus, also gave a more viscous and stickier characteristic to the solution. This feature helps the eye drop to spread and be more retained on the eye surface as it can be seen during eye drop instillation. PET/CT studies showed that the formulations were mucoadhesive and had an adequate consistency to remain on the ocular surface for a long time.

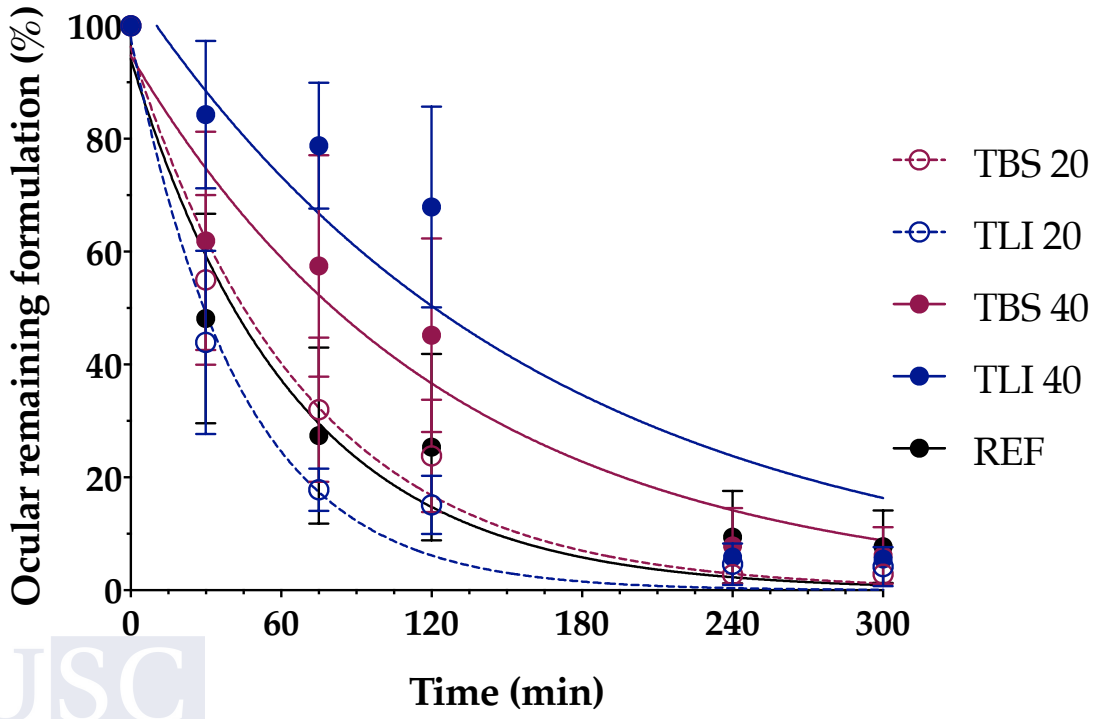


Figure 15. Tacrolimus eyedrop clearance rate (TBS 20, TLI 20, TBS 40, TLI 40 and REF) from the ocular surface determined by PET. Resulting data are represented in eye remaining radioactivity uptake (%) vs time after instillation.

A two-way ANOVA was applied to the % of remaining formulation on the ocular surface parameter in order to evaluate the influence of the time and the formulation in its clearance to determine whether there were differences in the permanence of the formulations on the corneal surface. No statistically significant differences were observed between 20% (*w*/*v*) HPβCD formulations compared to REF. Nevertheless, statistically significant differences were observed ($\alpha < 0.05$) between 20% (*w*/*v*) HPβCD formulations and 40% (*w*/*v*) HPβCD formulations as well as between 40% (*w*/*v*) HPβCD formulations and REF for times beyond 30 minutes post-administration.

Table 4. Pharmacokinetics parameters (*K*, $t_{1/2}$, $AUC_{0^{\infty}}$, mean residence time (MRT) and % remaining formulation at 75 min) of the tacrolimus eye drops obtained by the fitting of the percentage formulation remaining on ocular surface.

Formulation	*K* (min^{-1})		$t_{1/2}$ (min)		AUC $_{0^{\infty}}$ (%*min)		MRT (min)		Remaining Formulation at 75 min (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TBS 20	0.014	0.003	49.70	10.34	74.96	7.70	72.90	6.02	31.99	12.79
TLI 20	0.025	0.008	29.40	8.51	59.16	11.18	73.94	22.62	17.79	3.74
TBS 40	0.024	0.023	61.79	46.16	89.61	57.41	76.43	35.70	57.46	19.65
TLI 40	0.011	0.007	86.22	38.93	123.31	51.18	90.51	17.67	78.82	11.15
REF	0.018	0.010	46.34	22.88	82.22	30.2	93.28	37.55	27.44	15.65

The TLI 40 vehicle (Liquifilm®) is composed of PVA; this polymer 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) [82]. The mucoadhesion study assumptions related to TLI 40 formulation were confirmed by the PET/CT imaging technique.

The conventional pharmacological treatments for uveitis are associated with strict patient compliance, limited efficacy due to the appearance of refractory processes and different severe side effects. HPDs have resorted to a reformulation process of intravenous formulations as a way to obtain new topical ophthalmic pharmacy compounding as a pharmacological alternative. One of the most used pharmacy compounding products was based on Prograf® reformulation, this being an hydroalcoholic eye drop containing 0.03% (*w*/*v*) tacrolimus. Nevertheless, this type of treatment has shown different disadvantages, including high osmolality and patient discomfort due to the eye drop composition.

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Different formulations are proposed in the present work as new pharmacological alternatives, specifically intended for topical ophthalmic administration for uveitis treatment. These formulations have proven to be adequate for this administration pathway, showing several advantages compared to the currently used treatments, including I) patient comfort improvement, II) great efficacy with a simple preparation method, III) easy translational research to HPDs and IV) a health expenditure reduction in uveitis treatment.

4. CONCLUSIONS

In this work, a consistent tacrolimus solubilization study was carried out as a way to deeply understand this drug behavior in future topical ophthalmic formulations. Tacrolimus/HPβCD interaction in solution was confirmed by phase solubility diagram, NMR and molecular modeling studies, and the influence of the vehicle was also studied. The use of 40% (*w*/*v*) HPβCD allowed to prepare eye drop solutions with a 0.02% (*w*/*v*) tacrolimus concentration that could be in the therapeutic range for uveitis treatment.

The developed HPβCD-based formulations showed pH, osmolality, surface tension and safety values in the optimum range for topical ophthalmic administration. Stability studies showed no changes in the eye drops kept in refrigeration condition for at least 3 months, which could facilitate the preparation programming and improve the patient comfort.
Additionally, *ex vivo* mucoadhesion and *in vivo* ocular permanence studies showed good mucoadhesive properties and lower ocular clearance for TBS 40 and TLI 40, almost doubling the permanence half-life time in the ocular surface compared to the REF pharmacy compounding.

Taking into account all of the obtained results, TLI 40 was proposed as the best candidate. This eye drop allowed reaching a 0.02% (*w*/*v*) drug dose, which was safe and showed the best mucoadhesive and ocular permanence properties. In addition, the presence of benzalkonium chloride in Liquifilm® could help to prevent microbial growth once the eye drops were opened.

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CHAPTER 4

ANTI-INFLAMMATORY EFFECT OF TACROLIMUS/HYDROXYPROPYL-β-CYCLODEXTRIN EYE DROPS IN AN ENDOTOXIN-INDUCED UVEITIS MODEL

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ANTI-INFLAMMATORY EFFECT OF TACROLIMUS/HYDROXYPROPYL-β-CYCLODEXTRIN EYE DROPS IN AN ENDOTOXIN-INDUCED UVEITIS MODEL

ABSTRACT

Uveitis is an infrequent disease which constitutes a major cause of ocular morbidity. Correct management is essential, being corticosteroids its cornerstone. In case of contraindication to corticosteroids or treatment failure, the use of topical tacrolimus (TAC) could be an alternative which has already demonstrated safety and effectiveness in other ocular pathologies. However, TAC eye drops are not marketed, thus their elaboration must be carried out in Hospital Pharmacy Departments (HPDs). Thirty-two Sprague-Dawley rats were divided into 4 groups of 8 rats each: a) untreated healthy rats (Healthy); b) untreated Endotoxin-Induced Uveitis model-rats (EIU); c) EIU-rats treated with standard treatment of dexamethasone ophthalmic drops (DXM) and d) EIU-rats treated with TAC-hydroxypropyl-β-cyclodextrin eye drops previously developed by our group (TAC-HPβCD). The mRNA expression levels of IL-6, IL-8, MIP-1α and TNF-α, quantitative analysis of leucocytes in aqueous humor and histological evaluation were performed. TAC-HPβCD eye drops demonstrated to reduce ocular inflammation, expression of IL-6, TNF-α, MIP-1α and leukocyte infiltration in aqueous humor. TAC-HPβCD eye drops showed beneficial effect in EIU model in rats, positioning as an alternative for uveitis treatment in case of corticosteroids resistance or intolerance.

Keywords: endotoxin-induced uveitis; tacrolimus; eye drops; hydroxypropyl-β-cyclodextrin; interleukins; immunosuppressants.

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1. INTRODUCTION

Uveitis constitutes a heterogeneous group of intraocular inflammatory diseases which is defined as inflammation of the uveal tract that can secondarily affect adjacent structures, such as the retina or the optical nerve [1,2]. The overall prevalence and incidence of these conditions range from 5.81 to 19.42 per 10.000 subjects, and 2.51 to 11.12 per 10.000 person-years, respectively. [3,4]. Although it is an infrequent disease, it constitutes a major cause of ocular morbidity, since it is the third cause of blindness in developed countries, representing an important health problem [5,6].

From the pathophysiological point of view, the immune system plays a fundamental role in the development of uveitis, whether or not it is secondary to infectious processes [7]. In this sense, the origin of inflammation can be attributed to an endogenous mechanism, either as part of autoimmune systemic diseases or as an isolated ocular condition [8]. Concerning the etiology of non-infectious uveitis, in recent years there have been significant advances in the understanding of their pathogenic mechanisms, pointing to the interaction between environmental factors and a complex genetic background, triggering a deregulated immune response able to overcome the ocular immune privilege [9,10]. In this regard, different proinflammatory molecules are increased depending on the specific origin of the disease [11,12]. In addition, several genetic determinants which might predispose to uveitis have been reported, either focusing on a particular disease [9,13,14], or analyzing the shared genomic basis, regardless the diagnosis [15].

Uveitis correct management is essential for preserving visual function and avoiding ocular and extra-ocular morbidity [16]. In this way, patients are usually treated based on the presence of signs of inflammatory activity, being corticosteroids its cornerstone, due to their immediate efficacy [17]. Location and severity of the inflammation will influence the change from topical to oral administration route. However, long-term administration of these agents can cause serious systemic and ocular adverse effects, such as hypertension, diabetes, and glaucoma [18]. In addition, when patients do not respond to this therapy or have a contraindication for the use of corticosteroids, it is necessary to resort to ophthalmic topical immunosuppressants in order to achieve a sustained control of the inflammatory process [17,19]. In this context, the use of topical tacrolimus (TAC) could be an alternative which has already demonstrated safety and effectiveness in other ocular pathologies including corneal graft rejection [20], vernal keratoconjunctivitis (VKC) [21–24], dry eye [25] or scleritis [26,27] among others.

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TAC is a calcineurin inhibitor with a potent immunosuppressive effect which has a mechanism of action similar to that of cyclosporine, but it is 10–50 times more potent, having demonstrated in clinical studies more effectiveness at lower concentrations and a better safety profile [19,28,29].

Regarding uveitis, TAC has showed a positive effect on the progression of the disease through reduction of inflammatory activity and suppression of the entire pathogenic pathway of the disease [28]. It inhibits the expression of Toll-like receptors (TLRs), macrophage inflammatory protein-1 alpha (MIP-1α), T-cell proliferation and the release of inflammatory cytokines such as interleukine-6 (IL-6), interleukine-8 (IL-8), and tumor necrosis factor alpha (TNFα), which have a key role in the development of uveitis [7,28,30–32]. Although TAC has demonstrated to be effective in patients with refractory uveitis through oral [33] or intravitreal administration [34,35], topical application would be preferred because it avoids adverse effects related to systemic exposition to this drug and local complications after intraocular injections.

Despite all the above, the use of topical TAC for the treatment of uveitis has been limited because, nowadays TAC eye drops are not globally marketed. Consequently, this elaboration must be carried out in Hospital Pharmacy Departments (HPDs). Due to the deficient physicochemical properties of TAC, including low aqueous solubility and high molecular weight (804.02 g/mol), TAC eye drops must be prepared by reformulation from intravenous drug presentation (Prograf®) which contains ethanol and other compounds which usually cause discomfort and unpleasantness to patients [22].

In the last decade, several formulations of TAC for topical ophthalmic administration have been synthetized, including niosomes [36], nanovesicles [37], micelles [38] and nanocapsules [39]. However, it is not possible their translation to clinical practice because their synthesis is very complex to be carried out in HPDs. Most services do not have the necessary infrastructure to be able to develop these systems. The absence of specific equipment in production and quality control makes it impossible to produce these formulations optimally, and the personnel in charge of their production are not adequately trained to perform the necessary techniques in these processes.

In this sense, our group has previously developed and characterized a topical ophthalmic formulation of tacrolimus-hydroxypropyl-β-cyclodextrin (TAC-HPβCD) eye drops containing a 0.02% (*w*/*v*) of tacrolimus and a 40% (*w*/*v*) of HPβCD in Liquifilm®, demonstrating positive effects in terms of drug solubility, stability in aqueous solution and retention time on the ocular surface [40]. This formulation improves the currently prepared eyedrops in HPDs by replacing the irritating compounds of the intravenous drug presentation for HPβCD, which is

the most appropriated cyclodextrin for topical ophthalmic administration according to the European Medicines Agency (EMA) [40,41].

The aim of this work is to demonstrate the anti-inflammatory effect of a TAC-HPβCD eye drops formulation, previously developed by our group, in an endotoxin-induced uveitis model in rats to provide for the first time a translational alternative for the elaboration of TAC eyedrops in HPDs.

2. MATERIALS AND METHODS

Tacrolimus powder was acquired from Guinama® S.L.U. (La Pobla de Vallbona, Spain), 2-hydroxypropyl-β-cyclodextrin Kleptose® HPB (HPCD; MW = 1399 Da, substitution degree = 0.65 molar ratio) was provided from Roquette Laisa S.A.® (Valencia, Spain), Liquifilm® was purchased from Allergan® Pharmaceuticals Ireland (Mayo, Ireland), Maxidex® (1 mg/mL, eye drops) was purchased from Alcon Cusí, S.A.® (Barcelona, Spain) and Lipopolysaccharide endotoxin (LPS; *Escherichia coli.*) was obtained from Sigma-Aldrich (San Louis, MO, USA). All other chemicals and reagents were of the highest purity grade commercially available.

2.1. Formulation Elaboration Procedure

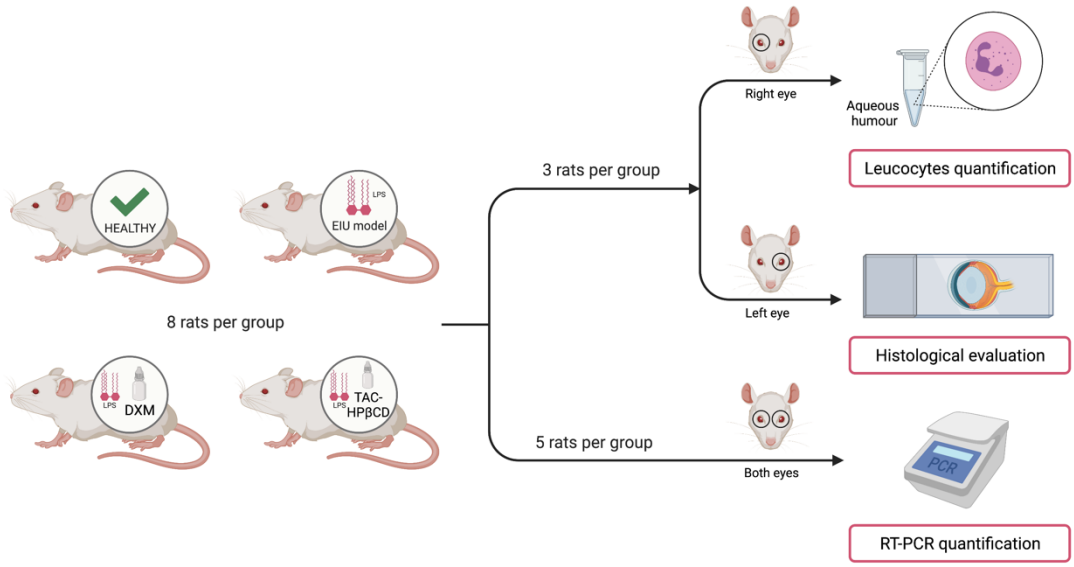
The entire elaboration process of TAC-HPβCD ophthalmic eyedrops was carried out under sterile conditions in a flow laminar cabinet in the HPD of the University Clinical Hospital of Santiago de Compostela. First, the indicated proportion of hydroxypropyl-β-cyclodextrin (40% *w*/*v*) is dissolved in Liquifilm® by stirring, when it was completely solubilized tacrolimus powder was added at a proportion of 0.02% (*w*/*v*) and left for 72 h under agitation (>750 rpm). Once this time has elapsed, the formulation is filtered through a 0.2 μm polyether sulfone (PES) filter (Stericup filtration system), and then packaged in sterilized polypropylene eyedropper bottles, showing to be stable for at least 3 months under refrigeration [40].

2.2. In Vivo Endotoxin-Induced Uveitis Model (EIU Model)

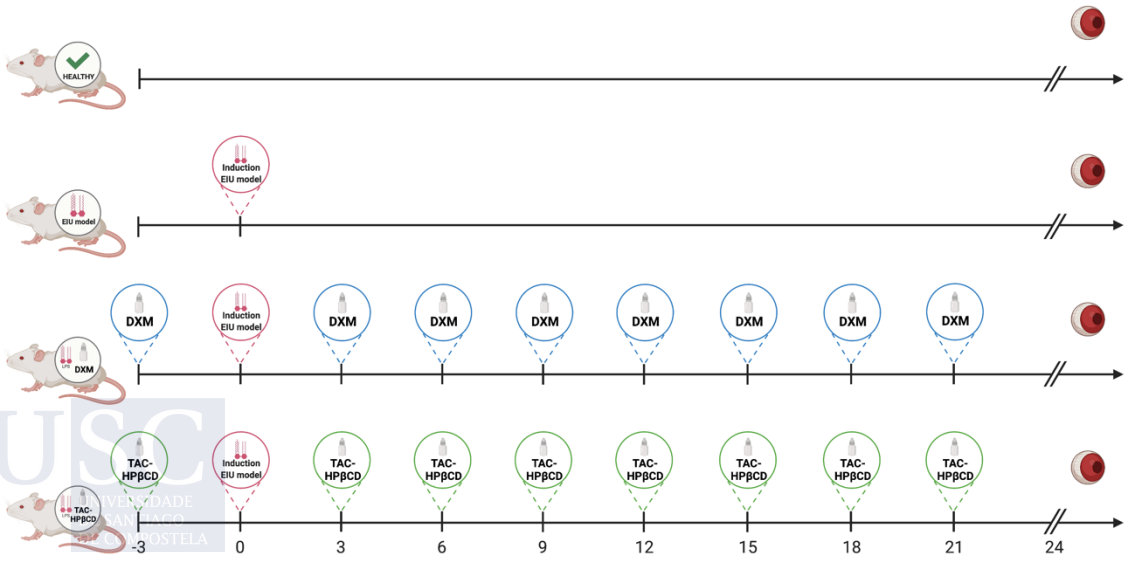
In vivo studies were carried out on male Sprague-Dawley rats with an average weight of 250 g supplied by the animal facility at the University of Santiago de Compostela (CE- BEGA) (Santiago de Compostela, Spain). The animals were kept in cages under controlled temperature (22 ± 1 ◦C) and humidity (60 ± 5%), with day–night cycles regulated by artificial light (12/12 h) and feeding ad libitum. The animals were treated according to the ARVO statement for the use of animals in ophthalmic and vision research as well as the approved guidelines for laboratory animals [42,43].

The EIU was induced inoculating 1 mg/kg *Escherichia coli.* LPS diluted in 0.1 mL balance salt solution (BSS) into the rat's right paw as previously described by other authors [44,45]. Once injected, the ocular inflammation peak reaches the maximum after 24 h [46].

In this case, 32 rats were divided into 4 groups of 8 rats each, as indicated in Figure 1. (**a**) untreated healthy rats (Healthy); (**b**) untreated EIU-rats (EIU); (**c**) EIU-rats treated with standard treatment of dexamethasone ophthalmic drops (DXM) (Maxidex®, dexamethasone 1 mg/mL, Alcon Cusí, Barcelona, Spain) and (**d**) EIU-rats treated with TAC-HPβCD eye drops (TAC-HPβCD). Animals were anesthetized with 2.5% (*v*/*v*) isoflurane/oxygen and treatments were topically instilled (20µL) on each eye 3 h before EIU model induction and every 3 h until 24 h later. Then, rats were euthanatized with carbon dioxide and all required samples were extracted.



(**a**)



(**b**)

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Figure 1. a) Study groups scheme and distribution of eyes for their posterior analysis. **b)** Study timeline: Induction EIU model, treatments, euthanasia and eyes removal. Created with BioRender.com.

2.3. RNA isolation and real-time PCR analysis

The mRNA expression levels of IL-6, IL-8, MIP-1α and TNFα were evaluated in both eyes of the remaining 5 rats of each group by real time-PCR. After extraction, eyeballs were frozen at -20°C and total RNA was isolated using TRIzol reagent (Invitrogen, Barcelona, Spain). Real-time PCR was made with 2 μg cDNA in a 20 μl volume using Luminaris Color HiGreenqPCR Master Mix (Fisher Scientific, Rockford, USA). Samples were denatured at 94 °C for 10 s, annealed at 58 °C for 10 s and extended at 72 °C for 10 s for a total of 35 cycles. The amount of PCR products formed in each cycle was evaluated based on SYBR Green fluorescence with 18S as the endogenous control. Oligonucleotide sequences are detailed in Table 1.

Table 1. Primer sets for real-time PCR.

Primers	Forward (5´-3´)	Reverse (5´-3´)
IL-6	CTTCAGGCCAAGTTCAGGAG	AGTGG ATCGTGGTCGTCTTC
IL-8	TCCAGCCAGTTGCCTTCTTG	GGTCTGTTGTGGGTGGTATCC
MIP-1α	CGTCCTCATCCTGATCACCT	GATACATCCCCGTGAACACC
TNFα	CCAGATGGTCACCCTCAGAT	CCTTGACCG CTGAAGAGAAC
18S	GTAACCGCTTGAACCCCATT	CCATCCAATCGCTAGTAGCG

2.4. Quantitative analysis of leucocytes in aqueous humor

For leucocyte determination, an expert ophthalmologist collected aqueous humor (10 μL) from the anterior chamber of the left eye of 3 rats from each group using a 28-gauge needle. Aqueous humor was stained (1:5) with Turk's solution (Merk, Darmstadt, Germany) for leucocyte counting in a Neubauer chamber (Brand GMBH þ CO KG, Wertheim, Germany). Each aqueous humor sample was evaluated in triplicate.

2.5. Histological evaluation

For histological evaluation, right eyes of 3 rats of each group were fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin routinely. Sections of 4 μm thick were stained with hematoxylin and eosin using a CoverStainer system (Dako-Agilent, Glostrup, Denmark). Six sections from each eye were examined to perform semiquantitative analysis by an

anatomopathologist expert. Leucocytes surrounded ciliary processes were taken into account for counting and photographed using an Olympus BX51 microscope equipped with a MC170 camera (Leica, Wetzlar, Germany).

2.6. Statistical analysis

Values were expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). Average values were compared using one-way ANOVA with Tukey´s multiple comparison test. P values of less than 0.05 were considered statistically significant. Graph Pad Prism (Version 8.0.1) software was used for all calculations.

3. RESULTS

3.1. Evaluation of the effect of tacrolimus on IL-6, IL-8, MIP-1α, and TNFα

The mRNA presence of the pro-inflammatory cytokines (IL-6, IL-8, MIP-1α, and TNFα) in the eye tissues was assessed by real time-PCR in order to evaluate the effect of TAC-HPβCD on factors involved in the inflammatory process. The levels of cytokines in the eye tissues of healthy, EIU, DXM and TAC-HPβCD groups are shown in Figure 2 and the statistical significance of the different levels detected among groups is depicted in Table 2.

EIU group had considerable high levels of IL-6 (138.89 ± 89.71), IL-8 (6.53 ± 5.36), MIP-1α (217.37 ± 113.15), and TNFα (12.58 ± 3.25) compared to healthy group [IL-6 (0.78 ± 0.68), IL-8 (1.14 ± 0.14), MIP-1α (1.20 ± 0.18) and TNFα (1.22 ± 0.34)]. Consequently, the augmentation of all these factors was statistically significant in the untreated EIU group as compared with healthy rats, which means that the EIU model was properly induced.

On the other hand, DXM group [IL-6 (1.29 ± 1.59), IL-8 (5.18 ± 5.09), MIP-1α (56.88 ± 54.70); TNFα (7.31 ± 5.52)] and TAC-HPβCD group [IL-6 (28.69 ± 21.24); IL-8 (2.92 ± 2.73); MIP-1α (59.26 ± 41.43); TNFα (3,57 ± 2.74)] had substantial fewer values of all the analyzed pro-inflammatory cytokines compared to EIU group. Hence, treatment with TAC-HPβCD and DMX significantly decreased levels of all these factors as compared with EIU group except for IL-8 whose reduction did not achieve statistical significance neither TAC-HPβCD group nor DMX.

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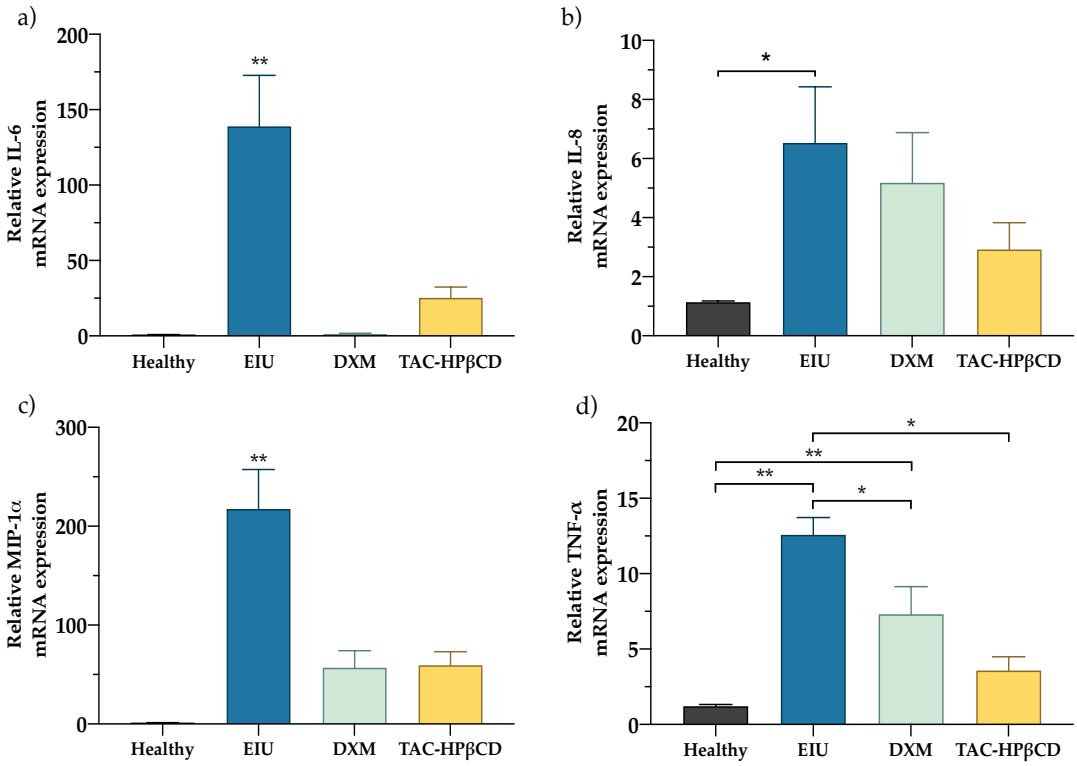


Figure 2. Anti-inflammatory *in vivo* effect (a) IL-6 (b) IL-8 (c) MIP-1α and (d) TNFα mRNA expression in healthy eyes (Healthy), EIU untreated rats (EIU), EIU rats treated with dexamethasone (DXM) and EIU rats treated with TAC-HPβCD (TAC-HPβCD). (n = 10 eyes per group). Bars represent mean ± SD (*α <0.05; ** α <0.005).

Table 2. Existence (YES) or absence (NO) of statistical significance calculated through a Tukey´s multiple comparison test among Healthy, EIU, DXM and TAC-HPβCD groups (*α <0.05; ** α <0.005).

Tukey´s multiple comparison test	**IL-6**	**IL-8**	**MIP-1α**	**TNFα**
Healthy vs EIU	YES**	YES*	YES**	YES**
Healthy vs TAC-HPβCD	NO	NO	NO	NO
Healthy vs DXM	NO	NO	NO	YES**
EIU vs TAC-HPβCD	YES**	NO	YES**	YES**
EIU vs DXM	YES**	NO	YES**	YES*
TAC-HPβCD vs DXM	NO	NO	NO	NO

Regarding the comparison of the detected levels of pro-inflammatory cytokines between healthy rats and TAC-HPβCD no statistically significant differences were found for IL-6, IL-8, MIP-1α levels, as well as between healthy rats and DMX group. This, along with the fact that no statistically significant differences were neither found among pro-inflammatory cytokines levels of TAC-HPβCD and DXM group demonstrate that TAC-HPβCD eye drops could be an alternative to topical corticosteroid therapy in the treatment of this model of EIU and their clinical translational use should be studied in future studies. In

addition, it should be highlighted that with regard to TNFα, statistically significant differences were detected between healthy rats and DXM group but no between healthy rats and TAC-HPβCD group. This fact suggest that TAC could be a better option than DXM when the uveitis is related with an elevation of TNFα.

3.2. Quantitative analysis of leucocytes in aqueous humor

Leukocyte counts in aqueous humor of healthy, EIU, DXM and TAC-HPβCD groups are depicted in Figure 3 and 4. In this sense, EIU group showed a significantly higher number of leukocytes (44.9 ± 11.0 cells/mL) compared to healthy group (0.2 ± 0.4 cells/mL) (α < 0.005), supporting the adequate development of the uveitis model. On the other hand, regarding leucocytes count of TAC-HPβCD group (27.55 ± 5.19 cells/mL), there is a considerable reduction in the number of leucocytes as compared with EIU group, being this difference statistically significant (α < 0.005) and positioning this therapy as a possible alternative to DXM treatment in uveitis. However, the decrease in the number of leucocytes is significantly greater in DXM group with regard to TAC-HPβCD group (α < 0.005), achieving a number of leucocytes comparable to healthy group.

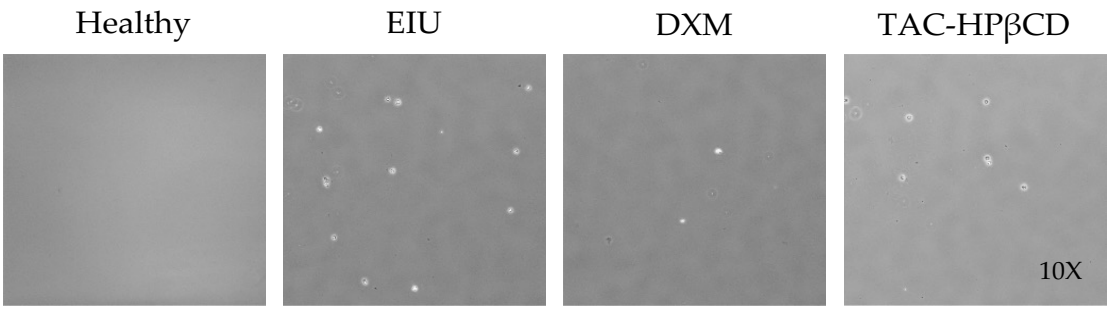
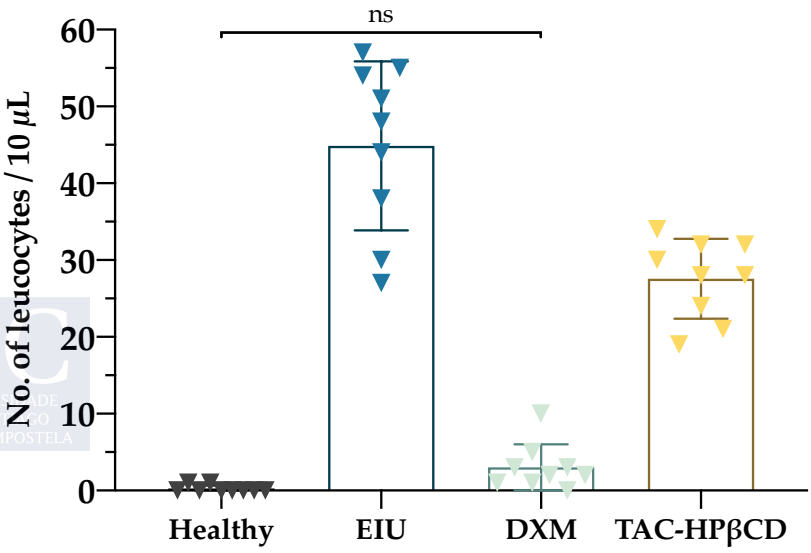


Figure 3. Leucocyte counting in aqueous humor 24 h after LPS injection in a Neubauer chamber. Groups were healthy eyes (Healthy), EIU untreated rats (EIU), EIU rats treated with dexamethasone (DXM) and EIU rats treated with TAC-HPβCD (TAC-HPβCD) (n = 3 eyes).

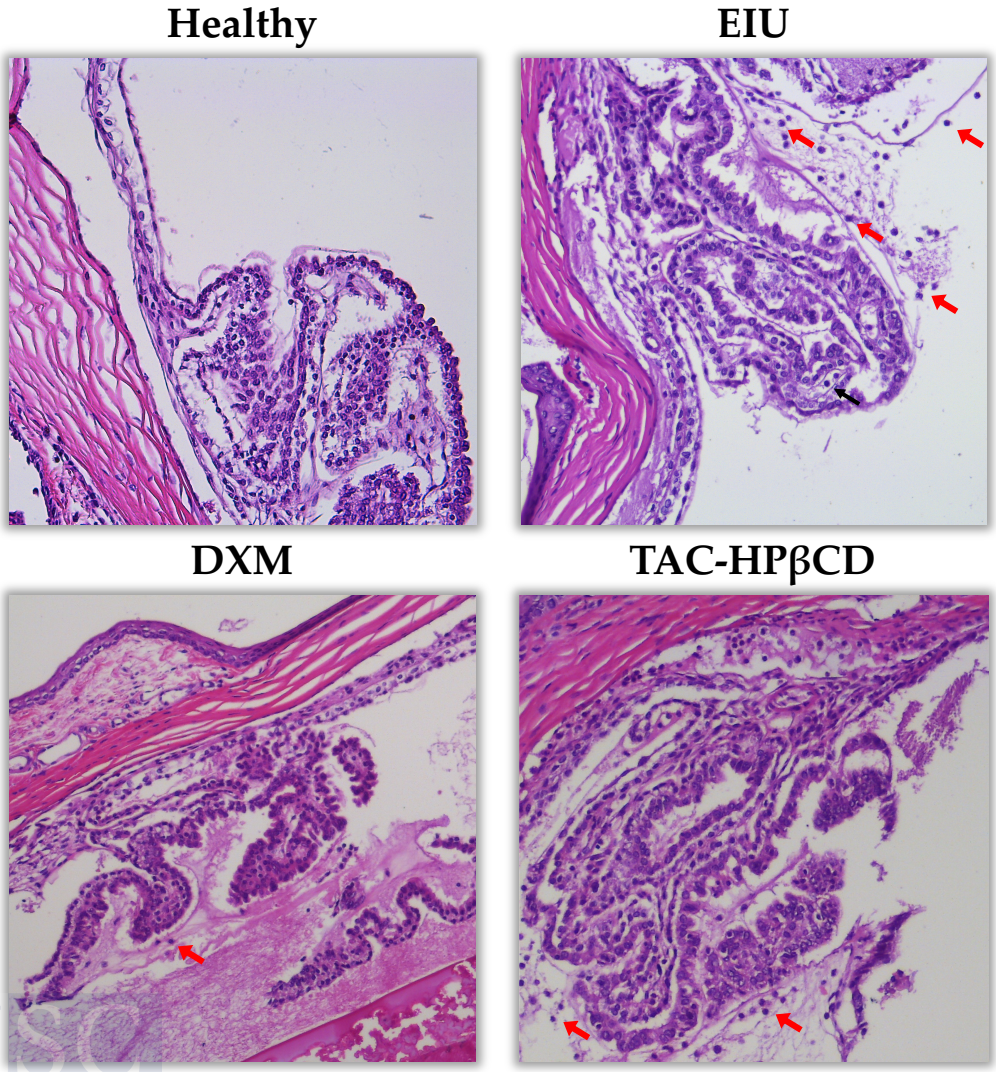


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Figure 4. Quantitative analysis of leucocytes in aqueous humor. Groups were healthy eyes (Healthy), EIU untreated rats (EIU), EIU rats treated with dexamethasone (DXM) and EIU rats treated with TAC-HPβCD (TAC-HPβCD) (n = 3 eyes per group measured in triplicate). Bars represent mean ± SD. There are statistically significant differences among all groups except between Healthy and DXM group whose difference is non-significant (ns).

3.3. Histological evaluation

As it can be seen in Figure 5, the histological evaluation of retinas and ciliary processes showed no infiltration of leucocytes in healthy rats with no LPS injection. Very few or no leucocytes were found in DXM group while some leukocytes can be observed in TAC-HPβCD group. However, there was a considerable higher infiltration of leucocytes in untreated EIU-rats as compared with DXM and TAC-HPβCD groups.



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Figure 5. Representative hematoxylin-eosin staining showing infiltrated leucocytes (marked with arrows) in retina and ciliary processes of healthy, EIU, DXM and TAC-HPβCD groups. The histology study was done 24 h after LPS injection.

4. DISCUSSION

Nowadays, TAC is commercially available in oral and intravenous formulations, used in transplanted patients, and as an ointment for the treatment of atopic dermatitis [47]. TAC eyedrops can constitute an alternative in the treatment of uveitis [48,49] because it has been successfully used for various immune-mediated ocular disorders such as graft rejection [20], vernal keratoconjunctivitis (VKC) [21–24], dry eye [25] or scleritis [26,27]. However, TAC eyedrops are not marketed and they must be elaborated in HPDs. In this sense, it is necessary to highlight that TAC is a highly lipophilic macrolide lactone with a very poor water-solubility (1–2 μg/ml) [50]. This fact constitutes an important hurdle because TAC must be dissolved to allow efficient transport or permeation and reach the target site to become therapeutically effective [51].

Another inconvenience of TAC is that it has a high susceptibility to hydrolysis which leads to a very low stability in aqueous solutions [28,29]. For these reasons, TAC eye drops must be elaborated in HPDs by reformulation of the intravenous drug presentation (Prograf®), containing ethanol which have showed to disrupt the integrity of corneal epithelium and induce inflammation in corneal cells [52]. Based on this situation, in recent years several formulations of TAC for topical ophthalmic administration have been developed. In this sense, niosomes [29] and micelles [31] have been synthetized but they were not tested *in vivo*. However, positive effects of ophthalmic TAC were recently reported for nanovesicles [30] and nanocapsules [32].

Our group has previously developed a formulation of TAC-HPβCD eye drops containing a 0.02% (w/v) of TAC and a 40% (w/v) of HPβCD in Liquifilm®, which can be safely prepared in HPDs and showed to be stable for at least 3 months under refrigeration [40]. The major advantage of TAC-HPβCD eye drops is that there is no need of using irritating excipients since our formulation strategy increases the solubilization of poorly soluble active ingredients and reduces the use of toxic products.

In order to evaluate our formulation, a EIU model was selected because it is easily reproducible, quantifiable and clinical changes are similar to those of human acute uveitis [37]. Some authors stated that after LPS administration there is an activation of TLRs, increasing levels of proinflammatory mediators, developing inflammation of anterior uvea, choroid and retina and producing the breakdown of blood-humor barrier, with exudation of leukocytes into the aqueous humor [53,54]. In this sense, significantly higher levels of TNFα, IL-6, IL-8, MIP-1α and leukocytes, as well as histological evaluation of EIU group, compared to healthy group confirmed the correct development of the uveitis model.

According to its mechanism of action, TAC inhibits calcineurin, blocks the production of proinflammatory cytokines and leads to inhibition of Th1 and Th2 cell activation [28,55]. In this regard, significantly lower levels of TNFα, IL-6, MIP-1α and leukocytes, as well as histological evaluation of TAC-HPβCD compared to EIU group confirms the effectiveness of this formulation in the treatment of uveitis. Other authors stated that ophthalmic treatment with TAC solution in light mineral oil, a vehicle where this active substance is not adequately dissolved, was not able to improve any parameter related with clinical manifestation of uveitis comparing to a EIU model [37]. Consequently, our positive therapeutic effects using TAC-HPβCD demonstrate the proper solubilization of TAC in our formulation.

Similarly, TAC proglycosome nanovesicles were synthetized by Garg et al., showing promising results in a rabbit EIU model since they achieved suppression of expression of TNFα and IL-6 in aqueous humor, and reduction in ocular inflammation, leukocyte infiltration and protein leakage into aqueous humor comparing with the administration of ophthalmic TAC solution [30]. Besides, Rebibo et al. elaborated TAC-loaded nanocapsules enabling a superior anti-inflammatory effect on an anterior chamber LPS-induced murine model of keratitis in comparison to the drug in oil solution and with a significant decrease in macrophage inflammatory protein 2 and IL-6, among other evaluated inflammatory markers [32].

Regarding the comparison between the efficacy of our formulation and the standard treatment with dexamethasone, no statistically significant differences were found among pro-inflammatory cytokines levels of TAC-HPβCD and DXM group demonstrating that TAC-HPβCD eye drops could be an alternative to topical corticosteroid therapy in the treatment of this model of EIU and their clinical translational use should be studied in future studies. It is necessary to highlight that Rebibo et al. and Garg et al., unlike us, did not compared their formulations with corticosteroid standard treatment [30,32].

In addition, concerning TNFα mRNA expression, its reduction is statistically significant only between TAC-HPβCD and EIU group but no between DXM and EIU group, which is consistent with TAC mechanism of action [56] and could be especially beneficial in uveitis related to the augmentation of this factor, such as HLA-B27-associated uveitis, sarcoid uveitis and uveitis associated with Behcet's disease among other noninfectious uveitis entities [11,12,57].

This possible personalization of the therapy based on the elevation of certain proinflammatory molecules could constitute an important advance, above all if this local increase could be correlated with systemic increases and detected through a blood test.

With respect to the limitations of our study, the fact that EIU model achieves the maximum inflammation 24 h after injection [36,37] makes necessary to carry out an accelerated dosage regimen and only permits a short-term evaluation which could constitute a hurdle regarding the translation of these results to the clinical setting. Another limitation is the lack of knowledge of the amount and rate of tacrolimus transcorneal permeation. Therefore, it will be necessary to study the intraocular pharmacokinetics of the TAC-HPβCD formulation with a suitable analytical method.

In spite of the fact that other ophthalmic TAC formulations have shown *in vivo* efficacy [37,39], as long as they are not commercialized, their translation to clinical practice is very difficult because of the high complexity of their synthesis. However, our TAC-HPβCD formulation has demonstrated *in vivo* efficacy and it can be easily elaborated in HPDs due to the simplicity of the elaboration process.

Regarding the difference of cost associated between TAC eye drops usually prepared in HPDs by reformulation of Prograf® and TAC-HPβCD eye drops, an estimate of the expenditure has been made in the Hospital Pharmacy Service for the two formulations in which the cost would be reduced by 200 euros for each patient and year with the implementation of the TAC-HPβCD formulation.

Future studies are needed to confirm the clinical effectiveness of TAC-HPβCD in order to consider this formulation as an alternative for the treatment of some forms of uveitis. The next step will be to conduct a study with patients in which the TAC-HPβCD formulation will be tested by comparing it with the results obtained in this group's previous work [22] with the TAC formulation used in HPDs.

5. CONCLUSIONS

TAC-HPβCD eyedrops showed beneficial effect in EIU model in rats, thus they could be considered an alternative for uveitis treatment in case of corticosteroids resistance or intolerance. This formulation demonstrated to reduce ocular inflammation, expression of IL-6, TNFα, MIP-1α and leukocyte infiltration in aqueous humor. In addition, the simple elaboration process of TAC-HPβCD makes possible to prepare this formulation in HPDs, improving in terms of safety the current elaborated TAC eyedrops by reformulation from intravenous drug presentation which contains ethanol and other irritating excipients. Further clinical studies are needed in order to evaluate long-term effectiveness of TAC-HPβCD.

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CHAPTER 5

PET STUDY OF INTRAVITREAL ADALIMUMAB PHARMACOKINETICS IN A UVEITIS RAT MODEL

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CHAPTER 5

PET STUDY OF INTRAVITREAL ADALIMUMAB PHARMACOKINETICS IN A UVEITIS RAT MODEL

ABSTRACT

Adalimumab is an anti-TNFα drug approved for uveitis treatment by subcutaneous injection. This administration route exposes patients to systemic adverse effects and makes difficult to obtain therapeutic drug concentrations in the site of action due to the anatomical and physiological barriers of the eye. These inconveniences could be avoided by intravitreal injection. The aim of this study is to evaluate the pharmacokinetic profile and the distribution of the intravitreal administration of ^{89}Zr-adalimumab in a uveitis rat model using PET imaging. Adalimumab was radiolabeled to ^{89}Zr with a maximum specific activity of 10 MBq/mg. Four μL containing ≃1.74 MBq of ^{89}Zr-labelled adalimumab were injected into the vitreous. A microPET acquisition was carried out immediately after the injection and at different time points through a 10-day study and blood samples were obtained through the tail vein. Radiolabeling was successfully performed with a radiochemical purity after ultrafiltration of 99.69%. The antibody ocular pharmacokinetics followed a one-compartment model, showing an intraocular elimination half-life of 15.57 hours for healthy rats and 33.64 hours for rats with uveitis, implying that ^{89}Zr-adalimumab remains around two times longer in rats with the disease compared to healthy ones. However, blood concentration half-life had similar values in both groups. In conclusion, this study shows for the first time the ocular and blood pharmacokinetic analysis of adalimumab in a uveitis model in rats.

Keywords: adalimumab; anti-TNFα; pharmacokinetics; molecular imaging; Positron Emission Tomography (PET); endotoxin-induced uveitis

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1. INTRODUCTION

Uveitis includes multiple heterogeneous clinical entities of ocular diseases which are characterized by the inflammation of the uveal tissue, involving iris, ciliary body and choroid [1,2]. With regard to epidemiology, there is a wide variability in reported data with an estimated incidence between 17 and 52 per 100 000 of population per year, and a prevalence of 38–714 cases per 100 000 of population [2–4]. In spite of its rarity, this eye disease is an important cause of ocular morbidity, being the third leading cause of blindness in developed countries [5,6].

Non-infectious uveitis requires in many cases corticosteroids and immunosuppressive therapy, which can effectively control the inflammation. Depending on the location and severity of the inflammation, topical eye drops may not be enough and systemic oral administration has to be performed [7]. Long-term treatment with these agents can cause severe side effects, such as renal and hepatic toxicity, hypertension, and hematologic abnormalities [8]. Tumor necrosis factor alpha (TNFα) inhibitors constitute an option in these patients due to its promising mechanism of action [9,10]. The presence of TNFα in the serum and aqueous humor of subjects with active uveitis has been demonstrated in clinical and preclinical studies [8,11–14]. Specifically, it has been reported that TNFα may induce the expression of adhesion molecules, chemokines and cytokines which are involved in inflammation. Consequently, inhibition of TNFα produce results in suppression of infiltrating macrophages, hence preventing tissue destruction [8–10].

At present, there is already an approved anti-TNFα drug (adalimumab) for the treatment of uveitis that has shown promising results [15]. However, its subcutaneous administration entails two major inconveniences. On the one hand, the patient is exposed to adverse effects associated with the systemic exposition to the drug and, on the other hand, it is difficult to obtain therapeutic drug concentrations in the site of action due to the anatomical and physiological barriers of the eye, which is relatively isolated from the systemic circulation by the tight blood-retinal barrier [16–18].

To circumvent these limitations, a clinical trial has been carried out in order to compare intravitreal vs subcutaneous administration of adalimumab in uveitis patients, but without reporting results [19]. Furthermore, intravitreal administration has been explored in other ocular diseases such as choroidal neovascularization secondary or diabetic macular retinopathy [20,21].

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In the clinical setting, the dose and frequency of adalimumab administration are based on concentrations obtained from plasma and not on intravitreal

pharmacokinetic studies due to the invasiveness of collecting vitreous samples. In this sense, the study of intravitreal adalimumab pharmacokinetics in humans should be preceded by a detailed characterization of its pharmacokinetics in preclinical models of uveitis providing information on drug concentration not only in the blood, but more importantly at the site of action (eye) and different organs and tissues of the whole body.

In this regard, intravitreal pharmacokinetic preclinical studies are usually carried out collecting vitreous samples over time on animals sacrificed at different time points. According to the 3Rs principles in animal experimentation [22], these studies can be efficiently performed using Positron Emission Tomography (PET), which represents a promising non-invasive imaging tool for the evaluation of the pharmacokinetic and distribution of antibodies. PET pharmacokinetic studies provide images showing the *in vivo* targeting and off-targeting of the antibodies and a follow-up over time in the same animal, avoiding the collection of multiple vitreous samples over time and other tedious procedures, and drastically reducing the number of animals needed.

Furthermore, these studies provide quantitative information in real time, enabling to estimate the pharmacokinetic profile by carrying out longitudinal studies with several measures of each animal over time [23]. However, this technique requires previous steps that can be challenging, mainly those related to the radiolabeling of the antibodies. On this basis, Zirconium-89 (^{89}Zr) is the PET radionuclide of choice for the radiolabeling of antibodies because of its long half-life (3.3 days), which is favorable for evaluating the *in vivo* distribution during several days, and its chemical properties suitable for the radiolabeling of antibodies using different chelators. To date, although multiple antibodies have been radiolabeled in order to conduct PET pharmacokinetic studies in different clinical areas, mainly in oncology [24], the radiolabeling of adalimumab has not been still described.

The aim of this study is to evaluate the pharmacokinetic profile and the distribution of the intravitreal administration of ^{89}Zr-adalimumab in a uveitis rat model using PET imaging.

2. MATERIALS AND METHODS

2.1. Conjugation, radiolabeling and quality control

The radiolabeling of adalimumab has been carried out according to the procedures previously developed by Verel et al. for other monoclonal antibodies [24], which consist of two phases:

2.1.1. Adalimumab conjugation

Adalimumab (Imraldi®; Biogen S.L., Madrid, Spain) was purified against MilliQ water using Amicon® Ultra-2 mL (NMWL 30 kDa) centrifugal filters (Merck®Millipore®, Burlington, Massachussets, USA) to remove other excipients of the commercial product. Once the purified antibody was obtained, a 2-fold molar excess of tetrafluorphenil-N-succinyldeferoxamine-B-Fe^{3+} (TFP-N-sucDf-Fe) (ABX®, Radeberg, Germany) was added in order to conjugate the molecule. Conjugation was carried out at 25 °C for 30 minutes in constant motion, adjusting the solution to pH 9.0 - 9.5 with 0.1 M Na_2CO_3. Once this time has elapsed, the pH was again reduced to pH 4.0 - 4.5 with 0.25 M H_2SO_4 and a 50-fold excess of 25 mg/mL EDTA (ethylenediaminetetraacetic acid) was added to trap the iron found in the TFP-N-sucDf-Fe molecule, and then, they were incubated at 30 °C for 30 minutes. Conjugated adalimumab was again purified adjusting the antibody concentration to the same as the commercial solution (50 mg/mL). Once the antibody conjugation was completed, the N-sucDf-adalimumab was stored at -80 °C to maintain it in optimal conditions.

2.1.2. Adalimumab radiolabeling

Prior to radiolabeling, adalimumab was kept at room temperature until completely thawed. The required amount of clinical grade ^{89}Zr-oxalate solution (BV cyclotron VU, PerkinElmer, Inc., Waltham, Massachusetts, USA) was collected with 1 M oxalic acid. To perform the proper labelling reaction, the pH was raised to a range of pH 4.0 - 4.5 with 2 M Na_2CO_3 and then HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was added until a solution at pH 7 was obtained. Once the pH was achieved, the N-sucDf-adalimumab was added to the solution and incubated for 1.5 h at room temperature. Adalimumab was labelled with a maximum specific activity of 10 MBq/mg. Afterwards, an ultrafiltration with Amicon® Ultra-0.5 mL Centrifugal Filters, NMWL 100 kDa (Merck®Millipore®, Burlington, Massachusetts, USA) was carried out in order to concentrate and purify the solution.

2.1.3. Quality control

The adalimumab conjugation quality control was performed with a size-exclusion high-performance liquid chromatography (SE-HPLC) using an HPLC system (Agilent 1260 series; Agilent Technologies®, Santa Clara, CA, USA) to check whether antibodies aggregation and fragmentation processes occurred during conjugation, as well as to calculate the conjugation ratio between the N-sucDf and the adalimumab. An Agilent Bio SEC-5, 5 μm, 300 Å, 7.8 × 150 mm column was used with a flow rate of 0.7 mL/min, the mobile phase consisted of

phosphate-buffered saline (PBS; pH 7.4). The UV-detector wavelengths were set to 220, 280 and 430 nm. The ratio of conjugated TFP-N-sucDf to the protein was determined by the antibody-bound versus unbound 430 nm signal of Fe^{3+} on SE-HPLC [25]. The radiochemical purity (RCP) of labelled adalimumab was evaluated by trichloroacetic acid (TCA) precipitation test. It was performed by mixing equal amounts of 1% HSA (Human Serum Albumin) (Albunorm® 20%, Octapharma®, Lachen, Switzerland) in PBS and 20% trichloroacetic acid in MilliQ water, an aliquot of the labelled compound is added to this solution. The sample was centrifuged to separate the precipitate (protein fraction) from the supernatant, and the radioactivity in both fractions were measured by a well counter (Atomlab® Wipe Test Counter, Biodex®, New York, USA) to determine the RCP [25].

2.2. Endotoxin-induced uveitis model (EIU)

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 (CEBEGA, Santiago de Compostela, Spain). The animals were kept in cages under controlled temperature (22 ± 1 °C) and humidity (60 ± 5%), with day–night cycles regulated by artificial light (12/12 h) and feeding *ad libitum*. The animals were treated according to the ARVO statement for the use of animals in ophthalmic and vision research as well as the approved guidelines for laboratory animals [26,27].

Experiments were approved by the Committee for Ethical Research of the Health Research Institute of Santiago de Compostela (IDIS) (15012/2021/001) and followed the Spanish and European Union (EU) rules (86/609/CEE, 2003/ 65/CE, 2010/63/EU, RD 1201/2005, and RD53/2013). Our study design consisted of a control group of healthy rats (n=3; 6 eyes) and a uveitis group of endotoxin-induced uveitis (EIU) rats (n=6, 12 eyes). EIU model has demonstrated being highly appropriate due to its reproducibility and its similar clinical changes to those appearing in human uveitis [28]. This model was induced inoculating 1 mg/kg *Escherichia coli* lipopolysaccharide endotoxin (LPS) diluted in 0.1 mL balance salt solution (BSS) into the rat's right paw as previously performed by our group [29] and by other authors [30,31].

2.3. Intravitreal administration of ^{89}Zr-adalimumab

Rats were kept in a veterinary gas chamber containing 3% (v/v) isoflurane/oxygen (Baxter®, Deerfield, Illinois, USA). Once anaesthetized, rats were placed in a surface where they continued receiving anesthesia with a face mask (2.5% isoflurane/oxygen). Intravitreal injection was performed according to the procedure described in our previous articles [23,25]. Topical anesthetic eye

drops (1 mg/mL tetracaine hydrochloride, 4 mg/mL oxybuprocaine Hydrochloride; Colircusí anestésico doble®, Alcon Healthcare, Texas, USA) were instilled in both eyes followed by mydriatic eye drops (10 mg/mL cyclopentolate hydrochloride; Colircusí Ciclopléjico®, Alcon Healthcare, Texas, USA) in order to visualize the eye fundus.

The injection procedure was carried out 12 hours after model induction by using a surgical microscope (Takagi OM-5 220-2; Takagi, Tokyo, Japan). A volume of 4 μL of the ^{89}Zr-labelled adalimumab was injected into the vitreous through pars plana using a NanoFil® syringe (WPI, Friedberg, Germany) attached to a 35 G needle. Concerning the activity of the radiolabeled compound, ≃ 1.74 MBq (≃ 0.17 mg of ^{89}Zr-adalimumab) was injected into each rat eye (≃ 3.48 MBq total activity injected per rat). Eyes with lens damage, or with significant bleeding when the intravitreal injection was made, were discarded from the study.

2.4. PET acquisition and image analysis

A preclinical PET/CT system (Albira model by Bruker Biospin, Billerica, Massachusetts, USA) was used to perform PET acquisitions immediately after intravitreal administration. Two-bed position of 10 min sequential acquisitions were carried out to include the whole body of rats. All animals were kept anaesthetized with 2.5% isoflurane/oxygen through a face mask during the acquisition and the respiration frequency was continuously monitored. Each animal was scanned at different time points (0, 2, 4, 8, 12, 24, 36 h and every day from 2 to 10 days) following the same process to obtain the pharmacokinetic profile.

Dynamic PET images were reconstructed using the maximum likelihood expectation maximization (MLEM) algorithm with 12 iterations and an image pixel size of 0.5 × 0.5 × 0.5 mm^3. All images were analyzed using Amide's Medical Image Data Analysis Tool. Quantitative analysis was carried out on the different image frames by manually drawing ellipsoidal regions of interest (ROIs) of 12 x 12 x 12 mm (904 mm^3), significantly larger than the volume of whole rat eye in order to include all the radiotracer uptake of each eye (the contour of the radiotracer distribution is commonly blurred due to the limited spatial resolution of the PET scanners). The mean radiotracer concentration obtained from the first image frame after intravitreal administration was taken as reference, and the subsequent measures from the following frames were expressed as a percentage of this initial uptake. Additional ROIs (7 x 7 x 7 mm, 343 mm^3) were delineated in the heart, liver, spleen and cervical lymph nodes for each time. The mean radiotracer concentration derived from the different ROIs was corrected by the radioactive decay of the ^{89}Zr radioisotope (half-life of 3.3 days).

The standardized uptake value (SUV) was calculated as the mean uptake of ^{89}Zr-adalimumab normalized by the injected activity and the bodyweight of the animal, as shown in the following Equation 1.

$$SUV = \frac{measured\ radioactivity\ concentration\ (Bq/mL)}{injected\ radioactivity\ (Bq)\ /\ body\ weight\ (Kg)} \quad (3)$$

Finally, a blood sample was obtained through the tail vein of each rat immediately after every PET acquisition while maintaining the anesthesia. Specifically, two aliquots of a known volume (between 20 and 100 µL) were extracted and measured in a well counter (Atomlab® Wipe Test Counter, Biodex®, New York, USA). Measured activity from the blood samples was also corrected for the radioactive decay of ^{89}Zr. Total blood activity was calculated taking into account the theoretical rat blood volume calculated based on body weight using the expression: (Blood volume = 0.06 x Body weight + 0.77 [32]).

Finally, blood activity levels after intravitreal injection of ^{89}Zr-adalimumab were reported as the ^{89}Zr-adalimumab uptake value normalized by the injected activity and the bodyweight of the animal (Activity (%)). The scheme of the experimental design of the study is shown in Figure 1.

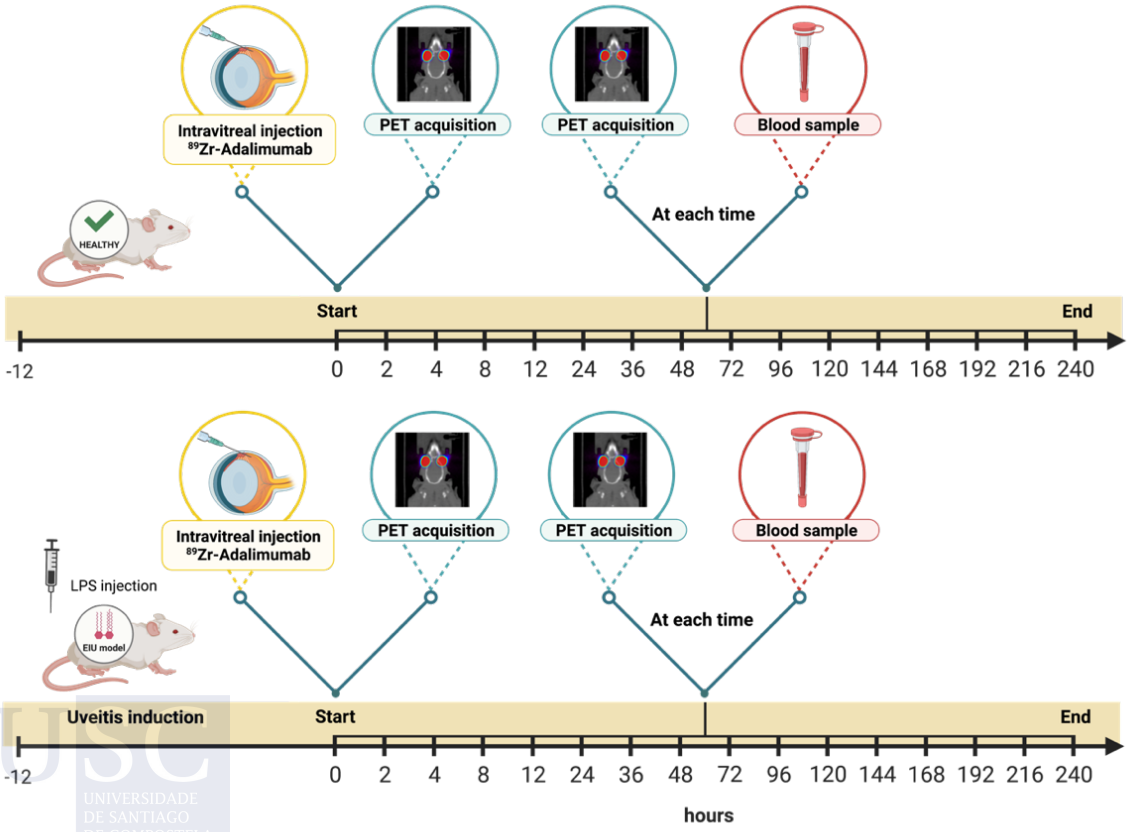


Figure 1. Study timeline for both groups (Healthy and Uveitis group): EIU model induction, intravitreal injections of ^{89}Zr-adalimumab, PET acquisitions and blood sample collection. Created with BioRender.com.

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2.5. Pharmacokinetic analysis

Compartmental data analyses were carried out using GraphPad Prism 8 v.8.2.1 software (GraphPad Software, San Diego, CA, USA) to calculate the pharmacokinetic parameters of the intravitreally injected radiolabeled adalimumab. One- and two-compartment models were used to fit the concentration vs time. Nonlinear regression methodology was used to fit experimental data. Nonlinear weighting method $1/Y^2$ was used to fit the eye ^{89}Zr-adalimumab concentration decay. No weighting method was used for blood % activity fitting. To quantify the goodness of nonlinear fit, SE (Equation 2), RMSE (Equation 3) and Akaike method (AIC) parameters were used.

$$SE = \sqrt{\frac{\sum(residual^2)}{n-k}} \tag{2}$$

$$RMSE = \sqrt{\frac{\sum(residual^2)}{n-1}} \tag{3}$$

where k is the number of fitted parameters by regression.

The compartmental analysis of ^{89}Zr-adalimumab distribution in eye was performed using models a) and b) of Figure 2 and the Equations 5 and 8. In the case of blood distribution models b) and c) and Equations 9 and 13 were used:

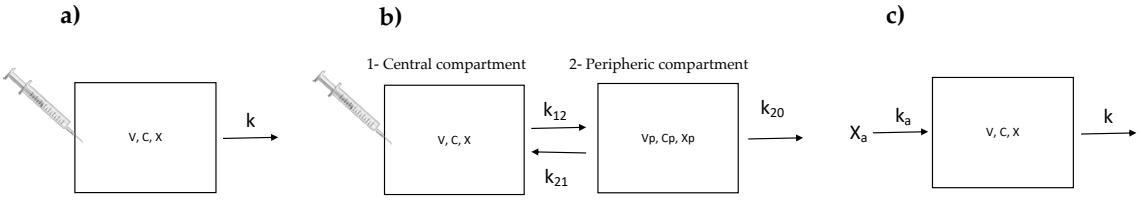


Figure 2. Compartmental analysis of ^{89}Zr-adalimumab distribution. **a)** Open intravitreal one-compartment model with first order elimination kinetics from eye, **b)** Open two-compartment model with intravitreal administration and linear elimination from peripheric compartment and **c)** Open one-compartment model with first order incorporation kinetic and linear elimination (blood).

Open intravitreal one-compartment model with a first order elimination kinetics from eye.

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$$\frac{dX}{dt} = -k \cdot X \tag{4}$$

$$X = De^{-kt} \tag{5}$$

where D is the dose, X the adalimumab amount and k the elimination constant.

Open two-compartment model with intravitreal administration and linear elimination from peripheric compartment.

$$\frac{\mathrm{dX_1}}{\mathrm{dt}} = \mathrm{k_{21}X_2} - \mathrm{k_{12}} \cdot \mathrm{X_1} \quad (6)$$

$$\frac{dX_2}{dt} = k_{12}X_1 - k_{21} \cdot X_2 - k_{20} \cdot X_2 \quad (7)$$

$$X_1 = \frac{D(\beta - k_{12})}{\beta - \alpha} e^{-\alpha t} + \frac{D(k_{12} - \alpha)}{\beta - \alpha} e^{-\beta t} \quad (8)$$

$$X_2 = \frac{Dk_{12}}{\beta - \alpha}\left(e^{-\alpha t} - e^{-\beta t}\right) \quad (9)$$

where

$$\alpha + \beta = k_{12} + k_{21} + k_{20} \quad (10)$$

$$\alpha \cdot \beta = \mathrm{k_{12}} \cdot \mathrm{k_{21}} \quad (11)$$

D is the dose, X_1 is the adalimumab amount in the central compartment (eye) and X_2 the amount in the peripheric compartment. k_{12} and k_{21} are the distribution constant, k_{20} the elimination constant, and α and β the hybrid constant of two-compartment model.

Open one-compartment model with first order incorporation kinetic and linear elimination (blood).

$$\frac{dX}{dt} = k_a X_a - k \cdot X \quad (12)$$

$$X = \frac{Dk_a}{k_a - k}(e^{-kt} - e^{-k_a t}) \quad (13)$$

where D is the dose, X_a is the adalimumab amount in the eyes, X the adalimumab amount in blood, k_a is the distribution constant from eyes to blood and k the elimination constant.

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The curve of remaining radioactivity in the eye (%) versus time and curves of the SUV in blood, heart, liver, spleen and cervical lymph nodes in each rat versus time after intravitreal injection of ^{89}Zr-adalimumab were generated using

GraphPad Prism 8 v.8.2.1 software (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed to verify if there were significant differences between the Healthy and the Uveitis group.

Two-tailed Mann-Whitney test and two-tailed parametric t-test were carried out for the different pharmacokinetic parameters, elimination and absorption constant (k and k_a), and also for the area under the curve (AUC_0^∞) and mean residence time (MRT), respectively. MRT gives information about the average time that adalimumab molecule remains in the eye or in the blood depending on the data. This parameter is calculated with the following Equation 14:

$$MRT = \frac{\int_0^\infty t \cdot C\ (t)dt}{\int_0^\infty C\ (t)dt} = \frac{AUMC_0^\infty}{AUC_0^\infty} \quad (14)$$

where C (t) is the concentration at each time t. The AUC_0^∞ and $AUMC_0^\infty$ were calculated using trapezoidal rules.

Since the volume of distribution (V) observed in rabbits and humans is similar to the vitreous anatomical volume, the same assumptions are made for rats [33–35]. Volume (V) value was calculated according to the experimental results obtained by Sha *et al.* [36] and taking into account that the rats used in this study were 10 weeks old. The clearance (CL) can be calculated according to the following equation CL = k · V, where k corresponds to the rate elimination constant, and the vitreous anatomical volume (V) is 47 μL. The same distribution volume was used for both healthy and uveitis rats.

3. RESULTS AND DISCUSSION

3.1. Conjugation, radiolabeling and quality control

The conjugation result between the chelating agent (N-sucDf) and the antibody was 1.466 chelating groups per adalimumab molecule. The N-sucDf group percentage bound to the antibody that is available for ^{89}Zr chelation was 94.37% for N-sucDf-adalimumab.

The absence of adalimumab dimerization processes during conjugation was achieved, avoiding a loss of antibody activity in this step. The result obtained for the radiolabeling efficiency of ^{89}Zr-adalimumab was 90.36%. This radiolabeled compound was subjected to an ultrafiltration process in which a Radiochemical purity (RCP) of 99.69% was obtained.

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3.2. Pharmacokinetics after intravitreal administration

3.2.1. Ocular levels

The radioactive uptake of ^{89}Zr-adalimumab at different times through PET/CT images in the group of healthy rats and uveitis rats is shown in Figure 3. It is possible to appreciate that the uptake in the eye declines as the study progresses in both groups. Nevertheless, there is a greater uptake at all times in the case of the Uveitis group except at the initial time, since it starts from the same activity. The information that can be obtained in these images is shown in more detail in Figure 4.

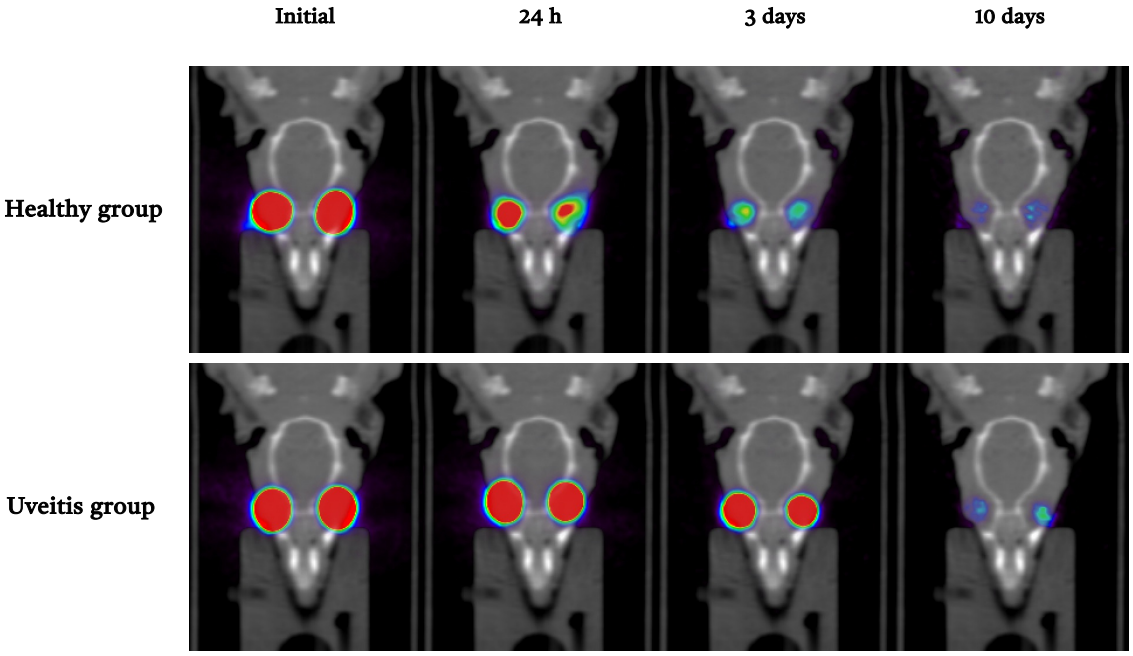


Figure 3. Fused PET/CT images displayed in coronal plane showing rat´s head at different time points (initial, 24 h, 3 days and 10 days) following intravitreal administration of ^{89}Zr-adalimumab in both groups (Healthy and Uveitis). The color scale represents the radioactive uptake of ^{89}Zr-adalimumab from lower (blue) to higher (red) intensity.

As can be seen in Figure 4, the percentage of remaining activity in the eye after intravitreal injection shows significant differences between healthy rats and uveitis rats. In Healthy group, there is a much more pronounced ocular elimination during the first timepoints post-injection compared to the Uveitis group. In contrast, in the Uveitis group, the antibody clearance occurs more slowly.

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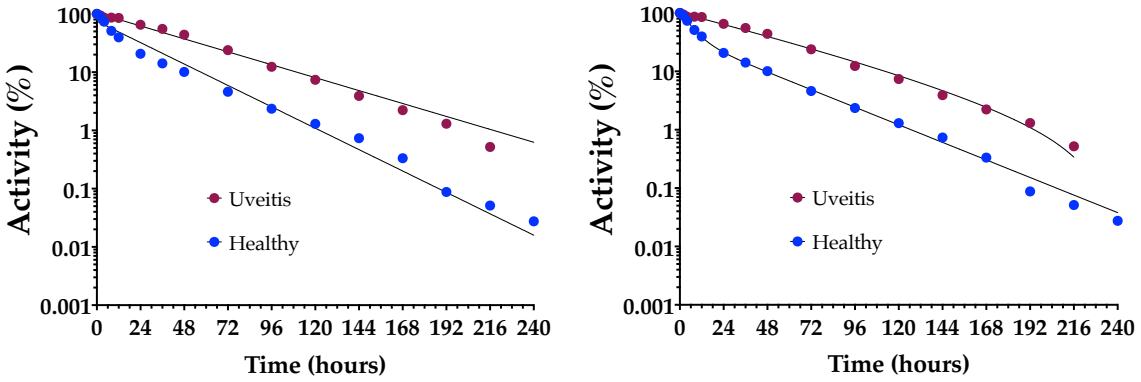


Figure 4. Percentage of remaining radioactivity in the eye versus time after intravitreal injection of ^{89}Zr-adalimumab in both rat groups (Healthy n=6 eyes and Uveitis n=12 eyes). Filled dots (measured by PET) and error bars represent the mean ± SD of the observed values, whereas the solid lines represent the predicted values obtained by a one-compartment model (left, Equation 5) and two-compartment model with intravitreal administration (right, Equation 8 central compartment).

Fitting parameters of eye pharmacokinetic data to one- or two-compartment models are shown in Table 1. Both models had a well adjustment to the experimental concentrations with similar fit parameter goodness. The two-compartment model has a very low k_{21} constant value in both groups suggesting that there is a minimal ^{89}Zr-adalimumab distribution from blood to the eye. As it can be seen in the semilogarithmic plots of Figure 4, healthy animals show a slight two-compartment behavior with the presence of a small initial distribution phase. However, this phase is not detected in the case of animals with uveitis. For this reason and for the fact that the k_{21} values are 12 times smaller than k_{12} the simplest one-compartment model can be selected to predict drug concentration.

Table 1. Results of the fitting of the eye ^{89}Zr-adalimumab concentration to the one-compartment model (Equation 5) and two-compartment model with intravitreal administration (Equation 8 central compartment).

	Healthy group		**Uveitis group**	
Parameter	One-compartment model	Two-compartment model	One-compartment model	Two-compartment model
k	0.0445 h^{-1}		0.0206 h^{-1}	
alfa		0.0041 h^{-1}		0.0206 h^{-1}
beta		0.0566 h^{-1}		4.9x10-32 h^{-1}
k12		0.0525 h^{-1}		0.0194 h^{-1}
k21		0.0044 h^{-1}		5.3x10-32 h^{-1}
k20		0.0038 h^{-1}		0.0016 h^{-1}
Se	0.1538	0,1141	0.06728	0.07003
RMSE	0.1431	0.1041	0.06264	0.06264
AICs	-51.60	-58.07	-78,05	-73.69

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Table 2 shows the results of some pharmacokinetic parameters concerning the elimination of intravitreal adalimumab. Half-life was 15.57 ± 2.64 hours for healthy rats and 33.64 ± 6.69 hours for uveitis rats, this implies that ^{89}Zr-adalimumab remains approximately two times longer in diseased rats compared to healthy ones. The area under the ocular activity-time curve (AUC_0^∞) values was again higher for the Uveitis group (2540.66 ± 476.14 %·hours) than for the Healthy group (6624.59 ± 732.84 %·hours), this means that the adalimumab quantity inside the eye during the whole study is around three times higher in the diseased group. Finally, the mean residence time (MRT) was (56.07 ± 3.99 hours) and (72.32 ± 10.86 hours) for healthy rats and uveitis rats, respectively.

A two-tailed Mann-Whitney test was performed to compare the elimination constant (k) of both groups showing significant differences ($\alpha < 0.05$) between them. In addition, a two-tailed parametric t-test was performed in order to compare the remaining adalimumab amount in the eye throughout the study (AUC_0^∞) and the residence time in the eye (MRT) of the two groups, confirming significant differences ($\alpha < 0.05$) in both parameters.

Table 2. Ocular pharmacokinetic parameters (mean ± SD) for the intraocular percentage of remaining activity using a one-compartment model after intravitreal injection of ^{89}Zr-adalimumab for both rat groups (Healthy n=6 eyes and Uveitis n=12 eyes).

Pharmacokinetic parameters	**Healthy**		**Uveitis**	
	Mean	SD	Mean	SD
k (hours^{-1})	0.0445	0.007	0.0206	0.0083
$t_{1/2}$ (hours)	15.57	2.64	33.64	6.69
AUC_0^∞ (%·hour)	2540.66	476.14	6624.59	1397.37
MRT (hours)	56.07	3.99	72.32	10.86

Regarding the ocular adalimumab clearance, it was 2.094 μL·h^{-1} for Healthy rats and 1.030 μL·h^{-1} for Uveitis rats (2 times faster in Healthy rats).

3.2.2. Systemic distribution

Figure 5 shows the distribution of ^{89}Zr-adalimumab once it is eliminated from the eye into the systemic circulation. It is possible to observe that the uptake is increased sharply in heart, liver, spleen and cervical lymph nodes. In addition, two videos showing the whole-body distribution of ^{89}Zr-adalimumab in healthy and uveitis rats can be found as Supplementary material 1.

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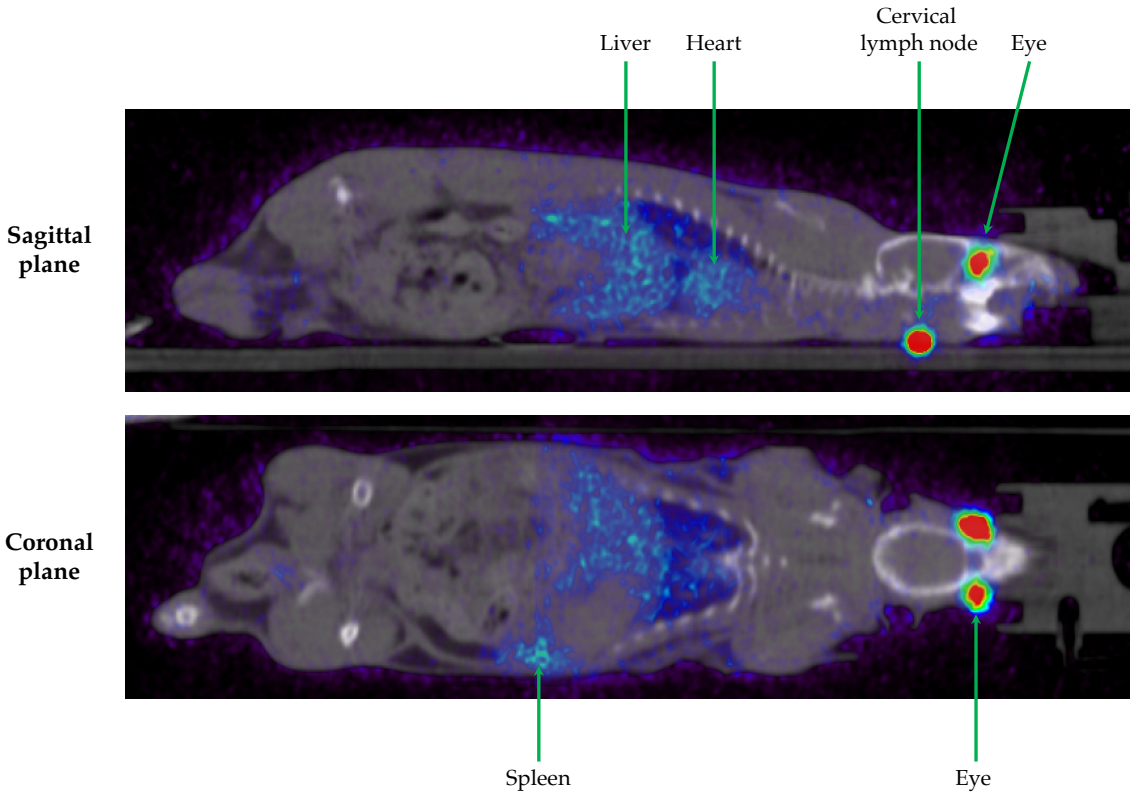


Figure 5. Rat whole-body PET/CT images displayed in coronal plane (down) and sagittal plane (up) representing the ^{89}Zr-adalimumab distribution in the organs with the highest uptake. The color scale represents the radioactive uptake of ^{89}Zr-adalimumab from lower (blue) to higher (red) intensity.

Figure 6 represents the blood uptake values (Activity (%)) of ^{89}Zr-adalimumab for both groups during the entire study period. The rate of adalimumab distribution into the bloodstream was found to be higher in Healthy rats compared to Uveitis ones, while at longer times the antibody amount in blood is equalized.

As previously performed in the eye case, the blood ^{89}Zr-adalimumab distribution values were adjusted to a one- and two-compartment model (Table 3 and Figure 6 solid lines). The values of SE, RMSE and AIC and the graphs in Figure 6 show that healthy animals fit better the ^{89}Zr-adalimumab blood concentration to the two-compartment model. On the contrary, for the uveitis group, better fitting values were obtained for the one-compartment model. Additionally, the α and β hybrid constants estimated for two-compartment analysis show identical values. In consequence one-compartment model can be assumed for uveitis rats.

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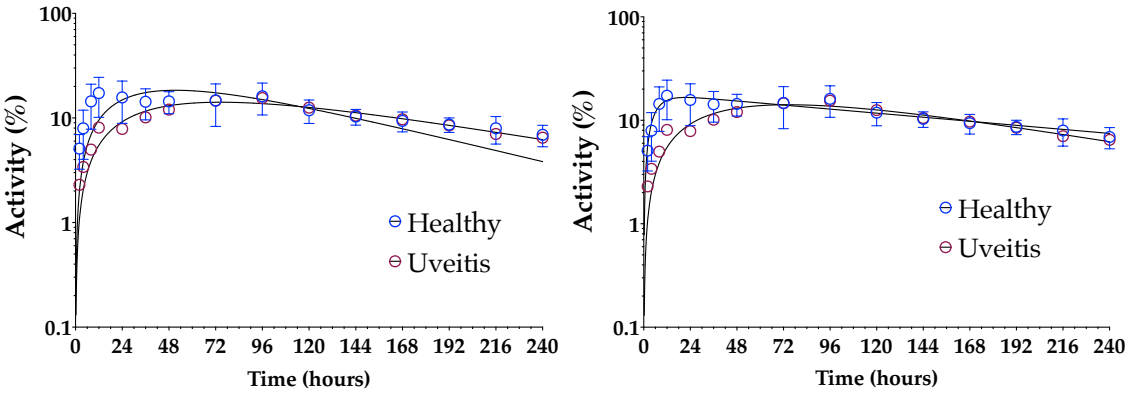


Figure 6. Blood concentration (%) versus time after intravitreal injection of ^{89}Zr-adalimumab in both rat groups (Healthy and Uveitis). Unfilled dots (measured with well counter) and error bars represent the mean ± SD of the observed values, whereas the solid lines represent the predicted values obtained by the first-order absorption one-compartment model (left, Equation 4) and two-compartment model (right, Equation 3 peripheric compartment).

Table 3. Results of the fitting of the eye ^{89}Zr-adalimumab blood concentration to the one-compartment model (Equation 13) and two-compartment model with intravitreal administration (Equation 9 peripherical compartment).

	Healthy group		**Uveitis group**	
Parameter	One-compartment model	Two-compartment model	One-compartment model	Two-compartment model
k	0.0411h^{-1}		0.006212 h^{-1}	
ka	0.0147h^{-1}		0.02511h^{-1}	
alfa		0.1957 h^{-1}		0.0127 h^{-1}
beta		0.0055 h^{-1}		0.0127 h^{-1}
k12		0.0414 h^{-1}		0.007 h^{-1}
k21		0.0265 h^{-1}		0.029 h^{-1}
k20		0.1397 h^{-1}		-0.009 h^{-1}
Se	4.604	1.094	2.439	2.378
RMSE	4.448	1.019	2.356	2.214
AICs	54.73	11.20	34.39	36.04

In order to compare the main pharmacokinetics parameters between Healthy and Uveitis groups, a non-compartmental analysis was made. The terminal half-life, maximum concentration (C_{max}) and time for maximum concentration (t_{max}) were estimated and the AUC_{∞}^{0} and MRT were calculated as it is shown in Table 4. ^{89}Zr-adalimumab had a blood terminal half-life of 339 ± 96.90 hours for the Healthy group and 295.93 ± 46.92 hours for the Uveitis group. t_{max} were 12 ± 0 hours for the healthy rats vs 84 ± 13.15 hours for uveitis rats and C_{max} were 20.56 ± 3.61 % vs ± 21. 27 % respectively. The blood AUC_{0}^{∞} values for both groups were 6026.15 ± 1727.21 (Healthy) and 5711.35 ± 1545.35 (Uveitis). MRT results were 172.97 ±

18.67 for the Healthy group and 175.18 ± 17.54 for the Uveitis group, respectively. The statistical differences between the pharmacokinetic parameters of the two groups were studied.

The two-tailed Mann-Whitney test was performed on the terminal half-life showing no significant differences (ns). A two-tailed parametric t-test was carried out on C_{max}, AUC_0^∞ and MRT. Significant differences were found in the t_{max} ($\alpha < 0.05$). Moreover, C_{max}, AUC_0^∞ and MRT show no significant differences (ns) between both groups.

Table 4. Blood pharmacokinetic non-compartmental parameters (terminal half-life, t_{max}, C_{max}, AUC_0^∞, mean residence time (MRT)) (mean ± SD) for blood concentration data using a first-order absorption one-compartment model after intravitreal injection of ^{89}Zr-adalimumab in both rat groups (Healthy and Uveitis).

Pharmacokinetic parameters	**Healthy**		**Uveitis**	
	Mean	SD	Mean	SD
Terminal half-life (hours)	339	96.9	295.93	46.92
t_{max} (hours)	12	0	84	13.15
C_{max} (%)	20.56	3.61	21.27	6.54
AUC_0^∞ (%·hour)	6026.15	1727.21	5711.35	1545.35
MRT (hours)	172.97	18.67	175.18	17.54

The pharmacokinetic results of the organs (heart, liver, spleen and cervical lymph nodes) were analyzed, a first-order absorption one-compartment model was the best pharmacokinetics fit for these results. As shown in Figure 7, in all the organs where there is uptake, it can be seen that the speed of adalimumab influx is faster in the Healthy rat group at short times. As time goes by, the curves overlap until they become equal in the last study stages for both groups, Healthy and Uveitis rats. During the study period, the elimination process of the monoclonal antibody is not clearly observed.

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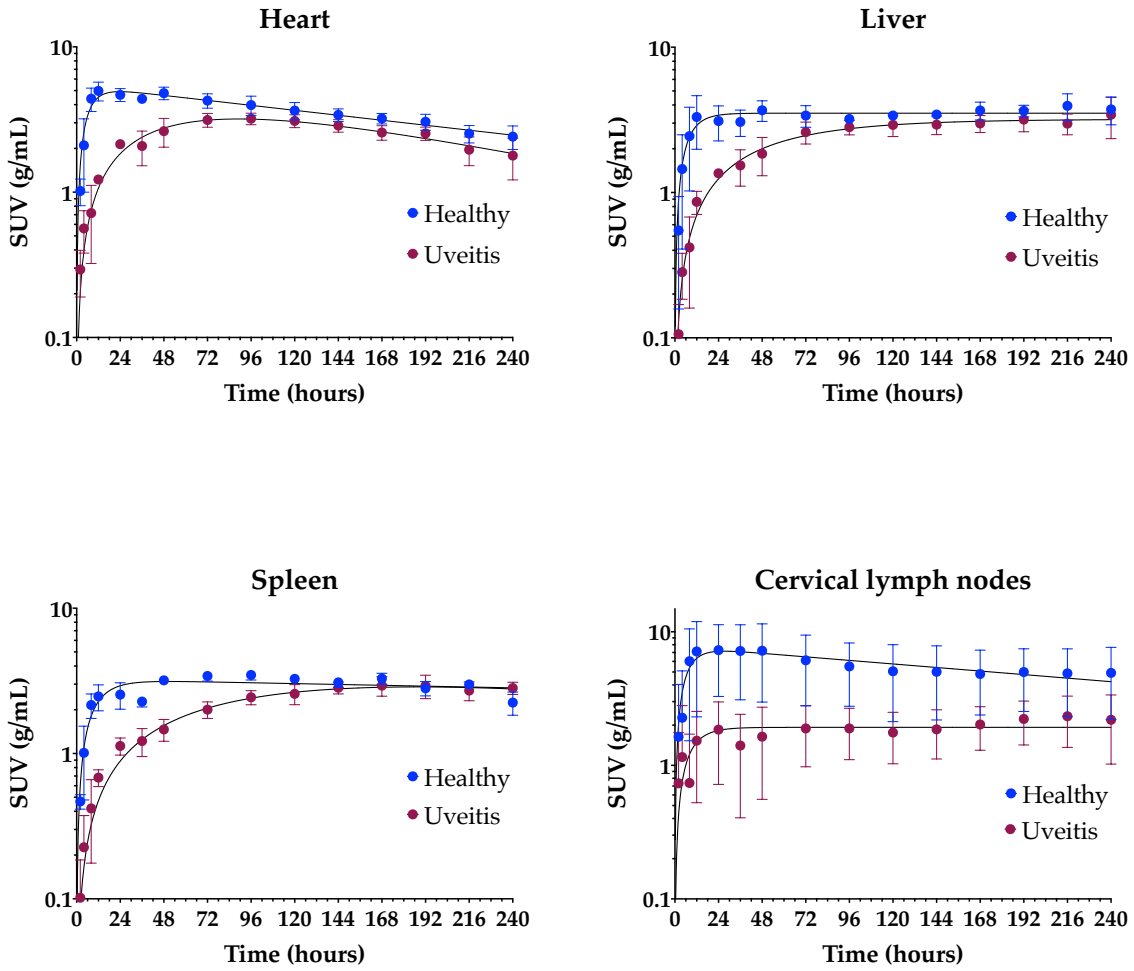


Figure 7. Activity (SUV) in the different organs (heart, liver, spleen and cervical lymph nodes) versus time after intravitreal injection of ^{89}Zr-adalimumab in both rat groups (Healthy and Uveitis). Filled dots (measured by PET) and error bars represent the mean ± SD of the observed values, whereas the solid lines represent the predicted values obtained by a first-order absorption one-compartment model.

4. DISCUSSION

To our knowledge, this is the first work addressing intravitreal pharmacokinetics of adalimumab. In this sense, regarding the ocular permanence of adalimumab, although the group of healthy animals shows a slight two-compartment behavior, data obtained from PET analysis fits well with a one-compartment model for both Healthy and Uveitis groups. The one-compartment model providing significant differences in the k elimination constant from the eye, and subsequently in the time adalimumab remains in the eye (half-life of 15.57 ± 2.64 hours for Healthy group and 33.64 ± 6.69 hours for Uveitis group). These results seem to be in contradiction with the behavior observed in a previous work comparing the pharmacokinetics of two radiopharmaceuticals, ^{18}F-NaF and ^{18}F-FDG [23]. In this earlier work, an increase in ^{18}F-FDG clearance caused by inflammation was observed, while ^{18}F-NaF remained unchanged. The

explanation for the possible causes of the observed increase in ^{18}F-FDG clearance in uveitis was the increased permeability of blood-retinal barrier and the GLUT transporter.

However, the ability of the adalimumab to selectively bind to the tumor necrosis factor is well known. Thus, this result is consistent with the overproduction of TNFα from macrophages and other cytokines in the uveitis process, which activates dendritic cells, starting the inflammatory cascade in which Th1 cells and Th17 cells migrate and infiltrate to the blood-retinal barrier causing damage [37]. This overproduction of TNFα was shown in a previous work of our group where rats with uveitis presented a higher presence of TNFα mRNA in the eye tissues than healthy rats [29]. These measures of TNFα levels have also been carried out by other authors in the preclinical and clinical setting [8,14]. Due to this biochemical mechanism, adalimumab is specifically blocking the binding of human TNFα to the receptors in the Uveitis group. Since adalimumab binds to TNFα receptors located in the uvea, its ocular clearance will be diminished.

The results of the pharmacokinetic compartmental analysis in blood show a different behavior between healthy and diseased animals. The activity vs time in healthy animals fits to a two-compartment model showing a rapid uptake of the antibody from the eye simultaneous to the tissue distribution process. However, in the case of animals with uveitis, the activity vs time fits better to a one-compartment model. This may be because the transfer process from the eye to the blood is much slower acting as a limiting step, so that the distribution process to the organs is not appreciated.

In order to compare the rate and magnitude of adalimumab transfer from the eye to the blood, a non-compartmental analysis was used. Regarding to this, our results provided similar blood terminal half-life between the Healthy (339.00 ± 96.90 hours) and Uveitis groups (295.93 ± 46.92 hours), as well as MRT (172.97 ± 18.67 hours and 175.18 ± 17.54 hours, respectively), but significant differences in the t_{max} between Healthy group and Uveitis group (12 ± 0.0 and 84 ± 13.15, respectively), probably due to the fact that, as can be observed in Figure 6, up to 72h the adalimumab amount in the blood of healthy rats is higher than that of uveitis rats. This could be caused by the binding between antibody and TNFα when the eyes are inflamed. Although ocular inflammation usually increases the permeability of ocular barriers and clearance of intravitreal drugs [23], our opposite results are due to the interaction between adalimumab and the TNFα [38] present in the ocular cavity, which achieves increased values in uveitis as demonstrated in previous studies [29]. After 72 h, blood values are equalized until the end of the study.

Intravitreal pharmacokinetic studies in patients are performed from the aqueous humor due to the impossibility of obtaining vitreous samples [39]. This procedure has also been carried out in the preclinical setting, specifically on animals sacrificed at different time points in order to obtain vitreous samples. In this sense, PET imaging has the advantage of avoiding invasive sampling and obtaining concentrations from the same animal over time, with the consequent decrease in the number of animals per study group [23]. Additionally, PET methodology avoids the intrusiveness of the pharmacokinetic studies, which is very important taking into account the invasiveness of the intravitreal injection itself. PET imaging requires the use of radioactive derivatives which are stable over time. In this study, ^{89}Zr was selected because it allows to visualize the distribution of antibodies for up to 10 days due to its long half-life. In order to obtain radiolabeled adalimumab, deferoxamine was previously bound to this drug to act as a chelator of ^{89}Zr. As studied in previous works of our group [25], ^{89}Zr-labelled deferoxamine without monoclonal antibody has a completely different kinetic behavior, the rate of elimination from both the vitreous cavity and blood is much faster (24h) and that vitreous elimination is independent of the deferoxamine chelator, only the molecule has an influence.

The main limitation of our study regarding intravitreal pharmacokinetics is that all ocular cavities are measured together, including aqueous and vitreous humor, as well as surrounding tissues. However, this is partially counteracted by the fact that drug concentration declines with the same decay rate in the vitreous humor, aqueous humor and retina, as several authors state [40,41]. As well as other species, rats represent a good model for intravitreal pharmacokinetic studies [42]. However, comparison with humans should be cautiously made due to the anatomical and physiological ocular differences. With this regard, rats have a larger len and a simpler inner limiting membrane, which could be a poorer barrier to diffusion [43,44], and around 50 μL of vitreous humor [45], which is well below that volume in the human eye (4 mL). In addition, it is necessary to take into account that the stability of the metal complex formed between ^{89}Zr and the bifunctional chelate used (TFP-N-sucDf) was studied by Perk et al. who reported a small loss of ^{89}Zr from conjugate over a 7-day period (4.7 ± 0.5%) [46], however, our study lasted 10 days so this loss could be slightly higher.

Regarding adalimumab pharmacokinetics outside the ocular cavity, interaction between the anti-TNFα that is removed from the eye and the major inflammatory agents is going to occur. The immune system is closely related to the lymphatic system, so an accumulation in the different lymph nodes close to the site of administration is expected, as can be confirmed by our results. In this sense, the highlighted area in the inferior part of the mandible (Figure 5-sagittal plane) corresponds to the cervical lymph nodes, as described by Suami *et al.* [47]. The elimination process of adalimumab in the body cannot be clearly appreciated by

observing the pharmacokinetic results of the main drug elimination organs. This is due to the fact that anti-TNFα has a large molecular weight (≃150 KDa), so filtration by the kidneys and elimination through urine is not possible, except under pathological conditions [48,49]. Thus, the main route of elimination occurs through intracellular catabolism by lysosomal degradation, transforming the monoclonal antibody into peptides and amino acids that can be re-used in the body for the *de novo* synthesis of proteins [50].

5. CONCLUSIONS

This study evaluates for the first time the pharmacokinetic profile of intravitreally injected adalimumab in a uveitis rat model, showing that this disease produces a major increase in the ocular permanence of this drug.

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CONCLUSIONS

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CONCLUSIONS

In this doctoral thesis, an investigation of therapeutic alternatives for the treatment of ocular inflammatory diseases has been performed, enabling us to draw the following conclusions:

1st - A detailed review of the different ocular pathways and the main aspects of ocular pharmacokinetics has been carried out. Different factors that are involved in the ocular drug delivery including the physiological barriers and drug transport pathways were described, as well as the advantages and drawbacks of the administration routes to the eye. Conventional administration pathways, such as topical or systemic routes, usually show important limitations, either by low ocular penetration or by the appearance of side effects linked to the posology, among others. New drug delivery systems (DDS) are needed to prolong the administration intervals for ocular pathologies, even though the development of novel DDS is particularly complicated due to several aspects that must be considered, such as pharmacokinetics, immunogenicity, biodegradation, tolerability and toxicity. In the last few decades, an exponential increase in the design and development of novel DDS intended for the treatment of ocular pathologies was observed. Unfortunately, knowledge about drug targeting into the eye is still sparse.

Likewise, huge advances were made in terms of research and development of new alternative administration routes for the different parts of the eye. All of them have specific pharmacokinetic characteristics that make them useful for the treatment of specific ocular pathologies. However, these pathways have shown several advantages and limitations, where the election of one or another is dependent on, not just the pathology itself, but the pharmaceutical form, the drug used and the patient´s adherence to treatment.

2nd - In order to provide information on the characteristics of the classic tacrolimus eye drops prepared in the Hospital Pharmacy Departments, a comparative study was carried out with two other vehicles, a PVA solution and a hyaluronic acid solution. A greater permanence on the ocular surface and greater stability at different temperature conditions of tacrolimus eye drops in polyvinyl alcohol was observed. Similarly, the results obtained in terms of cytotoxicity showed that tacrolimus eye drops are better tolerated than

cyclosporine eye drops. Although the results of the study in patients indicated good tolerability, some cases of mild irritation were reported after administration. This is probably caused by the high osmolality of the formulations and the presence of some excipients such as ethanol and hydrogenated and polyoxyethylated castor oil, contributed by the intravenous drug presentation with which the formulations are prepared.

3rd - The design and development of different topical ophthalmic formulations containing tacrolimus and cyclodextrins as an alternative to classical tacrolimus eye drops reformulated from the intravenous drug in Hospital Pharmacy Departments was carried out. Tacrolimus/HPβCD interaction in solution was confirmed by phase solubility diagram, NMR and molecular modeling studies, and the influence of the vehicle was also studied. The use of 40% (*w*/*v*) HPβCD allowed to prepare eye drop solutions with a 0.02% (*w*/*v*) tacrolimus concentration that could be in the therapeutic range for uveitis treatment.

The developed HPβCD-based formulations showed pH, osmolality, surface tension and safety values in the optimum range for topical ophthalmic administration, improving the properties of the eye drops studied in Chapter 2. Stability studies showed no changes in the eye drops kept in refrigeration condition for at least 3 months, which facilitate the preparation programming and improve the patient comfort. Additionally, *ex vivo* mucoadhesion and *in vivo* ocular permanence studies showed good mucoadhesive properties and lower ocular clearance for the mentioned tacrolimus/HPβCD formulation in Liquifilm®, almost doubling the permanence half-life time in the ocular surface compared to the classical tacrolimus eye drops. The simple elaboration process of TAC-HPβCD eye drops makes possible to prepare this formulation in HPDs, improving in terms of safety the current elaborated TAC eyedrops which contains ethanol and other irritating excipients.

4th - The effectiveness of the tacrolimus formulation developed in the third chapter was evaluated in a Endotoxin Induced Uveitis model in rats. TAC-HPβCD eyedrops showed beneficial effect against this model, thus they could be considered an alternative for uveitis treatment in case of corticosteroids resistance or intolerance. This formulation demonstrated to reduce ocular inflammation, expression of IL-6, TNFα, MIP-1α and leukocyte infiltration in aqueous humor.

5th - The evaluation of the pharmacokinetic profile of intravitreally injected adalimumab in a rat model was performed for the first time, showing that uveitis produces a reduction in ocular clearance of adalimumab. This is due to specific binding between the antibody and the TNFα receptors which are in greater quantities because of the inflammatory process produced by the disease. As a

result of the pharmacokinetic information obtained from this work, it will be possible to develop new drug delivery systems that allow a more controlled and prolonged release of the monoclonal antibody.

As overall conclusion, different tacrolimus formulations were proposed for the topical ophthalmic administration, supported by extensive preclinical studies in order to achieve a consistent base as an alternative to other pharmacological treatments for ocular inflammatory diseases. This was supported by extensive *in vitro, ex vivo,* and *in vivo* studies. Moreover, the intravitreal pharmacokinetics of adalimumab have also been elucidated in order to develop new delivery systems.

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CONCLUSIONES

En esta tesis doctoral se ha realizado una investigación de las alternativas terapéuticas para el tratamiento de las enfermedades inflamatorias oculares, lo que nos ha permitido extraer las siguientes conclusiones:

1º - Se ha realizado una revisión detallada de las diferentes vías oculares y de los principales aspectos de la farmacocinética ocular. Los diferentes factores que intervienen en la administración ocular de fármacos, incluidas las barreras fisiológicas y las vías de transporte de los mismos, así como las ventajas e inconvenientes de las vías de administración oftálmicas han sido también descritas. Las vías de administración convencionales, como la tópica o la sistémica, suelen presentar importantes limitaciones, ya sea por la baja penetración ocular o por la aparición de efectos secundarios ligados a la posología, entre otros. Se necesitan nuevos sistemas de administración de fármacos para prolongar los intervalos de administración en patologías oculares, aunque el desarrollo de estos nuevos sistemas es especialmente complicado debido a varios aspectos que deben considerarse, como la farmacocinética, la inmunogenicidad, la biodegradación, la tolerabilidad y la toxicidad. En las últimas décadas se ha observado un aumento exponencial en el diseño y desarrollo de nuevos sistemas de administración de fármacos destinados al tratamiento de patologías oculares. Desgraciadamente, los conocimientos sobre la vehiculización de los fármacos hacia el ojo siguen siendo escasos.

Asimismo, se han producido grandes avances en cuanto a la investigación y el desarrollo de nuevas vías de administración alternativas para las diferentes partes del ojo. Todas ellas tienen características farmacocinéticas específicas que las hacen útiles para el tratamiento de patologías oculares concretas. Sin embargo, estas vías han mostrado diversas ventajas y limitaciones, donde la elección de una u otra depende, no sólo de la propia patología, sino de la forma farmacéutica, del fármaco utilizado y de la adherencia del paciente al tratamiento.

2º - Con el fin de aportar información sobre las características del colirio clásico de tacrolimus preparado en los Servicios de Farmacia del Hospital, se realizó un estudio comparativo en otros dos vehículos, una solución de alcohol polivinílico y una solución de ácido hialurónico. Fue observada una mayor permanencia en

la superficie ocular y una mayor estabilidad a diferentes condiciones de temperatura del colirio de tacrolimus en alcohol polivinílico. Asimismo, los resultados obtenidos en términos de citotoxicidad demostraron que los colirios de tacrolimus son mejor tolerados que el de ciclosporina. Aunque los resultados del estudio en pacientes indicaron una buena tolerancia, se notificaron algunos casos de irritación leve tras la administración. Esto se debe probablemente a la elevada osmolalidad de las formulaciones y a la presencia de algunos excipientes, como el etanol y aceite de ricino hidrogenado y polioxietilenado, aportados por la presentación intravenosa del fármaco con la que se preparan las formulaciones.

3º - Fue llevado a cabo el diseño y desarrollo de diferentes formulaciones tópico-oftálmicas que contienen tacrolimus y ciclodextrina como alternativa a los colirios clásicos de tacrolimus reformulados a partir del fármaco intravenoso en los departamentos de farmacia de los hospitales. La interacción tacrolimus/HPβCD en solución se confirmó mediante el estudio de solubilidad, la RMN y los estudios de modelado molecular, estudiando también la influencia del vehículo. El uso del 40% *(p/v)* de HPβCD permitió preparar soluciones de colirio con una concentración de tacrolimus del 0,02% *(p/v)* que podría estar en el rango terapéutico para el tratamiento de la uveítis.

Las formulaciones desarrolladas a base de HPβCD mostraron valores de pH, osmolalidad, tensión superficial y seguridad en el rango óptimo para la administración tópico-oftálmica, mejorando las propiedades de los colirios estudiados en el capítulo 2. Los estudios de estabilidad no mostraron cambios en los colirios conservados en condiciones de refrigeración durante al menos 3 meses, lo que facilita la programación de la preparación en la farmacia hospitalaria y mejora la comodidad y adherencia del paciente al tratamiento. Además, los estudios de mucoadhesión *ex vivo* y de permanencia ocular *in vivo* mostraron buenas propiedades mucoadhesivas y un menor aclaramiento ocular para la mencionada formulación de tacrolimus/HPβCD en Liquifilm®, casi duplicando el tiempo de permanencia en la superficie ocular en comparación con el colirio de tacrolimus clásico. El sencillo proceso de elaboración de los colirios de TAC-HPβCD permite preparar esta formulación en los departamentos de farmacia hospitalaria, mejorando en términos de seguridad los actuales colirios de tacrolimus.

4º - La eficacia de la formulación de tacrolimus desarrollada en el capítulo 3 se evaluó en un modelo de uveítis inducida por endotoxina en ratas. El colirio de TAC-HPβCD mostró un efecto beneficioso en este modelo, por lo que podría considerarse una alternativa para el tratamiento de la uveítis en caso de resistencia o intolerancia a los corticosteroides. Esta formulación demostró reducir la inflamación ocular, la expresión de IL-6, TNFα, MIP-1α y la infiltración de leucocitos en el humor acuoso.

5º - Por primera vez, se ha realizado la evaluación del perfil farmacocinético del adalimumab inyectado por vía intravítrea en un modelo de rata, demostrando que la uveítis produce una reducción del aclaramiento ocular del adalimumab. Esto se debe a la unión específica entre el anticuerpo y los receptores del TNFα que se encuentran en mayor cantidad debido al proceso inflamatorio producido por la enfermedad. La información farmacocinética obtenida en este trabajo será de gran utilidad para desarrollar nuevos sistemas de administración de adalimumab que permitan una liberación más controlada y prolongada del anticuerpo monoclonal.

Como conclusión general, en esta tesis doctoral fueron propuestas diferentes formulaciones de tacrolimus para la administración tópico-oftálmica, respaldadas por amplios estudios preclínicos con el fin de lograr una base consistente como alternativa a otros tratamientos farmacológicos para las enfermedades inflamatorias oculares. Esto fue confirmado con amplios estudios *in vitro*, *ex vivo* e *in vivo*. Por otra parte, en lo que respecta al estudio de inyecciones intravítreas de adalimumab, esta tesis doctoral ha dilucidado la distribución y farmacocinética intravítrea del anticuerpo monoclonal que será de gran utilidad para el desarrollo de nuevos sistemas de administración.

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CONCLUSIÓNS

Nesta tese de doutoramento realizouse unha investigación das alternativas terapéuticas para o tratamento das enfermidades inflamatorias oculares, o que nos permitiu extraer as seguintes conclusións:

1º - Realizouse unha revisión detallada das diferentes vías oculares e dos principais aspectos da farmacocinética ocular. Os diferentes factores que interveñen na administración ocular de fármacos, incluídas as barreiras fisiolóxicas e as vías de transporte dos mesmos, así como as vantaxes e desvantaxes das vías de administración oftálmicas foron tamén descritas. As vías de administración convencionais, como a tópica ou a sistémica, normalmente presentan importantes limitacións, xa sexa pola baixa penetración ocular ou pola aparición de efectos secundarios ligados á posoloxía, entre outros. Necesítanse novos sistemas de administración de fármacos para prolongar os intervalos de administración en patoloxías oculares, aínda que o desenvolvemento destes novos sistemas é especialmente complicado debido a varios aspectos que deben considerarse, como a farmacocinética, a inmunoxenicidade, a biodegradación, a tolerabilidade e a toxicidade. Nas últimas décadas observouse un aumento exponencial no deseño e desenvolvemento de novos sistemas de administración de fármacos destinados ó tratamento de patoloxías oculares. Desafortunadamente, os coñecementos sobre a vehiculización dos fármacos cara ó ollo seguen sendo escasos.

Así mesmo, producíronse grandes avances en canto á investigación e o desenvolvemento de novas vías de administración alternativas para as diferentes partes do ollo. Todas elas teñen características farmacocinéticas específicas que as fan útiles para o tratamento de patoloxías oculares concretas. Sen embargo, estas vías mostraron diversas vantaxes e limitacións, onde a elección dunha ou outra depende, non só da propia patoloxía, senón da forma farmacéutica, do fármaco empregado e da adherencia do paciente ó tratamento**.**

2º - Co fin de aportar información sobre as características do colirio clásico de tacrolimus preparado nos Servizos de Farmacia do Hospital, realizouse un estudo comparativo noutros dous vehículos, unha solución de alcohol polivinílico e unha solución de ácido hialurónico. Foi observada unha maior permanencia na superficie ocular e unha maior estabilidade a diferentes

condicións de temperatura do colirio de tacrolimus en alcohol polivinílico. Así mesmo, os resultados obtidos en termos de citotoxicidade demostraron que os colirios de tacrolimus son mellor tolerados que o de ciclosporina. Aínda que os resultados do estudo en pacientes indicaron unha boa tolerancia, notificáronse algúns casos de irritación leve trala administración. Isto débese probablemente á elevada osmolalidade das formulacións e á presenza dalgúns excipientes, como o etanol e aceite de ricino hidroxenado e polioxietilenado, aportados pola presentación intravenosa do fármaco ca que se preparan as formulacións.

3º - Foi levado a cabo o deseño e desenvolvemento de diferentes formulacións tópico-oftálmicas que conteñen tacrolimus e ciclodextrina como alternativa ós colirios clásicos de tacrolimus reformulados a partir do fármaco intravenoso nos departamentos de farmacia dos hospitais. A interacción tacrolimus/HPβCD en solución confirmouse mediante o estudo de solubilidade, a RMN e os estudos de modelado molecular, estudando tamén a influencia do vehículo. O uso do 40% *(p/v)* de HPβCD permitiu preparar solucións de colirio cunha concentración de tacrolimus do 0,02% *(p/v)* que podería estar no rango terapéutico para o tratamento da uveíte.

As formulacións desenvolvidas a base de HPβCD mostraron valores de pH, osmolalidade, tensión superficial e seguridade no rango óptimo para a administración tópico-oftálmica, mellorando as propiedades dos colirios estudados no capítulo 2. Os estudos de estabilidade non mostraron cambios nos colirios conservados en condicións de refrixeración durante polo menos 3 meses, o que facilita a programación da preparación na farmacia hospitalaria e mellora a comodidade e adherencia do paciente ó tratamento. Ademais, os estudos de mucoadhesión *ex vivo* e de permanencia ocular *in vivo* mostraron boas propiedades mucoadhesivas e un menor aclaramento ocular para a mencionada formulación de tacrolimus/HPβCD en Liquifilm®, case duplicando o tempo de permanencia na superficie ocular en comparación co colirio de tacrolimus clásico. O sinxelo proceso de elaboración dos colirios de TAC-HPβCD permite preparar esta formulación nos departamentos de farmacia hospitalaria, mellorando en termos de seguridade os actuais colirios de tacrolimus.

4º - A eficacia da formulación de tacrolimus desenvolvida no capítulo 3 avaliouse nun modelo de uveíte inducida por endotoxina en ratas. O colirio de TAC-HPβCD mostrou un efecto beneficioso neste modelo, polo que podería considerarse unha alternativa para o tratamento da uveíte en caso de resistencia ou intolerancia ós corticosteroides. Esta formulación demostrou reducir a inflamación ocular, a expresión de IL-6, TNFα, MIP-1α e a infiltración de leucocitos no humor acuoso.

5º - Por primeira vez, realizouse a avaliación do perfil farmacocinético do adalimumab inxectado por vía intravítrea nun modelo de rata, demostrando que a uveíte produce unha redución do aclaramento ocular do adalimumab. Isto débese á unión específica entre o anticorpo e os receptores do TNFα que se encontran en maior cantidade debido ó proceso inflamatorio producido pola enfermidade. A información farmacocinética obtida neste traballo será de gran utilidade para desenvolver novos sistemas de administración de adalimumab que permitan unha liberación máis controlada e prolongada do anticorpo monoclonal.

Como conclusión xeral, nesta tese de doutoramento foron propostas diferentes formulacións de tacrolimus para a administración tópico-oftálmica, suxeitas por amplos estudos preclínicos co fin de lograr unha base consistente como alternativa a outros tratamentos farmacolóxicos para as enfermidades inflamatorias oculares. Isto foi confirmado con amplos estudos *in vitro*, *ex vivo* e *in vivo*. Por outra parte, no que respecta ó estudo de inxeccións intravítreas de adalimumab, esta tese de doutoramento dilucidou a distribución e farmacocinética intravítrea do anticorpo monoclonal que será de gran utilidade para o desenvolvemento de novos sistemas de administración.

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APPENDIX

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APPENDIX

CONFLICT OF INTEREST

The doctoral student declares that there is no conflict of interest regarding the doctoral thesis.

ANIMAL EXPERIMENTS

In the present thesis, male adult Sprague-Dawley rats with a 200-300 g average weight were used, supplied by the animal facility of the University of Santiago de Compostela (Santiago de Compostela, Spain). During the experiments, the animals were kept in individual cages with free access to food and water in a room under controlled temperature (22 ± 1°C) and humidity (60 ± 5%) and with day–night cycles regulated by artificial light (12/12 hours). The animals were treated according to the guidelines for the care and use of laboratory animals. Animal experiments complied with the ARRIVE guidelines (Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010 Jun 29;8(6):e1000412.). Experiments were performed at the Preclinical Imaging Service of the Experimental Biomedicine Center (CEBEGA) (REGA number: ES150780292901).

Experiments were approved by the Consellería de Medio Rural da Xunta de Galicia (15012/2021/001 and 15010/2019/006) and were authorized by the Animal Research Committee of the Health Research Institute of Santiago de Compostela (IDIS). They followed the Spanish and European Union (EU) regulations for animal experimentation (86/609/CEE, 2003/ 65/CE, 2010/63/EU, and RD53/2013).

CLINICAL STUDY

In the present thesis, the prospective observational clinical study was performed in 16 eyes of 8 patients with severe ocular disease. 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 and the Consellería de Sanidade da Xunta de Galicia (2019/204). The committee complies with current legal requirements applicable to Research Ethics Committee, following the Standards of Good Clinical Practice (CPMP/ICH/135/95).

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ARTICLES DERIVED FROM THIS THESIS

In compliance with the rules of doctoral studies at Universidade de Santiago de Compostela in Regulamento dos estudos de doutoramento na USC, DOG de 16 de Setembro de 2020, it was provided some information regarding the articles on which this work is based and the journals that published those articles. In particular it was given the names of the authors, the title of the journals, the year each article was published, the publisher, the DOI-type link, the Journal Impact Factor and the quartile from the Journal Citation Reports, some relevant information regarding copyright and use of the articles and the author contribution.

⇒ Chapter 1

TITLE: *Drug Delivery to the Posterior Segment of the Eye: Biopharmaceutic and Pharmacokinetic Considerations*
AUTHORS: *Varela-Fernández, R.; Díaz-Tomé, V.; Luaces-Rodríguez, A.; Conde-Penedo, A.;* ***García-Otero, X.****; Luzardo-Álvarez, A.; Fernández-Ferreiro, A.; Otero-Espinar, F.J.*
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AUTHOR CONTRIBUTION: The contributions of the Ph.D. candidate were essential in all the included articles. The candidate contributed to the information research and analysis, and the writing of the manuscripts.

⇒ **Chapter 2**

TITLE: *Preclinical characterization and clinical evaluation of tacrolimus eye drops*
AUTHORS: *Luaces-Rodríguez A.; Touriño-Peralba R.; Alonso-Rodríguez I.;* ***García-Otero X.****; González-Barcia M.; Rodríguez-Ares MT.; Martínez-Pérez L; Aguiar P.; Gómez-Lado N.; Silva-Rodríguez J.; Herranz M.; Ruibal-Morell Á.; Lamas MJ.; Otero-Espinar FJ.; Fernández-Ferreiro A.*


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AUTHOR CONTRIBUTION: The contributions of the Ph.D. candidate were essential in all the included articles. The candidate contributed to the design of the research and proofs, to the analysis of the results and to the writing of the manuscripts.

⇒ **Chapter 3**

TITLE: *Development and Characterization of a Tacrolimus/Hydroxypropyl-β-Cyclodextrin Eye Drop*
AUTHORS: ***García-Otero, X.****; Díaz-Tomé, V.; Varela-Fernández, R.; Martín-Pastor, M.; González-Barcia, M.; Blanco-Méndez, J.; Mondelo-García, C.; Bermudez, M.A.; Gonzalez, F.; Aguiar, P.; Fernández-Ferreiro, A.; Otero-Espinar, F.J.*
JOURNAL: *Pharmaceutics*
YEAR: *2021*
PUBLISHER: *MDPI*
LINK: https://doi.org/10.3390/pharmaceutics13020149
JOURNAL IMPACT FACTOR: *6.525 (Q1)*
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⇒ **Chapter 4**

TITLE: *Anti-Inflammatory Effect of Tacrolimus/Hydroxypropyl-β-Cyclodextrin Eye Drops in an Endotoxin-Induced Uveitis Model*

AUTHORS: ***García-Otero, X.;*** *Mondelo-García, C.; González, F.; Perez-Fernandez, R.; Avila, L.; Antúnez-López, J.R.; González-Barcia, M.; Adan, A.; Aguiar, P.; Otero-Espinar, F.J.; Bermúdez, M.A.; Fernández-Ferreiro, A.*

JOURNAL: *Pharmaceutics*

YEAR: *2021*

PUBLISHER: *MDPI*

LINK: https://doi.org/10.3390/pharmaceutics13101737

JOURNAL IMPACT FACTOR: *6.525 (Q1)*

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⇒ **Chapter 5**

TITLE: *PET Study of Intravitreal Adalimumab Pharmacokinetics in a Uveitis Rat Model*

AUTHORS: ***García-Otero, X.****; Mondelo-García, C.; Bandín-Vilar, E.; Gómez-Lado, N.; Silva-Rodríguez, J.; Rey-Bretal, D.; Victoria Otero-Espinar, M.; Adan, A.; González-Barcia, M.; Aguiar, P.; Otero-Espinar, F.J.; Fernández-Ferreiro, A.*

JOURNAL: *International Journal of Pharmaceutics*

YEAR: *2022*



PUBLISHER: *Elsevier*
LINK: https://doi.org/10.1016/j.ijpharm.2022.122261
JOURNAL IMPACT FACTOR: *6.51 (Q1)*
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FIGURES AUTHORSHIP

The author declares that all unreferenced figures of this thesis are of his authorship.

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RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTO DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 15012/2021/001
Persoa solicitante: Anxo Vidal Figueroa
Forma de inicio: solicitude da persoa interesada

Data de inicio do expediente: 28.2.2021
Procedemento: resolución de autorización

ANTECEDENTES

A persoa solicitante presentou con data 28.2.2021 unha solicitude para a realización do proxecto de experimentación animal (entrada no Rexistro Electrónico da Xunta de Galicia 2021/430700), cuxos datos se detallan a continuación:

Denominación do proxecto: *Estudo da farmacocinética intravítrea de anticorpos marcados con circonio-89 mediante inxección intravítrea en ratas*

Nome do centro usuario: Centro de Biomedicina Experimental (CEBEGA) da Universidade de Santiago de Compostela
Persoa responsable do proxecto: Anxo Fernández Ferreiro

Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): Centro de Biomedicina Experimental (CEBEGA)

Clasificación do proxecto : Tipo I [ ] Tipo II [X] Tipo III [ ]

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1 O Real decreto 53/2013, de 1 de febreiro (BOE 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

2 O artigo 88 da Lei 39/2015, de 1 de outubro, do procedemento administrativo común das administracións públicas (BOE 236, do 2 de outubro de 2015) establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

CVE: FM21k1NFg7
Verificación: https://sede.xunta.gal/cve

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3 O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto realizada polo órgano habilitado, a Sección de Experimentación Animal do Comité de Bioética da Universidade de Santiago de Compostela.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co Decreto 149/2018, do 5 de decembro, polo que se establece a estrutura orgánica da Consellería do Medio Rural e se modifica parcialmente o Decreto 177/2016, do 15 de decembro, polo que se fixa a estrutura orgánica da Vicepresidencia e das consellerías da Xunta de Galicia (DOG 235, do 11 de novembro).

De acordo con todo o indicado, RESOLVO:

1 Autorizar o proxecto solicitado.

2 O proxecto non precisa someterse a unha avaliación retrospectiva.

3 A autorización deste proxecto terá unha duración de tres anos e unha vez transcorrido este tempo deberá ser renovada.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpoñer un recurso de alzada ante o conselleiro de Medio Rural. O prazo comezará a contar dende o día seguinte ao da recepción desta resolución. Todo isto, segundo o disposto nos artigos 121 e 122 da citada Lei 39/2015.

Mediante este escrito notifícaselle a Anxo Vidal Figueroa esta resolución segundo o esixido no artigo 40.1 da antedita Lei 39/2015.

Asinado por: LOPEZ LOPEZ, MONICA
Cargo: Xefa Territorial
Data e hora: 12/03/2021 14:57:52

CVE: FM21k1NFg7
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DICTAMEN DEL Comité de ética de la investigación con medicamentos de Galicia (CEIm-G)

M.ª Belen Pérez Marcos, Vicesecretaria del Comité de ética de la investigación con medicamentos de Galicia (CEIm-G)

CERTIFICA:

Que este Comité evaluó en su reunión del día 25/04/19 :

Título: Evaluación prospectiva del uso, eficacia y seguridad de las formulaciones magistrales oftálmicas elaboradas en Servicios de Farmacia Hospitalaria
Promotor*: Anxo Fernández Ferreiro*
Tipo de estudio: EPA-SP
Version: v1
Código del Promotor: AFF-FOR-2019-01
Código de Registro: 2019/204

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIm-G de Galicia

Emite un **INFORME FAVORABLE*** para la realización del estudio por el/la investigador/a del centro:

Centros	Investigadores Principales
XXI Santiago	Anxo Fernández Ferreiro

NOTA: En el objetivo principal del estudio sustituir eficacia por efectividad dado que se trata de una evaluación en vida real (no en el contexto de un ensayo clínico).

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Belén Pérez Marcos (02/05/2019 12:20)
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Y HACE CONSTAR QUE:

1. El comité cumple los requisitos legales vigentes aplicables a los Comités de ética de investigación.
2. El comité tanto en su composición como en sus PNTs cumple las Normas de Buena Práctica Clínica (CPMP/ICH/135/95).
3. La composición actual del comité es:

Susana María Romero Yuste (Presidenta). Médico Especialista en Reumatología.
Irene Zarra Ferro. (Vicepresidenta). Farmacéutica de Atención Especializada.
Paula Mª López Vázquez, (Secretaria). Médico Especialista en Farmacología Clínica.
Belen Pérez Marcos (Vicesecretaria). Farmacéutica de Atención Especializada
Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.
Juan Casariego Rosón. Médico Especialista en Cardiología.
Nuria Carballeda Feijóo. Miembro lego. Representante de los intereses de los pacientes.
Juana Mª Cruz del Río. Trabajadora Social.
Ana Belen Cruz Valiño. Licenciada en Derecho.
Juan Fernando Cueva Bañuelos. Médico Especialista en Oncología Médica.
José Álvaro Fernández Rial. Médico Especialista en Medicina Interna.
José Luis Fernández Trisac. Médico Especialista en Pediatría.
Mª José Ferreira Díaz. Diplomada Universitaria de Enfermería.
Agustín Pía Morandeira. Farmacéutico de Atención Primaria
Jorge Prado Casal. Licenciado en CC. Físicas. Experto en Protección de Datos.
Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.
Diego Santos García. Médico Especialista en Neurología.
Juan Vázquez Lago. Médico Especialista en Medicina Preventiva y Salud Pública.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica..

En Santiago de Compostela,

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In the present thesis, different formulations of tacrolimus for topical-ophthalmic administration have been proposed, supported by extensive preclinical studies in order to achieve a consistent basis for the treatment of ocular inflammatory diseases. On the other hand, a pharmacokinetic and distribution study of an anti-TNFα monoclonal antibody (adalimumab) injected intravitreally was also carried out, allowing to know its behavior in the eye and thus develop new administration systems.