Universidade de Lisboa

Faculdade de Farmácia

Caracterização de nanossuspensões de itraconazol para administração oftálmica

Cláudia Patrica Inácio

Mestrado Integrado em Ciências Farmacêuticas

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Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

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This project was developed under the Erasmus+ Programme, at the pharmacology, pharmacy and Pharmaceutial Technology department at Santiago de Compostela's University Faculty of Farmacy, in Santiago de Compostela, Spain, under the supervison of Professor Francisco Otero Espinar.

Resumo

A utilização da via tópica na administração de fármacos tem vindo a crescer ao longo dos tempos, possuindo na atualidade um papel essencial na administração de alguns manipulados farmacêuticos. Como papel principal, os manipulados farmacêuticos preenchem falhas no mercado, especialmente quando não existe dose ou forma farmacêutica disponível para o tratamento de um determinado doente.

A queratite fúngica é uma infeção da camada frontal do olho, a córnea, causada por um fungo. Este tipo de infeção ocular é caraterizada por dor, fotofobia, sugerindo inflamação grave, que pode levar à úlcera da córnea e possível perda da visão. Por estes motivos, um diagnóstico precoce e um tratamento imediato são extremamente importantes para evitar complicações a longo prazo. Apesar da existência de alguns fármacos para o tratamento da queratite fúngica, devido à sua baixa estabilidade e custos elevados existe ainda um largo número de doentes não tratados, o que tem levado a alguns oftalmologistas a procurar alternativas terapêuticas como antifúngicos em gotas para aplicação tópica produzidas na farmácia hospitalar. A aplicação tópica é a via não invasiva mais usada na administração de medicamentos no tratamento de doenças no segmento anterior do olho. No entanto, esta via possui uma baixa biodisponibilidade ocular com menos de 1 a 7% de absorção do fármaco, uma vez que o seu transporte e permanência são reduzidos devido ao mecanismo de defesa do olho contra agentes externos. Com este fim, foi utilizado um novo sistema de administração ocular tendo sido estudadas três nanossuspensões de itraconazol com diferentes métodos de produção. Cada nanossuspensão foi caraterizada e avaliado o perfil de liberação e difusão passiva *in vitro*, mucoadesividade, toxicidade em córneas bovinas e membranas coriônicas de ovos de galinha fertilizados.

Os resultados obtidos permitem concluir que é possível preparar uma formulação oftálmica de itraconazol basada em nanosuspensões estabilizadas que quanto às suas características de tamanho de partícula, pH, potencial zeta e mucoadesividade se enquadram nas especificações para uso oftálmico, não induzindo desconforto pela opacidade da nanosuspensão, nem toxicidade em estudos *ex vivo*.

Palavras-chave: Queratite fúngica, Administração ocular de fármacos, Nanosuspensão, Itraconazol

Abstract

The use of the topical route in drug administration has been growing over time and currently has an essential role in the administration of some pharmaceutical compounding. As a primary role, pharmaceutical compounding fill gaps in the market, especially when there is no dose or pharmaceutical form available for treating a particular patient.

The fungal keratitis is an infection of the clear, front layer of the eye (the cornea) which is caused by a fungus. This type of ocular infection is characterized by pain, photophobia, reduced vision, suggesting severe inflammation, which can lead to corneal ulcer and possible vision loss. For this reason, an earlier diagnosis and prompt treatment are extremely important to prevent long-term complications. Despite the existence of some drugs for the treatment of fungal keratitis, due to their low stability and high costs, there are still a large number of untreated patients, which has led some ophthalmologists to look for therapeutic alternatives as topical drop antifungals produced at the hospital pharmacy. Topical application is the most non-invasive route of drug administration used to treat diseases at the anterior segment of the eye. However, this drug delivery route has a low ocular bioavailability of topically applied drugs with less than 1-7% of the applied drug being absorbed, since the reduced drug transport and permanence in the eye due to eye deflating mechanisms against external agents. For this purpose, a novel ocular drug delivery system was used and three itraconazole nanosuspensions with different production methods were studied. Each nanosuspension characterized and evaluated for the in vitro release and passive diffusion profile, mucoadhesiveness, toxicity in bovine corneas and chorionic membranes of fertilized chicken eggs.

The results allow to conclude that it is possible to prepare an ophthalmic formulation of itraconazole based on stabilized nanosuspensions which, in terms of particle size, pH, zeta potential and mucoadhesive characteristics, meet the specifications for ophthalmic use, not inducing discomfort due to nanosuspension opacity and neither toxicity in ex vivo studies.

Keywords: Fungal Keratitis, Ocular drug delivery, Nanosuspension, Itraconazol

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Abbreviations

AH - Hyaluronic acid

BCOP - Bovine Cornea Opacity/Permeability

BM - Bowman’s membrane

BSC - Biopharmaceutics classification scheme

CAM - Chorioallantoic membrane

CDC - Controle diseased center and prevention

CE - Corneal epithelium

DM - Descemet´s membrane

FK - Fungal keratitis

HET-CAM - Hen’s egg test chorioallantoic membrane

HP-CD - Hydroxypropyl-cyclodextrin

HPB-CD - β-Hydroxypropyl-cyclodextrin

ITR - Itraconazole

ITR-βCD - Itraconazole with β-Hydroxypropyl-cyclodextrin

ITR AH-CARBO centri - Itraconazole with solution of Acid Hyaluronic and Carbopol centrifuge

ITR AH-CARBO - Itraconazole with solution of Acid Hyaluronic and Carbopol

ITR AH+CARBO - Itraconazole with Acid Hyaluronic and Carbopol added in separated

NS - Nanosuspension

PBS - Phosphate buffer solution

PC - Phosphatidylcholine

PDI – Polydispersion index

TF - Tear film

TFLL - Tear film lipid layer

ZP - Potential Zeta

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1. Introduction

1.1. Fungal keratitis

Fungal keratitis (FK) was first documented in 1879 and the incidents reported has been increasing overtime. In 2015, FK gained momentum importance due to an outbreak among contact lens wearers in many developed countries (1). Studies report 40% to 50% from all isolated keratitis cases were caused by fungi, and since the outcome of fungal keratitis is worse than that of bacterial keratitis, FK must be underscored due to limited availability of antifungal agents as well as the poor response to the therapy (1–3).

According to the Center of Diseased Control and Prevention (CDC), a fungal keratitis is an infection of the clear, front layer of the eye (the cornea) which is caused by a fungus (4). Usually, both of cornea and conjunctive form a protective anatomic barrier against external agents but since the main fungi access into corneal stroma is through a defect in the epithelium, FK is usually associated with trauma in those two structures. Once in the tissue, the fungi start to replicate in the anterior chamber, then by the iris diaphragm and pupil leading to an extremely difficult infection to eradicate and diagnosis. This invasion results in mostly innate and adaptative immune response with damage tissue, scarring and opacification of the cornea (3,5). This type of ocular infection is characterized by pain, photophobia, reduced vision, opacification of the corneal surface, suggesting severe inflammation, which can lead to corneal ulcer, vision loss and hypopyon, with the presence of fungal hyphae within the corneal stroma (figure 1). For this reason, an earlier diagnosis and prompt treatment are extremely important to prevent long-term complications (3,5,6).

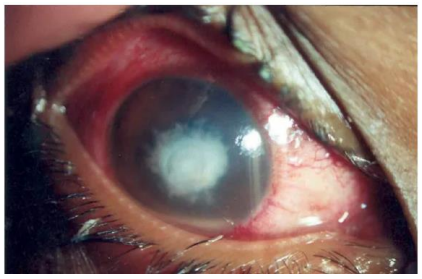


Figure 1- Human eye with fungal keratitis (Adapted from Thomas, 2003) (7)

In the past, in both developed and developing countries, the usual cause of FK was a trauma with vegetative matter or objects contaminated with soil. However, nowadays with the farms

‘industrialization , the use of contact lenses became the major cause of FK (37%) in the number of patients who has ocular trauma (25%) (5,8).

To prevent the major complications associated with FK, an effective and earlier diagnosis is very important. For that reason, tissue sampling and culture are imperative to the diagnostic of fungal keratitis (5). The most common isolated report cases of FK were caused by *Aspergillus* species (27% to 64%), followed by *Fusarium* (6% to 32%) and *Penicillium* (2% to 29%) and like most of infection diseases, the prevalence is influenced by some factors like geographical location and socioeconomic status (9).

Pharmaceutical treatment of FK is based on topical ocular drugs and the type of antifungal used depends on their corneal penetration activity and effectiveness (10). The most used antifungal to treat FK consist in polyene drugs such as Natamycin, but drugs like Anfotericine B, Voriconazole, Econazole, Fluconazole and Ketoconazol are also used (5). It is important to notice that none of these drugs are available in the Portugal market (11). Despite of the progresses made in the FK treatment, nowadays still exists a significant lack of commercial ophthalmic formulations due to their low stability and high costs and for that reason there are still a large number of patients untreated. To bridge this gap, many ophthalmologists were force to seek alternatives such as antifungal eye drops produced in hospital pharmacy (12).

1.2. Ocular drug delivery

Human eye anatomy and physiology makes this organ unique, complex and a major challenge to drug delivery. Ocular drug delivery system should have three key properties to be an ideal one. These key properties are (13):

- Controlled and sustained release profile to reduce the frequency of administration with the therapeutic concentration of the drug over time;
- Prolonged retention and specific targeting in the diseased tissues to improve therapeutic efficiency and avoid side effects;
- Patient-friendly delivery routes.

To improve the drug delivery at the eye, it is very important to know its structure, the different pathways available and the challenges which pathway faces.

1.2.1. Eye structure

Human eye is one of the most important sensory organs in the human body. This organ is responsible for the capture of images which are transmitted to the brain through the optic nerves as signals (14).

The ocular structure can be divided into two different segments, the anterior segment and the posterior segment. The first one consists of the cornea, lens, conjunctiva, iris, aqueous humor and ciliary body. The posterior segment mainly consists of the vitreous humor, choroid, retina, and optic nerve (Figure 2). In order to find a treatment to fungal keratitis, it is important to know the structure of cornea and their properties and also the structure and properties of the tear (15).

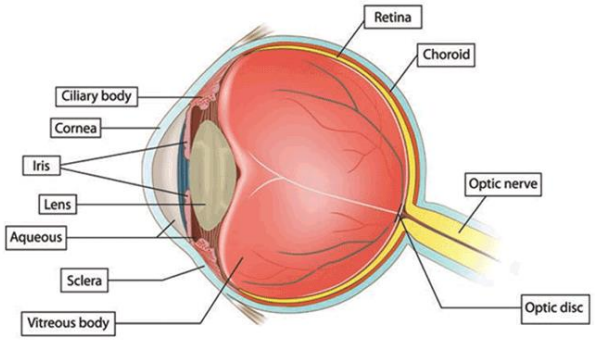


Figure 2- Human eye structures (Adapted from Glaucoma Research Foundation,2017) (16)

1.2.2. Cornea

Being the most anterior segment in the eye, the cornea is one of the most sensitive tissues in the body due to be highly innervated. Cornea function is to refract light rays in order to get them catch by the retina (14,17). This organ is a transparent tissue which has no blood vessels, unlike the most of the tissues in the body but instead of blood to nourish and protect against external agents, cornea is bathed by tears at the anterior surface and at the posterior surface is bathed by aqueous humor (17,18).

Cornea's tissues are organised into three layers which are the epithelium, the stroma, and the endothelium. These layers are separated by Bowman's membrane and Descemet's membrane as shown in figure 3. The composition of each layer is different from each other which gives the cornea some specific properties (19).

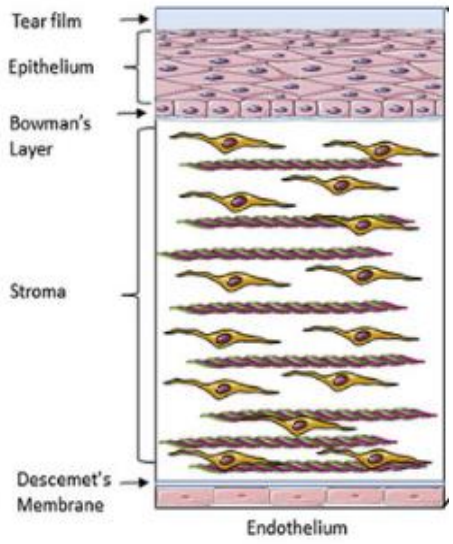


Figure 3- Cornea's structure (Adapted from Masterton, et al.,2018) (20)

Corneal Epithelium (CE) is the most anterior layer of the cornea and has the function of preventing the passage of foreign materials trough the eye and also the absorption of oxygen and nutrients from tears which will be delivered to the other layers. This type of epithelium is stratified squamous, non-keratinized in five or six cells layers and a high lipid content which restricts the permeability of polar, water-soluble compounds. The lipid content is localized apically at the epithelium and it is composed of glycoproteins, such as mucins, which forms a complex, the glycocalyx. This mucin are made up for sialic acid (pKa=2.6) which gives the epithelium the characteristic corneal negative charge at physiological pH (18,21–23).

Bowman's membrane (BM) is a transparent tissue film constituted by collagen fibres (type I) implanted in the proteoglycan matrix. This membrane is the next layer after the epithelium and has as function the maintenance of the corneal shape. BM has some specific properties like being non-regenerative and making the connection between corneal stroma and epithelial cells (14,18).

Stroma is the central corneal layer, rich in collagen type I and also constituted by 70–80% water which represents 90% of the thickness of the cornea. Stroma's collagen gives to the layer strength, elasticity and form and its specific proteins' arrangement and shape are responsible for the light-conducting transparency. This layer consists of keratocytes, fibroblastic cells, neural tissue and Schwann cells which gave to stroma hydrophilic properties acting like barrier against liposoluble compounds (14,18,22,24).

Descemet´s membrane (DM) is the layer located behind the stroma and is composed of two layers, an anterior banded layer and a posterior non-banded layer. The anterior one is developed by collagen lamellae and proteoglycans which can be found in fetal corneas as early as 12 weeks of gestation. The posterior non-banded layer is laid down by endothelial cells and thickens over decades. Collagen lamellae is constituted by collagen type IV and VIII fibrils, and the type IV collagen fibrils are specific to DM. This thin but strong layer, protects the eye against infection and injuries (18,25,26).

Endothelium is a thin monolayer' cells which secretes collagen used in the formation of DM. This layer maintains the health and clarity of the stroma by controlling the stromal through hydration and also allows the passage of nutrients and other molecules from the aquous humor. Controlling the stromal deturgecence, the endothelium's layer controls stroma's water, maintaining its fineness and transparent characteristics. Having a high lipid content, the endothelium represents a hydrosoluble compounds barrier (18,22,27).

1.2.3. Tear

Ocular surface is the most exposed mucosal surface in the human body. To prevent infections, allergens, extremes temperature or dryness, the eye has a liquid layer called tear film (TF). TF is produced in the lacrimal gland and usually has a pH similar to the blood plasma but the osmotic pressure is a slightly higher. The tear film'constituition has usually antimicronial components including peroxidades, lysosies, in three distinct layers, the lipid layer (TFLL), an aquous layer and the mucin layer which is the closest layer to the surface of cornea (Figure 4)(28).

TFLL is the outer layer from the TF which is in contact to the environment. This layer is mostly composed by lipids and has as functions, the reduction of surface tension of the tear film, help in the TF re-spreading after blinks and prevention of water evaporation. The aqueous layer of the tear film is the second layer and as main role this layer provide an optically smooth surface for light refraction, improve the lubrication during the blinks and eye movements and also the prevention of eye surface dehydration. At least, the last layer of the tear film, and the one that is in direct contact with the cornea is the mucin layer which provides to the cornea an easily lubricated surface and assists the water re-spreading after blinks (28–30).

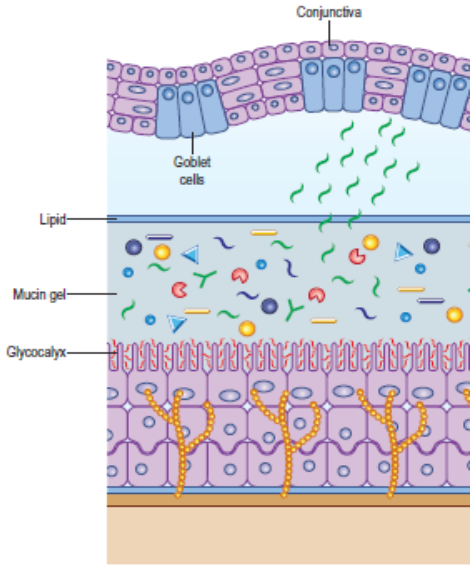


Figure 4- Tear Film Structure (Adapted from Foster, et al.,2013) (28)

1.3. Ocular topical formulations

Topical application is the most non-invasive route of drug administration used to treat diseases at the anterior segment of the eye. Conventional dosage forms applied to treat diseases in this segment are solutions (62.4%), suspensions (8.7%), and ointments (17.4%), which compose an estimated 90% of marketed ophthalmic formulations (15,26).

However, topical drug administration demonstrates poor ocular bioavailability due to the body's natural mechanisms and barriers. Despite the easy access of the topical treatment, topical drugs delivery to the ocular surface face reduced drug transport, which is influenced by lacrimation and tear turnover, nasolacrimal drainage, spillage from the eye, metabolic degradation and non-productive adsorption/absorption, usually resulting in low ocular bioavailability of topically applied drugs with less than 1-7% of the applied drug being absorbed (31).

To improve the efficiency of ocular drug delivery there are two types of delivery systems, the conventional ocular drug delivery and the novel. The conventional one is related to topical liquid/solution eye drops, emulsions, suspensions and ointments and the novel one is related to ocular drug delivery based in nanotechnology, such as nanomicelles, nanoparticles, nanosuspensions, lipossomes, dendrimers, in-situ gelling systems, contact lens, implants and microneedles (32).

1.4. Nanosuspensions

Nanomaterial used in the eye' drug delivery systems have been increasing since this type of systems represent a huge potential with wide range of applications. Ophthalmic formulations containing nanoparticles drug show some advantages such as less limitations surrounding ocular drug penetration, decreased direct cellular stimulation and reduced amount drug used by increased its bioavailability. Another advantage is the fact that nanoparticles decreased the suspension'opacity and the light scatter which made them more comfortable, less sensed and irritate for the eye. This type of technology can be used in both anterior and posterior segments and the particle size is ranging from 1-1000 nm (13,33–37).

From the past two decades, the nanocrystal technology has been growing in the pharmaceutical field, once one of its major advantage is the ability to formulate poorly soluble drugs, resulting in nanosuspensions (NS). NSs consist essentially in a dispersion of pure drug nanocrystals in a liquid medium which are typically water. According to the authors Nangare KA, Powar SD, Kate VK, Patwekar SR, Payghan SA, a "nanosuspensions can be defined as a very finely colloid, biphasic, dispersed, solid drug particles in aqueous vehicle, size below 1μm, without any matrix material, stabilized by surfactants or polymers, prepared by suitable methods for drug delivery applications, through various routes of administration like oral, topical, parenteral, ocular and pulmonary routes" (36). NSs have some key properties which make them a versatile drug delivery platform. Some of these properties are the ability to enhance permeability, increase the bioavailability, have a specific site of delivery, enhance adhesiveness, have nanocarrier behaviour, easy scale up and long-term stability. The stability and therapeutic efficacy of nanosuspensions is influenced by the physicochemical characteristics of the system itself (36).

Nanosuspensions' particles size influences the ocular delivery in terms of residence in circulation, its uptake, adherence, clearance and degradation. In these field, nanosuspensions with size range 10 to 1000 nm can improve the topical passage of large water insoluble molecules through the barriers of the ocular system (13).

Some studies using nanosuspensions in the ocular delivery reported increased residence time for eye drops, increased ability of the drug to penetrate into the deeper layers of the ocular structure and aqueous humor which minimized the precorneal drug loss caused by rapid tear fluid turnover and decreased toxicity (13,38).

1.5. Itraconazole

Itraconazole (ITR) is a triazole antifungal agent with activity against a broad spectrum of fungal species including *Cryptococcus, Candida, Aspergillus, Blastomyces* and Histoplasma capsulatum var. Capsulatum. Triazole antifungal agents have in common a characteristic structure with five-membered azole ring and a complex side chain which gives to the molecule a fungistatic activity (39–41).

According to biopharmaceutics classification scheme (BCS), ITR is classified as a class II drug due to its characteristics of lipophilic weak base with a calculated logP of 6.2. Aqueous solubility is estimated at approximately 1 ng/ml at neutral pH and 5.66 at a pH of 8.1 which is practically insoluble in water (~ 4 ng/ml) (40,42). To improve the solubilising of ITR, it is very common add the Hydroxypropyl-cyclodextrin (HP-CD) to the formulations. The HP-CP has a special structure with one ring of substituted glucose molecules that form a cylindrical structure which is hydrophobic on the inside and hydrophilic on the outside. The hydrophobic cavity forms an ideal chamber for lipophilic molecules improving their solubility (39).

Regarding to the mechanism of action of ITR, this compound inhibits the synthesis of ergosterol, which is a vital component of the fungal cell membrane. Under normal fungi growth, the precursor of ergosterol, the lanosterol is catalysed from 14α-demethylation by fungal cytochrome P450 (CYP). When the Itraconazole is in contact with the fungi, this compound interacts with the substrate-binding site of fungal CYP and blocks this reaction. As a result, instead of ergosterol, lanosterol and the others 14α-methyl sterols are accumulated in the cell membrane. This impairment of ergosterol synthesis leads to abnormalities in the fungal membrane permeability, membrane-bound enzyme activity and the coordination of chitin synthesis (39).

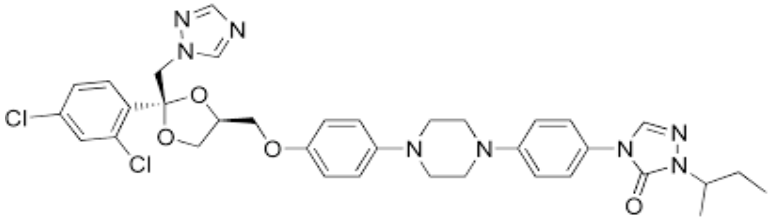


Figure 5 – Molecular structure of Itraconazole

2. Objective

Continuing the studies of Itraconazole nanosuspensions for topical application with the objective of preparation pharmaceutical compounding in hospital and community pharmacies. More specifically, by developing a formulation which could be an alternative cheaper, stable and more efficient compared to those already in the market, using a different antifungal, like itraconazole.

It is intended to characterize physico-chemically a formulation, namely, pH, particle size, Zeta potencial, concentration of ITR and transparency of each formulation, see the morphology using Transmission electronic microscopy (TEM), the mucoadhesive properties, in vitro drug release studies and ex vivo toxicity studies.

3. Materials and Methods

3.1. Materials

Itraconazol (ITR) was purchased from Roig Farma S.A (Spain), Hyaluronic acid (AH) (Acofarma® (Spain)), B-Hydroxypropyl-cyclodextrin (HPB-CD) Molar substitution degree of 0.65 and molecular weight of 1399 Da, (Kleptose HPB, Roquette Laisa (Spain)), Carbopol® 974 P (CARBO) (BFGoodrich, (Brecksville)), Polymer F127, Dodecane anhydrous 99% (Sigma-Aldrich-Merck (Darmstad (USA)), Acetone 99,6% GLR, Sodium chloride (Labken (Spain)), Dihydrogen phosphate (GPR Rectapur (Belgium)), Sodium dihydrogen dodecahydrate (Scharlau (Spain)), L-αPhosphatidylcholine (PC) (Sigma® (USA)).

3.2. Methods

3.2.1. Preparation of the nanosuspensions

The ITR nanosuspensions were prepared according to the nanoprecipation technique and the composition of NS were made according to previous work (43,44). First, ITR was dissolved with Pluronic F127 in acetone in ice bath during 2-3 min and after the solution was heated to obtain a transparent solution. The resultant solution was injected drop by drop into cold water (4ºC) which was stirred continuously on a magnetic stirrer at 1000 rpm for 10 min at 4ºC. The obtained nanosuspension was subject to a cycle of ultrasounds Bandelin electronic (GM 3200, Berlin, Germany) at amplitude 20 during 10 min and after 10 min at the rotavapor Buchi (R-200, Germany) at 85ºC. In order to produce three different ITR NS, three different solutions made with water were added to the initial nanosuspension, the solution of AH, AH with CARBO in the same solution and the solution of CARBO. After the addition of each solution, the resulting nanosuspension was subjected to magnetic stirrer at 500 rpm for 20 min at 4ºC, followed by one cycle of ultrasounds for 10 min and with amplitude 20.

From this process result two different NS, the ITR AH-CARBO which has the AH and CARBO in the same solution, and the ITR AH+CARBO which was added separately the solution of AH at first and after the solution of CARBO.

Another formulation was studied, the ITR AH-CARBO centri. This last formulation is the same as the ITR AH-CARBO but it was centrifugated at 13000 during 30 min at 10ºC and the

precipitate was resuspended with 200 µl water each Eppendorf. All NS had the pH adjusted to eye pH physiologic.

3.2.2. Characterization of nanosuspensions

3.2.2.1. Particle size, Zeta potential, pH, concentration of ITR and NS transparency

The mean particle diameter, polydispersity index (PDI) and zeta potential (ZP) of NSs formulations analysed in this work were determined by Zetasizer® 3000 HS (Malvern Instruments, UK) equipped with the Malvern PCS software (version 7.11).

Another important factor involved in the NSs formulation process is the pH because that this parameter has in the solubility and stability. NSs should have a pH compatible with the ophthalmic delivery so that there is no eye irritation but also keep the formulation stable over time. The pH of the prepared formulations was checked by using pH meter (HI5221 HANNA®, Woonsocket, USA).

Concentration of ITR evaluation test was performed by UV- spectrometer Agilent® technologies (Cary 60, USA) and it was obtained thought a calibration line. The percentage transmittance of the nanosuspensions was recorded between 200 and 800 nm wavelength using UV- spectrometer Agilent technologies.

3.2.2.2. Morphology using Transmission Electron Microscopy (TEM)

The morphologic examination of the NSs was performed by a transmission electron microscope (TEM) (JEOL JEM-F200CF-HR, Japan) with a camera Gatan one view high resolution. One drop of the diluted vesicular dispersion was deposited on the surface of a carbon-coated copper grid, negatively stained with 2% phosphotungstic acid then allowed to dry at room temperature for 10 min for investigation.

3.2.3. Corneal mucoadhesive properties of the nanosuspensions

Mucoadhesive properties of NSs were evaluated by SHIMADZU (AGS-X 1KN, Japan) using fresh bovine corneas. Plaster supports were made with the curved shape of the anterior part of the eyeball which was obtained through a mold made by eyeball' contact printing on alginate paste. Cornea was stick on supports with cyanoacrylate adhesive (Permabond 2050)

and these to the upper probe with double-sided tape. Formulations were deposited in weighing bottles (Ø×h: 40x) and placed in the lower part of the analyser. Then, the cornea was lowered into the formulation at a speed of 1 mm/s and maximum force at 0.001 N until contact was made. Cornea was introduced at a distance of 10 mm from the formulation and then the probe was pushed back at a speed of 1 mm/s until its complete separation from the formulation to be tested. Force-displacement curve was recorded, and the work was calculated from the area under the curve obtained during the traction phase.

3.2.4. *In vitro* drug release studies

Itraconazole *in vitro* release from the nanosuspensions systems (ITR AH-CARBO; ITR AH+CARBO; ITR AH-CARBO centri) and the itraconazole with hydroxypropyl-cyclodextrin (ITR-HPB) was estimated by using Franz diffusion cells and Visking dialysis tubings of a cut off of 12-14KDa (Medicell membranes Ltd). The itraconazole with hydroxypropyl-cyclodextrin (ITR-HPB) was used as control solution. The dissolution medium used was freshly prepared phosphate buffer solution with hydroxypropyl-cyclodextrin (PBS-HPB). PBS was prepared according with the 9th European Pharmacopeia, from dihydrogen phosphate, sodium chloride and sodium dihydrogen dodecahydrate and adjusted to pH 7.4 using 0.1M solution of sodium hydroxide. Visking dialysis tubings was previously soaked overnight in the dissolution medium to hydrate the membrane. The volume of 2500 µl of formulation containing 0,5 mg of itraconazole were accurately placed into the upper compartment. Sink conditions were maintained in the receptor compartment which was filled with 7ml of PBS-HPB. During the experiment, cell compartments were continuously homogenized by magnetic stirring at high speed in a thermostated bath at 37±1°C. Serial sampling was performed at stipulated times intervals and replaced by an equal volume of the PBS (1ml). The aliquots were analysed by UV-Vis spectrophotometry at 262 nm.

3.2.5. Nanosuspensions passive diffusion with parallel artificial membrane permeation assay (PAMPA)

The parallel artificial membrane permeability assay (PAMPA) was performed according to protocol used already in corneas permeability studies and also according with the provider (45,46). Before starting the test, 300µl of 1% solution (w/v) of phosphatidylcholine (PC) in dodecane was sonicated. For each donor well, 5µl of the PC/dodecane mixture was pipetted carefully. The contact between pipette tip was avoided. Immediately after the application of the

lipidic membrane, 300 µl of ITR NSs was added into the donor well. Then, 300 µl of PBS-HPB was added in each well of the acceptor plate and after, the drug-filled Donor plate was placed into the acceptor plate taking into account that the membrane should be in contact with the buffer. The plates were incubated for 4h with shaking at 1330 rpm at 35 °C in the Agitator Heidolph (Unimax, Germany).This step was followed by the separation of PAMPA sandwich plates and determination of concentrations of ITR in the acceptor chamber by UV-Vis spectrophotometry at 262 nm.

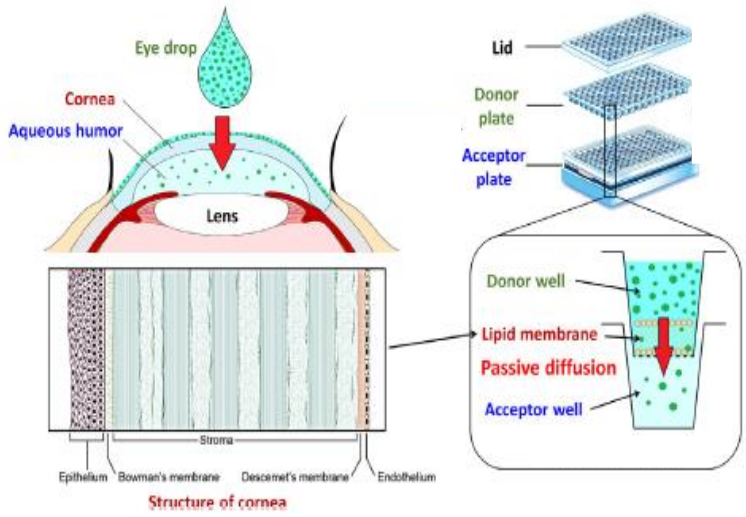


Figure 6 - The parallel artificial membrane permeability assay and cornea structure (Adapted from Dragó, et al., 2019) (45)

The membrane retention and effective permeability of ITR NSs were calculated using the following equation:

(1)

$$P_{am} = -2.303 \times \frac{V_{dn} \times V_{ac}}{V_{dn} + V_{ac}} \times \frac{1}{S \times t} \times \log\left(1 - \frac{flux\%}{100}\right)$$

Where,

P_{am} is the permeability coefficient through the membrane (cm/s); V_{dn} is the volume of donor compartment (ml); V_{ac} is the volume of the acceptor compartment (ml); *S* membrane area (cm^2) and *t* is the incubation time (s); *flux%* is the flux percentage, defined as:

(2)

$$flux\% = \frac{Q_f}{Q_{ref}} \times 100$$

Where,

Q_f is the ITR concentration in the acceptor compartment at the end of the assay and Q_{ref} is the final concentration in the donor and acceptor that will be obtained if the membrane barrier do not exists.

3.2.6. *Ex vivo* toxicity studies

3.2.6.1. Bovine Cornea Opacity/Permeability test (BCOP)

The Bovine Cornea Opacity/Permeability test (BCOP) test was performed according to the ECVAM DB-ALM: INVITTOX protocol nº127 (47). The corneas used in this test were obtained from the cows' eyes in the local slaughterhouse, 1 hour after the cows have been slaughtered and were brought at the laboratory in PBS at 4ºC. Since in the laboratory, the corneas were excised 2-4 mm of surrounding sclera with a scalpel. Illuminance was measured by using a luxometer (Gossen Mavolux 5032C USB) and Corneas were placed between two cylindrical supporting black holders (fabricated with polylactic acid filaments using a 3D print, Wilbox BQ) illuminated with Olympus Highlight 200 pipe ligtht in brightness position 5. Before the test began, a measurement of the illuminance in the support without cornea was made (Blank). Opacity was obtained from transmittance values obtained by UV-spectrometer from 800 nm to 200 nm and a transmittance spectrum was obtained versus wavelength. It was used as a negative control (PBS) and a positive control (Ethanol 100%). After illuminance and transparence initial readings, corneas were placed in Franz cells and the epithelial part of the cornea was placed towards the donor compartment. Receptor compartment was filled with PBS and was homogenized by magnetic stirring in a thermostated bath at 36°C during assay time. The assay consisted of two parts. At first, the undamaged bovine corneas were incubated for

60 min at Franz cells with 1 ml of PBS and then, PBS was removed and illuminance and transparency were measured. In the second part of assay, 1ml of each ITR NS (ITR AH-CARBO; ITR AH+CARBO; ITR AH-CARBO centri) was introduced at the epithelial part of the cornea and left there for 10 min. The donor compartment was cleaned and incubated with PBS for 120 minutes. Any corneal damage to the cornea was evaluated by transmittance and level of opacity. At the permeability test, 1ml of fluorescein 4mg/ml were added to the donor compartment during 90 min. Two samples were taken after 30 and 90 min and the concentration of fluorescein was measured by UV-Vis spectrophotometry at 262 nm in the UV- spectrometer.

To determine the *in vitro* irritation score produced in each cornea, the following formulation and table are used:

(3)

$$In\ vitro\ irritation\ score = Cov + (15 \times CovD_{490})$$

Where,

Cov is the corrected Opacity value (Lx); $CovD_{490}$ is the Corrected OD490 Value (Lx).

Table 1 - *In vitro* irritation scale

In vitro Irritation Score	In vitro Irritation Scale
0 - 3	Non eye irritant
3.1 – 25	Mild eye irritant
25.1 – 55	Moderate eye irritant
≥ 55.1	Severe/corrosive eye irritant

3.2.6.2. Irritation ocular Assay (HET-CAM)

The Hen's egg test chorioallantoic membrane (HET-CAM) was performed using the method previously described in ICCVAM (48). This test was made with fertile Broiler chicken eggs placed in an automatic rotation incubator for 8 days and incubation was kept at 37 ± 0.5°C with 65±7.5% of relative humidity. The last 24 hours in the incubator, the eggs' rotation process was stopped to maintain the air chamber in the widest part of the egg. The assay was made the 9th day of incubation and to open the eggs it was used a tiny drill (Dremel®). The inner membrane, chorioallantoic membrane (CAM) was soak with 0.9% NaCl and removed with

forceps. A volume of 0.3 mL of each formulation formulations (ITR AH-CARBO; ITR AH+CARBO; ITR AH-CARBO centri) was applied directly into the CAM surface and were compared with a negative control (NaCl 0.9%) and a positive control (NaOH 0.1%). The CAM was observed over a period of 300 seconds and the effects were measured by the appearance of haemorrhage, coagulation and vessel lysis. The scores are represented in table 2 (49).

Table 2- Scoring chart for HET-CAM test

Effect	Score	Inference
No visible hemorrhage	0	Non irritant
Just visible membrane discoloration	1	Mild irritant
Structures are covered partially due to membrane discoloration or hemorrhage	2	Moderately irritant
Structures are covered totally due to membrane discoloration or hemorrhages	3	Severely irritant

3.3. Statistical analyse of results

To compare the result of the different studies was used one way analyse of the variances (One way ANOVA) and turkey's multiple comparison test. The analysed was made using the Graph Pad Prim® version 6.

4. Results and Discussion

4.1. Preparation of the nanosuspensions

From previous studies, 3 different nanosuspensions of itraconazole were chosen to continue characterization, release and toxicity studies (44). The composition of ITR NS studied are represented in table 3.

Table 3 - Composition of ITR NS

NS	ITR (mg/ml)	F127 (% w/v)	AH (% w/v)	CARBO (% w/v)
ITR AH-CARBO	0.167	0.200	0.0025	0.02
ITR AH+CARBO				0.05
ITR AH-CARBO centri				0.02

4.2. Characterization of nanosuspensions

4.2.1. Particle size, Zeta potential, pH, Concentration of ITR

All three ITR NS were assessed for particle size, PDI, ZP, pH and concentration of ITR as presented in Table 4. The pH values obtained were near to 5 belonging to pH rage tolerated by the eye (pH 4-8) (50). The mean particle diameter for all NS formulations tested was found to be between 300 and 1000 which was reported suitable for ocular applications (13). This parameter along to PDI allows to understand some physicochemical properties like dissolution velocity, physical stability, saturation solubility and biological performance. In all ITR NS, the PDI was less than 0,5 which represents quite homogeneous and monodisperse size populations, suitable to have good physical stability (51).

Zeta potential values were closed to -30mV represent good long-term stability of NS preparations since to obtain an electrostatically stabilized nanosuspension the ideal minimum zeta potential is between ± 20 - 30mV (51). No significant differences were observed for Zeta potential between formulations. The negative charge surface has the origin in the ionized

carboxylic acid residues of both polymers, AH and Carbopol. The incorporation of Carbopol in the formulation do not modify the electric surface charge properties, because both polymers present negative charges at the pH of the formulation.

ITR concentration values in the nanosuspensions varies between formulations and the major ITR concentration was ITR AH-CARBO centri (0,55mg/ml). Although ITR AH-CARBO and ITR AH+CARBO have lower concentration of ITR in their constitution, when compared with a solution of ITR in water (s ~ 4 ng/ml), both of NSs have increased the solubility of ITR in water (40,42).

Table 4- Characterization of nanosuspensions

NS	pH	ZP	Size (nm)	PDI	[ITR] (mg/ml)
ITR AH-CARBO	5.0±0.01*	-30.8±1.08	877.4±65.0	0.262±0.09	0.178±0.02
ITR AH + CARBO	5.03±0.02*	-29.9±6.93	741.7±103.5	0.259±0.09	0.147±0.047
ITR AH-CARBO centri	5.9±0.9*	-31.4±6.99	668.5±139.7	0.19±0.11	0.55±0.29

* With pH adjustment (NaOH 0.1%)

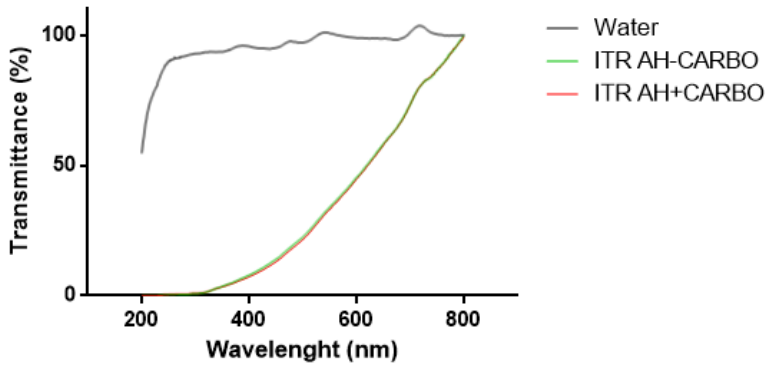
4.2.2. Transparency of nanosuspensions

For ocular drugs delivery, the transparency of a formulation is an important parameter because if the formulation has a low transmittance, it has a high opacity and the adherence to therapy may be compromised since this type of formulations induce discomfort in patients. According with figure 7a), both of ITR AH-CARBO and ITR AH+CARBO has a similar transmittance profile. However, the ITR AH-CARBO centri present a saturate transmittance profile may have been caused by the high content in nanoparticles of ITR, as is constated in the determination of the ITR concentration values in the nanosuspensions.

Although the transmittance indicates that developed formulations are not transparent, it is not expected that the administration of the nanosuspensions cause vision problems because the

low volume of the instilled solution and the small optical path length of the tear film prevent from important visual perturbances.

a)



b)

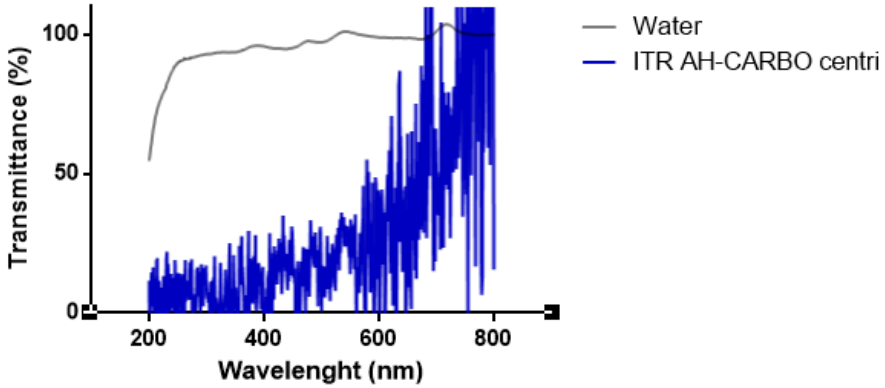
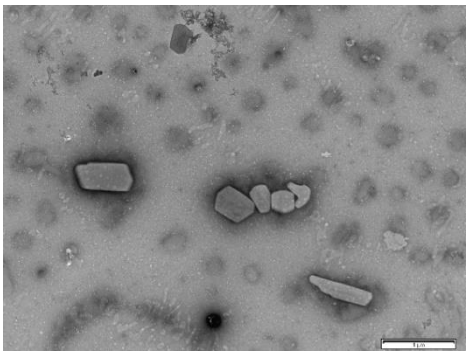


Figure 7 - Percentage of transmittance of ITR formulations a) ITR AH-CARBO and ITR AH+CARBO, b) ITR AH-CARBO centri

4.2.3. Morphology using Transmission Electron Microscopy (TEM)

The morphology of the prepared ITR NS was observed using TEM as shown in Figure 8. Most of the ITR crystals have a rectangular shape and regular surfaces with diameter less than 1µm. The TEM micrograph showed a low number of nanoparticles with irregular or polygonal shapes. These shapes are typical from particles obtained by precipitation and crystallization of drugs where the edges tend to be sharper. The dark areas and stains surrounding the particles in the TEM image may be attributed to the presence of the AH and CARBO. No differences in shapes of nanoparticles are observed between the three formulations.

a)



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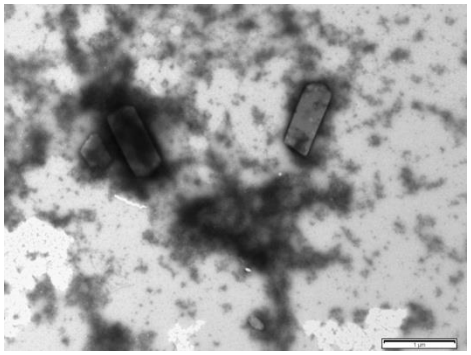


c)

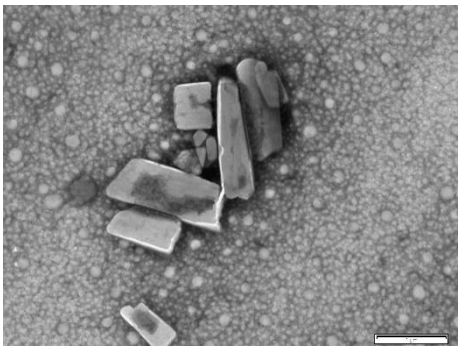


Figure 8- Transmission Electron Microscopy (TEM) images of a) ITR AH-CARBO, b) ITR AH+CARBO and c) ITR AH-CARBO centri

4.3. Mucoadhesive properties of nanosuspensions

Mucoadhesiveness studies were performed to measure the adhesive strength of the ITR nanosuspensions to the cornea. As shown in the table 5 and figure 9, the mucoadhesion of all NS were very similar. According to the analysis of the variances (One way ANOVA) no significant differences were observed in the mucoadhesion work (α ns) however differences were found significant in the maximum detachment force ($\alpha<0.05$). According with the Turkey's multiple test comparison of maximum detachment force, the ITR AH-CARBO centri is significantly lower than ITR AH-CARBO (Table 6). This difference between those two nanosuspensions can be justified by the fact that ITR AH-CARBO centri has less AH because of the change of AH medium for water after the centrifugation process, which decreases the bio-adhesiveness of the nanosuspension. Additionally, the incorporation of Carbopol in the medium do not increase the bioadhesive properties of the nanosuspension. The values of mucoadhesion are in the same order to other ophthalmic bioadhesive systems (52).

Table 5- Mucoadhesive properties of NSs

Formulations	Work of mucoadhesion (mJ)	Maximun force (N)
ITR AH-CARBO	0.0507±0.0138	0.0102±0.0006
ITR AH+CARBO	0.0357±0.0072	0.0091±0.0006
ITR AH - CARBO centri	0.0304±0.0070	0.0083±0.0001

a) b)

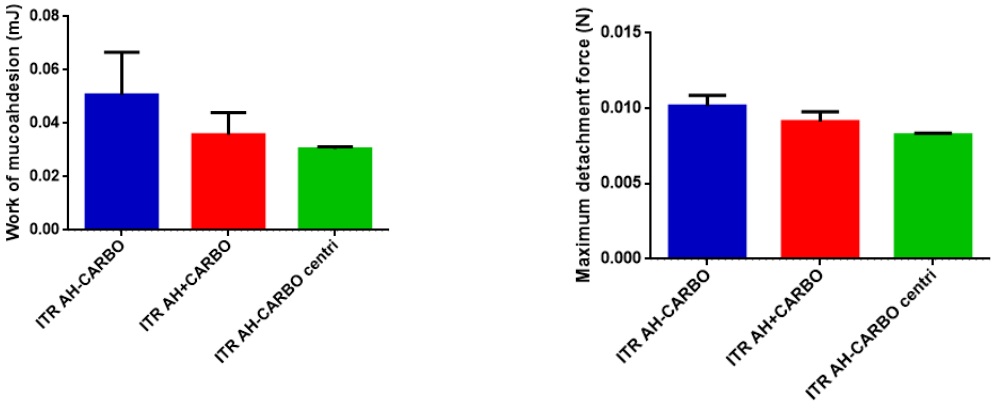


Figure 9 - Mucoadhesivesive properties a) Mucoadhesion' force of ITR NS and b) Maximum detachment force applied to ITR NS

Table 6 - Turkey's multiple comparison test related to the maximum detachment force applied to ITR NS

ITR NS	Mean Diff.	95% Cl of diff.	Significance
ITR AH-CARBO vs ITR AH+CARBO	0.001025	$-7.766 \times 10^{-0.005}$ to 0.002128	No
ITR AH-CARBO vs ITR AH-CARBO centri	0.0019	0.0007973 to 0.003003	Yes
ITR AH+CARBO vs ITR AH-CARBO centri	0.0008750	-0.0002277 to 0.001978	No

4.4. *In vitro* drug release and permeability studies

The in vitro release profile of the ITR AH-CARBO, ITR AH+CARBO and ITR AH-CARBO centri, are summarized in the concentration of the Itraconazole released shown in Figures 10 and 11. When evaluated the variance analysis of Itraconazole released concentration from each nanosuspension during 1440 min, the ITR NS with faster released was the ITR-HPB (control). The others ITR NS showed no significant differences at the ITR release when submitted at Turkey's multiple comparison test, except the ITR AH-CARBO centri with a lower ITR release when compared to ITR AH+CARBO (table 7).

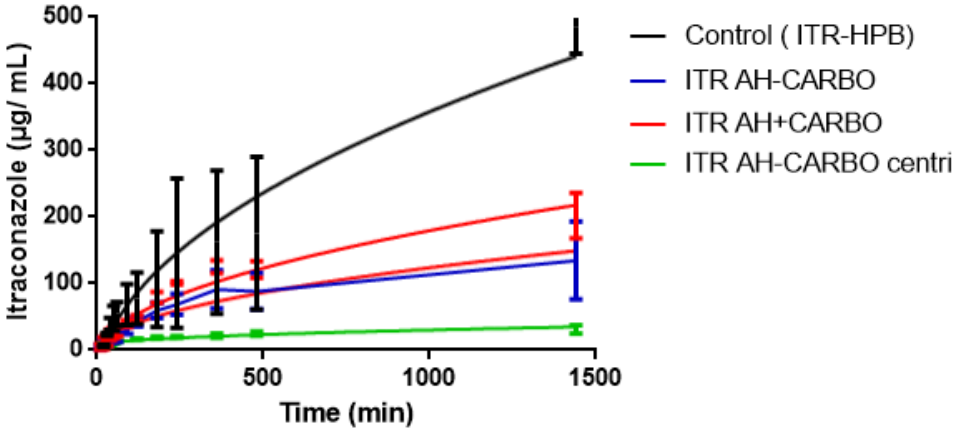
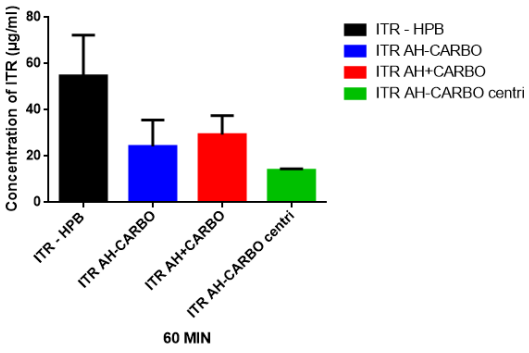
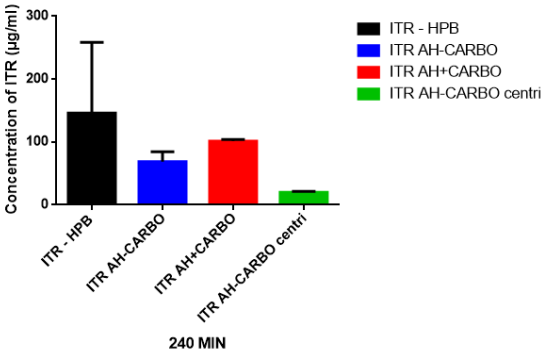


Figure 10 - *In vitro* release profiles of ITR nanosuspensions

a)



b)



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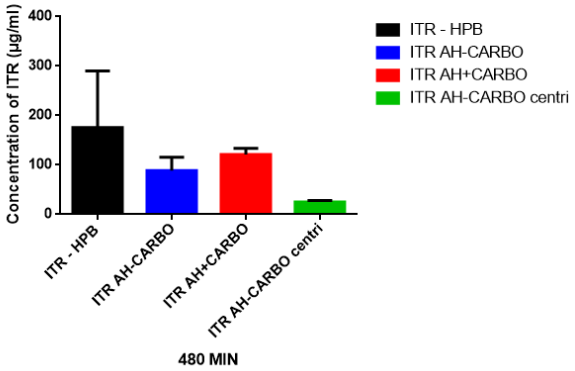


d)

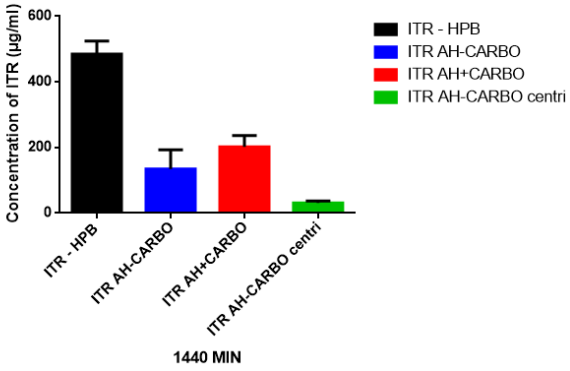


Figure 11 - Concentration of ITR a) 60min, b) 240min, c) 480min and d) 1440min

Table 7 - Turkey's multiple comparison test related to the in vitro ITR release of the ITR NS at 1440 min

ITR NS	Mean Diff.	95% CI of diff.	Significance
ITR - HPB vs. ITR AH-CARBO	350.1	216.6 to 483.6	Yes
ITR - HPB vs. ITR AH+CARBO	282.7	149.2 to 416.3	Yes
ITR - HPB vs. ITR AH-CARBO centri	453.6	307.3 to 599.8	Yes
ITR AH-CARBO vs. ITR AH+CARBO	-67.37	-186.8 to 52.04	No
ITR AH-CARBO vs. ITR AH-CARBO centri	103.4	-30.07 to 236.9	No
ITR AH+CARBO vs. ITR AH-CARBO centri	170.8	37.30 to 304.3	Yes

4.5. Nanosuspensions passive diffusion with parallel artificial membrane permeation assay (PAMPA)

The parallel artificial membrane permeability assay (PAMPA) was performed in order to obtained a prediction of ITR NS corneal permeability (45). To evaluate the permeability coefficient through the artificial membrane of ITR NS, the assay was tested in 1440 min with volume of 0.3ml in the acceptor and donor chambers with 0.32 cm^2 of membrane area. The results for each ITR NS are showed in table 8 and figure 12. The nanosuspension with higher permeability was ITR AH+CARBO with a percentage of flux of 63.9%. According to the analysis of the variances (One way ANOVA) there are not significant differences between ITR NS related to the PAMPA assay (P n.s.) (Table 8).

Generally, compounds which have a Pam < 10 $x10^{-6}$ cm/s are classified as having low permeability. Recently studies calculated the corneal drug permeability of different drugs using PAMPA membranes obtaining values in the 10^{-6} cm/s range (45). The values presented here are in the same range of 10^{-6} cm/s and in the limit of the values to be considered low permeability drugs. The results indicate that the presence of HA and Carbo do not modify the permeability of the ITR.

Table 8 - ITR NS passive diffusion with parallel artificial membrane

ITR NS	Flux %	P_{am} (cm/s)
ITR AH+CARBO	63.9±6.3	3.36 x 10^{-6}
ITR AH-CARBO	59.7±22.7	3.50 x 10^{-6}
ITR AH-CARBO centri	29.8±9.9	1.8 x 10^{-6}

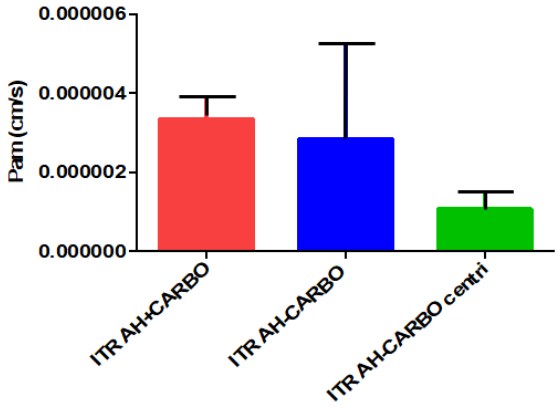


Figure 12 - ITR NS passive diffusion with parallel artificial membrane

4.6. *Ex vivo* toxicity studies

4.6.1. Bovine Cornea Opacity/Permeability test (BCOP)

The Bovine Cornea Opacity/Permeability test (BCOP) is an alternative ocular irritation assay designed to replace the rabbit eye test which is commonly used to test the effects of potential irritant substances on the opacity and permeability in bovine corneas (53). The opacity was directly measure with an opacimeter while corneal permeability was determined by sodium fluorescein which is a compound that usually cannot cross the epithelial cells of the cornea. As seen in figure 13, the ITR NS profile with major cornea light transmission, or less opacity was ITR AH-CARBO centri. When comparing the ITR NS with each other, according to percentage of transmittance, the profiles of both ITR AH-CARBO and ITR AH+CARBO are very similar. All formulations have more percentage of transmittance than the positive control (Etanol) representing less discomfort for the eye than the positive control (figure 14).

According to the variance analysis, there are no significative differences from each ITR NS tested at the opacity and permeability levels.

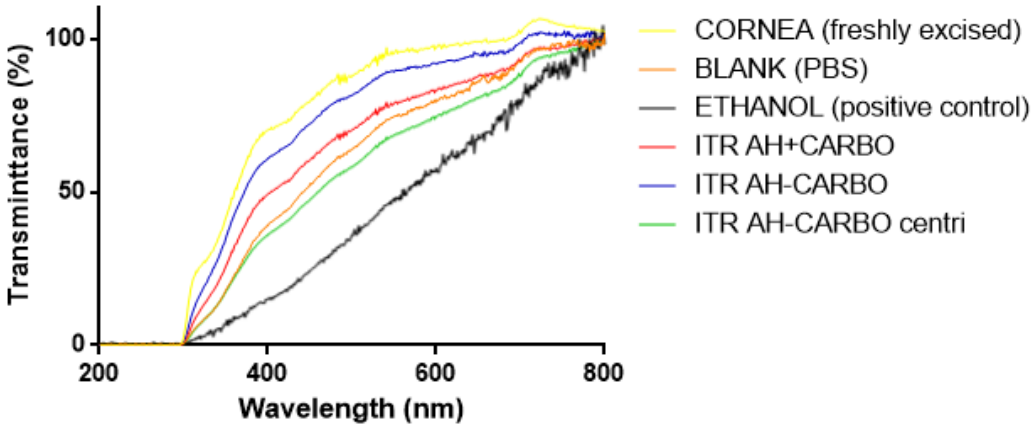


Figure 13 - Percentage of transmittance in corneas incubated in ITR NS for 10 min after 120 min of PBS

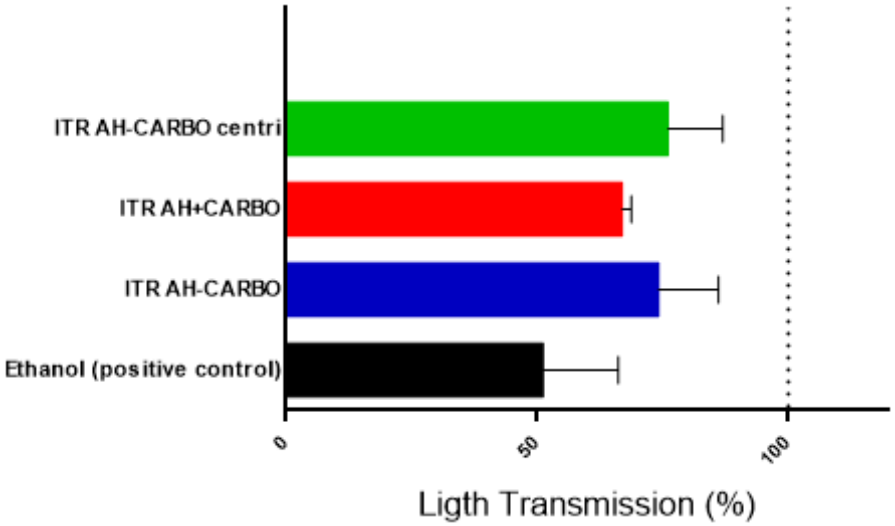


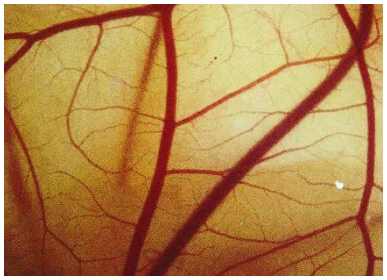
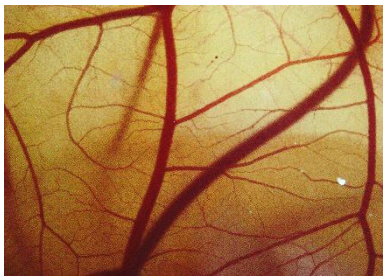
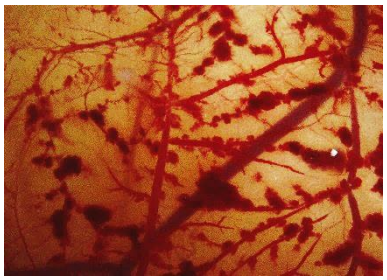
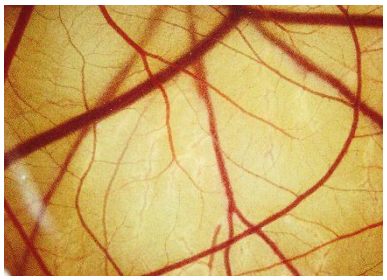
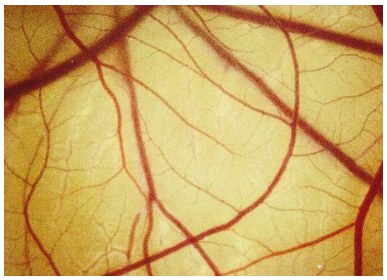
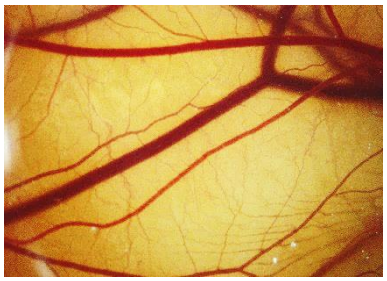
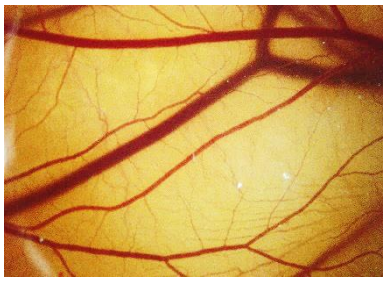
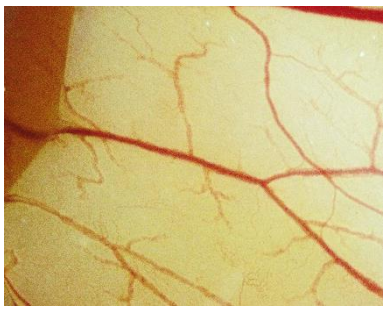
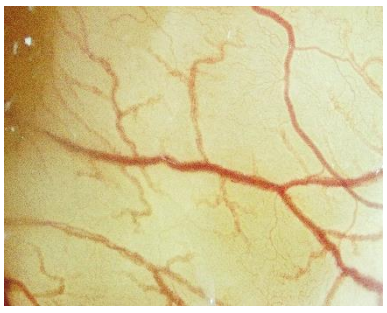
Figure 14 - Opacity of Itraconazole nanosuspensions

According with the equation 3 and the table 1, the in vitro irritation score is respectively, 0.77 for ITR AH+CARBO, 0.79 ITR AH-CARBO and 0.09 ITR AH-CARBO centri which represents that any formulation tested was irritating for the eye.

4.6.2. Irritation ocular Assay (HET-CAM)

The HET-CAM test is a widely used test because of its extensive vascularization and easy accessibility. The obtained results from the NS analysed were compared with those only used NaCl 0.9%, which was used as control supposed to be practically non-irritant (Negative control). When NSs were compared to the positive control (NaOH 0.1%), any of the formulations tested produce any injury in the part of chorioallantoic membrane so it was found to be non-irritant after 5 minutes of contact. Since none of the characteristics of irritation were found in this test (table 9), all of them were scoring with 0 according to the table 2. Therefore, it seems that these ITR NS are non-irritating for the ocular surface.

Table 9 - HET-CAM assay

	Without formulation	After application (5min)
Negative Control (NaCl 0.9%)		
Positive Control (NaOH 0.1%)		
ITR AH-CARBO		
ITR AH+CARBO		
ITR AH-CARBO centri		

5. Further Studies

Until any medicine goes to the market, there are still some important steps to guaranty the formulation's quality, safety and efficacy. As further studies for ITR formulations, it is important to study their physical and chemical stability over time and under extreme conditions in order to understand the viability and the possible shelf life of the formulations.

Being an ophthalmic medicine, ITR formulations need to be sterile to be able to apply in the eyes, so it is important to study how the formulations behave after a sterilization process. Corneal permeability *ex vivo* tests would be very helpful to understand the viability of the ITR formulations before the *in vivo* effectiveness test usually made on rabbits. The purpose of this last test is to evaluate the bioavailability of the ITR formulations in the eye and the ability to treat fungal keratitis.

6. Conclusions

The importance of fungal keratitis treatment has been growing since the increased of cases caused by the use of contact lenses. Despite the progresses made in the FK treatment, nowadays still exists a large number of patients untreated due to significant lack of commercial ophthalmic formulations. This lack in the market can be justified by low stability of ophthalmic formulations, high costs and problems found in the use conventional ocular drug delivery, like reduced ocular surface face drug transport and low ocular bioavailability of topically applied. To overcome this problem, a novel ocular drug delivery based in nanotechnology, nanosuspension of ITR was tested with three different constitutions based on previous works.

According to the characterization of the formulations, the mean particle diameter for all NS formulations tested was found to be between 300 and 1000 nm, reported suitable for ocular applications which was proven by TEM studies. Relatively to PDI, these values were less than 0.5 which represents good physical stability in all ITR NS and Zeta potential values were closed to -30mV which represent good long-term stability of NS preparations and ITR concentration values in the nanosuspensions varies between formulations. The major ITR concentration was found in ITR AH-CARBO centri (0.55mg/ml).

All ITR NS' transmittance indicates nontransparent formulations, yet it's not expected vision problems from the administration of the nanosuspensions. *In vitro* ITR release test showed the faster and the slower ITR release was obtained from ITR-HPB and ITR AH-CARBO centri, respectively. Relatively to ITR NS' mucoadhesion, these values are in the same order to other ophthalmic bioadhesive systems.

According to the *ex vivo* toxicity tests, BCOP and HET-CAM, none of ITR NS showed eye injury. Passive diffusion with a parallel artificial membrane test showed permeability values in the same range or other drugs compared with literature.

When comparing the three ITR NS, all formulations has very similar properties from each other, being suitable for eye application. However, because of the profitability in ITR AH-CARBO centri production, this formulation has probably the less interest when comparing with the other formulations according with the results presented in this thesis.

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