Desarrollo de formas farmacéuticas de liberación controlada para la administración ocular y transdérmica de fármacos

Development of ocular and transdermal drug delivery systems

Memoria que, para optar al grado de
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La memoria del trabajo de investigación para la obtención del grado de Doctor realizada por Dña. María Sebastián Morelló, titulada: "Desarrollo de formas farmacéuticas de liberación controlada para la administración ocular y transdérmica de fármacos. Development of ocular and transdermal drug delivery systems", ha sido realizada bajo la dirección de Dña. Alicia López Castellano, Catedrática del Departamento de Farmacia y Dña. Cristina Balaguer Fernández, Profesora Adjunta del Departamento de Farmacia de la Facultad de Ciencias de la Salud de la Universidad CEU Cardenal Herrera, y reúne todos los requisitos necesarios para su juicio y calificación.

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Abbreviations

ACM, Acetaminophen

ACN, Acetonitrile

Ag, Silver

AgCl, Silver chloride

ANOVA, Analysis of variance

b, Slop of linear regression

C, Concentration of diffuding substance

D, Diffusion coefficient of the drug in the stratum corneum

dC/dx, Concentration gradient

DL, Detection limit

DNFB, 1-fluoro-2, 4-dinitro benceno

EM, Electromigration

EO, Electroosmosis

F, Faraday's constant

FDA, Food and drug administration

GABA, Gamma-aminobutyric acid

GSH, Glutathione

h, Hour

H, Membrane thickness

HCl, Hydrochloric acid

HEC, HydroxyEthyl cellulose

HPLC, High performance liquid chromatography

HPMC, Hydroxypropyl methylcellulose

I, Current intensity

IF, Inhibition factor

J, Flux of the drug

J_{EM}, Electromigration flux

J_{EO}, Electroosmotic flux

J_{SS}, Steady-state flux of the durg

J_{TOT}, Total flux

$KHCO_3$, Potassium bicarbonate

KOH, Potassium hydroxide

log $P_{oct/wat}$, Octanol-water partition coefficient

MES, 2-Morpholino-ethanesulfonic acid monohydrate

min, Minutes

Mox, Moxifloxacin

NaCl, Sodium chloride

NaOH, Sodium hydroxide

OLZ, Olanzapine

P, Partition coefficient between the stratum corneum and the vehicle

PBS, Phosphate buffered solution

PCA, Perchloric acid

PEG, Polyethylen glycol

P.L.E.A.S.E., Painless laser epidermal system

PTFE, Polytetrafluoroehylene

PVP, Polyvinylpyrrolidone

Q, Accumulative amount

QL, Quantification limit

RP, Retinitis pigmentosa

RSD, Relative standard deviation

SD, Standard deviation

T80, Tween 80®

TS, Transdermal system

Tn, Transport number

UV/VIS, Ultraviolet/Visible

v, Solvent flow

Vit C, Vitamin C

x, Space co-ordinate

z, Valence of the molecule

Chapter 1: Introduction

In recent years, several new pharmaceutical controlled release forms have been developed. These forms provide better regimens, an improved pharmacokinetic profile and fewer adverse effects [1]. Control release forms allow for the release of the active compounds to be controlled by any administration route, but the most commonly used are oral, transdermal, ocular and parenteral.

Topical ocular drug delivery is the most common route of treatment in eye diseases, since it is the simplest application form [2]. The most common dosage form for this route is eye drops. However, the characteristics of ocular absorption result in a problem of bioavailability of the active ingredients in the eye [3]. Some new ocular administration forms have been developed to increase the residence time of the drug in the ophthalmic mucosa or corneal surface, with minimal side effects, maximum efficiency and stable therapeutic concentrations in the eye area. This group of dosage forms includes ophthalmic inserts.

Ocular inserts are solid or semisolid controlled-release devices that facilitate the delivery of drugs to the anterior segment of the eye. These inserts allow for the reduction of the systemic absorption of the drugs and in many cases improve patient compliance/adherence with dosage regimens, since they reduce the frequency of administration and, visual and systemic, side effects [4,5].

Conjunctivitis is one of the most common eye diseases. It is normally bilateral, benign and of self-limiting symptoms, but moreover the proximity of these symptoms to the cornea can cause corneal wounds (keratoconjuntivitis). The course of the disease distinguishes acute from chronic conjunctivitis, which refers to the duration of the symptoms. Conjunctivitis can be treated with moxifloxacin (Mox) [6], a hydrophilic molecule with a value of log $P_{oct/wat}$ 0.6 [7], which is currently marketed only as eye drops. However, the aforementioned anatomical barriers reduce the drug's bioavailability, resulting in less than 5 % of the drug penetrating the inner part of the eye [3]. In this context, the formulation of an ocular insert containing moxifloxacin could be an alternative to conventional treatment.

Retinitis Pigmentosa (RP) is a hereditary eye disease. The retina undergoes a progressive degeneration that causes a severe deterioration of the visual capacity and which in many cases leads to blindness. Several studies have suggested that

glutathione (GSH) metabolism and other related amino acids such as glutamate are altered in different models of retinal degeneration [8–11]. GSH is a peptide composed by three amino acids: cysteine, glutamate and glycine. It is present in most mammalian cells, and plays an important role in many biological processes, protecting cells from oxidative stress caused by free radicals and other reactive species, maintaining normal redox potential inside the cell. For that reasons, the use of GSH has been proposed to treat or to reduce the progression of this disease. Specifically, ocular administration of GSH is one of the possible routes; namely, this molecule could be incorporated into an ophthalmic insert for use in eye therapy.

Administration of drugs through the skin in order to obtain a systemic effect has led to the development of a pharmaceutical form known as transdermal systems. These systems allow for dosage control and a constant delivery of the drug. In recent years, they have been used in the administration of drugs used in long-term treatments, and there are several studies that have been carried out to attempt to incorporate different therapeutic agents. Given the difficulty with which drugs pass through the skin, different promoter techniques have been incorporated into transdermal systems. One of the principal techniques is iontophoresis, defined as the application of an electrical potential maintaining a constant electric current through the skin (typically ≤ 0.5 mA/cm^2), which improves the passage of ionized and non-ionized molecules [12]. Another enhancing technique is controlled laser microporation, which can be used to undermine the integrity of this barrier [13]. This laser emits μs pulses and excites the molecules of water in the epidermis and dermis, their explosive evaporation resulting in the formation of micropores.

Schizophrenia is a heterogeneous syndrome characterized by a disturbance of language, perception, social emotional and will. Antipsychotic drugs, especially atypical antipsychotic agents, are the mainstays of acute treatment and maintenance of symptoms of schizophrenia. Olanzapine (OLZ) is a highly efficient antipsychotic agent and is generally well tolerated. Its pharmacokinetics are linear and dose-proportional within the approved dosage range [14]. It is currently available for oral and intramuscular injection administration. OLZ possesses an extensive first pass metabolism, with approximately 40 % of the dose being lost before reaching the

systemic circulation, such as lack adherence to the treatment. Therefore, the administration of OLZ by the transdermal route may bring therapeutic progress in the treatment of these patients. For this aim, an OLZ transdermal system (TS) could be an effective option, and iontophoresis or microporation could be used as enhancer techniques to increase drug permeation through the skin.

Chapter 2: Aims of the thesis / Objetivos de la tesis

1. Aims of the thesis

The research described in this thesis focuses on the development of drug delivery systems for ocular administration of moxifloxacin and glutathione and transdermal administration of olanzapine.

The aims of the present work were:

1. To develop an ocular therapeutic system containing moxifloxacin for the treatment of conjunctivitis, and to study *ex-vivo* absorption of moxifloxacin through the cornea from the developed form.

2. To evaluate the possibility of administrating glutathione through the eye and to develop a formulation for its possible use as antioxidant treatment for retinal degenerative diseases.

3. To analyze the feasibility of transdermal administration of olanzapine as alternative treatment for schizophrenia.

    a. To validate an analytical method for the detection and quantification of olanzapine in samples obtained from transdermal diffusion studies.

    b. To study the *ex-vivo* absorption of olanzapine through the skin.

    c. To study the effect of enhancer techniques (microporation and iontophoresis) to increase the absorption of olanzapine through the skin.

    d. To develop transdermal drug delivery systems for the administration of olanzapine and to evaluate the effect of the enhancer technique selected on the permeation of olanzapine from these formulations.

2. Objetivos de la tesis

La investigación descrita en esta tesis se centra en el desarrollo de sistemas de liberación controlada para la administración ocular de moxifloxacino y glutatión, así como la administración transdérmica de olanzapina.

Los objetivos del presente trabajo fueron:

1. Desarrollar un sistema terapéutico ocular que contenga moxifloxacino para el tratamiento de la conjuntivitis, y estudiar la absorción *ex-vivo* del moxifloxacino a través de la córnea a partir de la formulación desarrollada.

2. Evaluar la posibilidad de administrar glutatión por vía ocular y desarrollar una formulación para su posible uso como tratamiento antioxidante en enfermedades degenerativas de la retina.

3. Analizar la viabilidad de la administración transdérmica de olanzapina como alternativa terapéutica para la esquizofrenia.

    a. Validar un método analítico para la detección y cuantificación de la olanzapina a partir de muestras obtenidas de estudios de difusión transdérmica.

    b. Estudiar la absorción *ex-vivo* de la olanzapina a través de la piel.

    c. Estudiar el efecto de técnicas promotoras (microporación e iontoforesis) para aumentar la absorción de la olanzapina a través de la piel.

    d. Desarrollar sistemas transdérmicos de liberación controlada con olanzapina para la administración de este fármaco y evaluar el efecto de la técnica seleccionada en la permeación de la olanzapina a partir de estas formulaciones.

Chapter 3: Background

1. Drug delivery systems

A drug delivery system is defined as a formulation or a device that enables the introduction of a therapeutic substance into the body and improves its efficacy and safety by controlling the rate, time, and place of release of the drug in the body. This process includes the administration of the formulation, the release of the active ingredient from formulation, and the subsequent transport of the active ingredients across the biological membranes to the site of action [1].

Drugs may be introduced into the human body by various anatomical routes. Sometimes the final aim is the systemic effects or targeted to various organs and diseases. The choice of route of administration depends on the disease, the effect desired, and the available commercialized formulations. The drug can be administrated directly to the target organ or systemically, being targeted at the affected organ(s).

The ocular route is normally used to treat ocular disease. In this case, drug delivery systems can increase the bioavailability of the drug and the effect of the treatment. Ophthalmic inserts are one of the options of drug delivery systems available for use in the eye [15].

Transdermal drug delivery is an approach used to deliver drugs through the skin for a systemic therapeutic effect, and is used as an alternative to oral, intravascular, subcutaneous and transmucosal routes. Transdermal systems (TS) are the most commonly used pharmaceutical drug delivery systems [1].

2. Ocular administration

2.1. Ocular structures

The adult eyeball measures about 2.5 cm in diameter. Of its total area, only a sixth of the front is exposed; the rest is hidden and protected by the orbit, within which is housed the eye [16] (Figure 1).

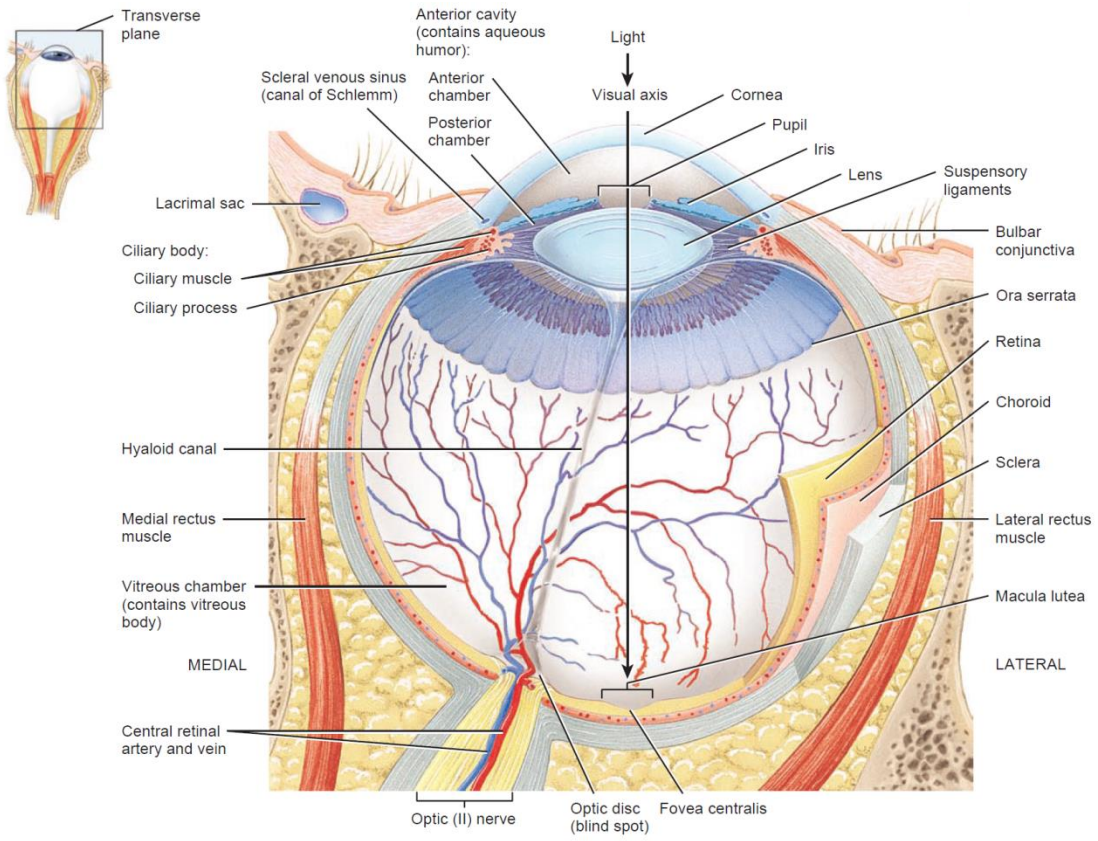


Figure 1. Schematic representation of human eye obtained from reference [16].

The **cornea** is a transparent layer that covers the colored iris. Its curvature helps focus light on the retina and a correct visual perception depends on its healthy state. It is a richly innervated avascular tissue consisting of five layers; namely, from outermost to innermost, the epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium [5] (Figure 2).

Epithelium constitutes 10 % of the total corneal thickness. It is a multilayered epithelium, and is not keratinized. It is constantly bathed by the tear, which provides the oxygen and metabolites it needs. It consists of 5 to 10 cell layers and their thickness varies depending on the area, being thinner in the center [16].

Bowman's membrane is a transparent and acellular layer composed of mucopolysaccharides and mucoprotein extensions. It acts as a defensive barrier against bacterial attack [16].

The **stroma** accounts for 85 % or 90 % of the total thickness of the cornea and consists of dense connective tissue. It is composed of cells (keratinocytes, lymphocytes,

macrophages and polymorphs), collagen fibers, mucopolysaccharides and glycoproteins [16].

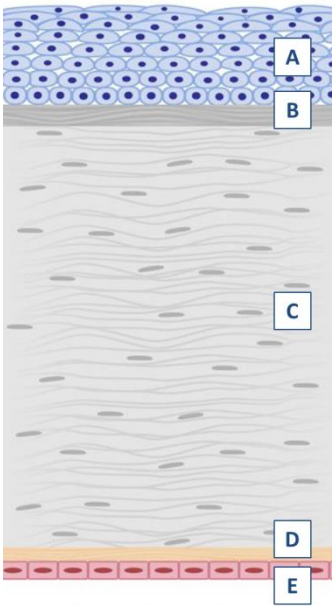


Figure 2. Schematic representation of the human cornea. **A**, Epithelium; **B**, Bowman's membrane; **C**, Stroma; **D**, Descemet's membrane; **E**, Endothelium.

Descemet's membrane is the basement membrane that lies between the stroma and the endothelial layer. It is composed of collagen and glycoprotein's [16].

The **endothelium** is the innermost layer of the cornea and it is made up of a monolayer of cells that regulate the transport of solutes between the aqueous humor of the anterior chamber and the stroma [16].

The **sclera** is the so-called "white" of the eye, a layer of dense connective tissue composed mainly of collagen fibers and fibroblasts, which makes it more rigid, gives it shape and protects the internal parts of the eye. The entire eyeball except the cornea is covered by sclera [16].

Anatomically it is constituted of three layers: the fibrous layer, the vascular layer and the retina.

The **fibrous layer** is the surface covering the eyeball and is constituted by the cornea and sclera.

The **vascular layer,** or uvea, is the middle layer of the eyeball, and is made up of three parts: the choroid, ciliary body and iris.

The **choroid** is highly vascular and constitutes the posterior portion of the vascular lining layer, accounting for and most of the posterior surface of the sclera.

The **ciliary body** is constituted by the ciliary processes and ciliary muscle; it is attached to the retina and reaches the junction between the cornea and sclera. It is brown because of its high content of melanin. Its functions are to secrete the aqueous humor and alter the shape of the lens to adapt it to near and far vision [16].

The **iris** is the colored portion of the eye. Its function is to regulate the amount of light entering the eyeball through the pupil.

The **retina** is the third layer and the innermost layer of the eye. It represents the beginning of the optical path. It is a specialized sensory organ capable of transforming light into electric signals that are transmitted via the optic nerve to the visual center of the brain. It is composed of a thin sheet of neural tissue covering 2/3 of the inner surface of the posterior part of the eye [17]. It is essentially a portion of the forebrain projected towards the frontal or anterior surface of the organism, and consists of highly specialized neurons with the exclusive function of light perception and first order signal processing.

The mature mammalian retina consists of two district tissues: the neural retina composed of neurons and glial cells, and the retinal pigmented epithelium, a single epithelial cell layer.

The retina is a seven-layer structure, with light entering the ganglion cell layer first. From there light must penetrate each layer and its cells (bipolar, amacrine and horizontal cells) before reaching the rods and cones, the two types of photoreceptors that absorb light and transform it into electrical signals that initiate downstream events that are essential for the sense of sight [17] (Figure 3).

The signal is transmitted to bipolar cells, responsible for processing and transmitting the signal. Amacrine and horizontal cells are also involved in perpetuating the signal from the photoreceptors. Ganglion cells receive the signal from the bipolar

cells and connect directly, via the optic nerve, with the brain for further processing and visual perception [17].

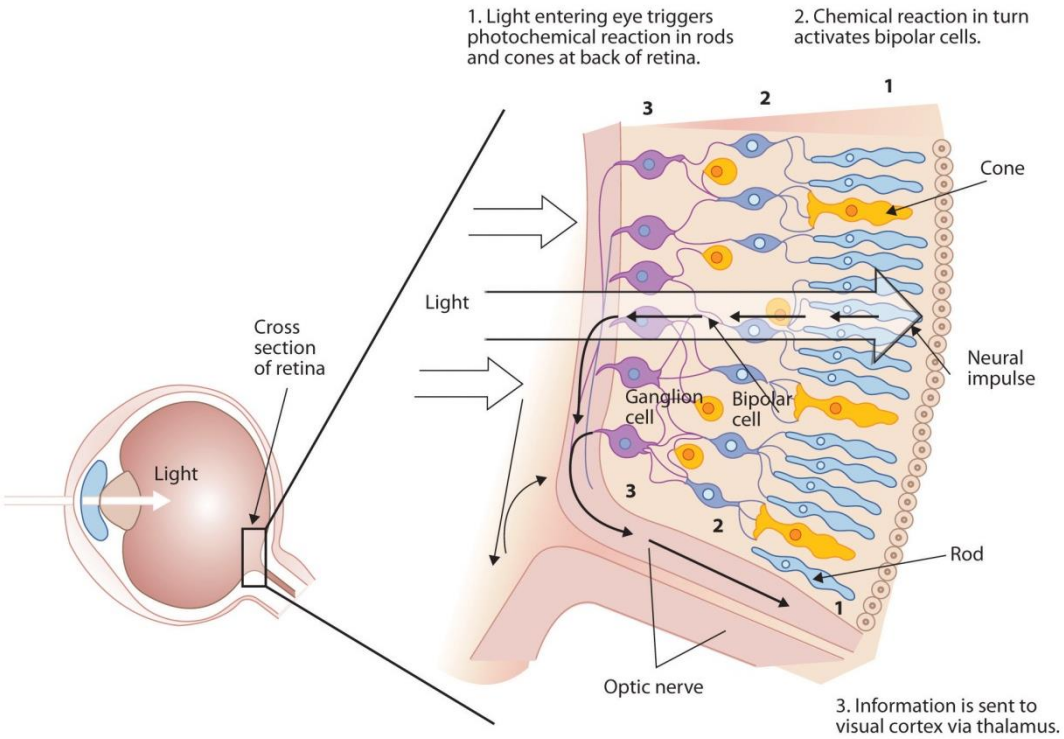


Figure 3. Schematic representation of human retina obtained from reference [18].

2.2. Routes of ocular absorption

The administration form most commonly used for ophthalmic treatment is the topical route [2]. To exert their action, most drugs must penetrate the cornea and/or sclera and reach the aqueous humour. However, ocular absorption of topically applied drugs is poor because of inherent anatomical and physiological barriers [3]. Absorption through the cornea is considered the main route for ocular administration of drugs [19]. The epithelium is the corneal layer that plays a critical role in the penetration of active substances, as is designed to protect ocular tissues that prevent foreign matter from entering the eye [20]. It represents 90 % of all corneal cells and is a barrier for hydrophilic molecules due to their hydrophobic properties. The stroma – the next layer – is composed of a highly hydrated extracellular matrix that limits the permeation of lipophilic substances [15]. Drug penetration through the corneal epithelium can occur by the transcellular or paracellular route depending on the properties of the drug (Figure

4). The transcellular route is favored for hydrophobic active substances of a small size, which can cross the epithelial cells by diffusion. The paracellular route, on the other hand, is used by hydrophilic substances, which pass through the spaces between cells [21].

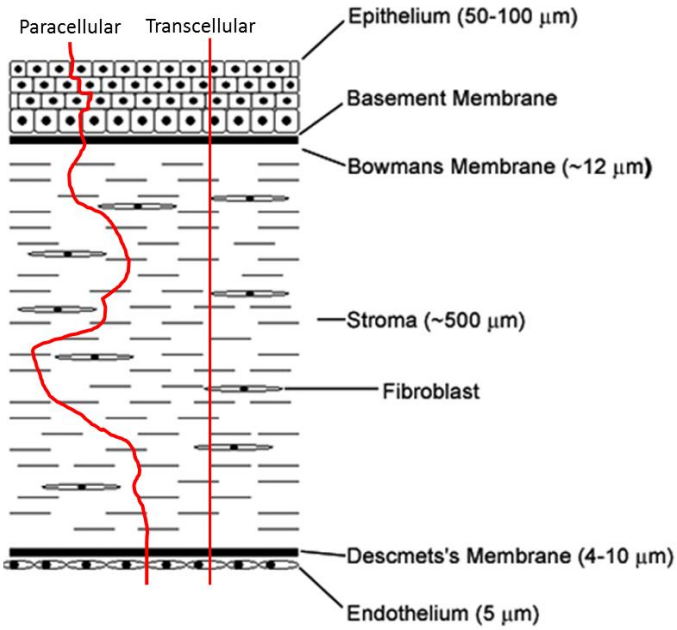


Figure 4. Penetration routes though the cornea. Adapted from reference [22].

The sclera is a minor absorption pathway compared to the corneal route, but for a few compounds its contribution is significant. Structurally, the sclera is very similar to the corneal stroma, containing numerous channels and consisting mainly of collagen and mucopolysaccharides [23]. The poorly vascularized sclera is significantly more permeable than the cornea. Its permeability is similar to that of the stroma. The pathway of drug molecules across the sclera is inversely proportional to the molecular radius [24]. Furthermore, the charge of the drug molecule also affects its permeability across the sclera. Positively charged molecules exhibit poor permeability, apparently due to their binding to the negatively charged proteoglycan matrix [23].

2.3. Ocular formulations

Ophthalmic topical drug forms are one of the most important and widely developed areas of pharmaceutical technology. This dosage form can be classified by physical properties as liquid, semisolid and solid.

The most common dosage forms are eye drops and semisolids, such as ointments or gels, but the number of solid formulations in the form of inserts is increasing and providing encouraging therapeutical results.

2.3.1. Eye drops

Eye drops are the most common ocular formulation currently available on the market. The vehicle used for its formulation can be aqueous or of an oily nature, and it can be a solution, emulsion or suspension of one or more drugs. When stored in multiuse packaging, it usually contains preservatives. These forms are sterile and isotonic. Ideally, the pH of the formulations is within the pH range of the eye; between 6.6 and 7.8 [25].

Eye drops afford low bioavailability of the active substances. In fact, it is known that of the total amount of drug administered, only up to 5 % reaches the aqueous humor [3]. Following administration, the precorneal film is the first zone to come into contact with the eyedrop. The volume of the precorneal film is between 7 µl and 10 µl [26]. When a drop is administered to the eye, results in most of the drop rolling down the cheek, while the rest flows into the nasolacrimal duct, through which is rapidly eliminated. Additionally, the administration of any preparation to the eye causes a tear reaction that dilutes the remaining volume [15].

2.3.2. Semisolid ophthalmic preparations

Semisolid ophthalmic preparations are sterile ointments, creams or gels designed to be applied to the conjunctiva. They contain one or more active principles dissolved or dispersed in an appropriate base. The vehicle of such preparations should meet a number of requirements; as the formulation comes into contact with the mucosa, especially in the cornea, the carrier needs to have good extensibility [27].

The major disadvantages of these preparations are the difficulty with which they are administered adequately, and the blurred vision generated when the formulation is on the ocular surface [27].

2.3.3. Ocular inserts

Inserts are solid or semisolid dosage forms placed in the upper or lower cul-de-sac and in some cases directly into the cornea [27]. They are generally composed of an active substance reservoir embedded in a matrix or attached to a membrane that controls the release rate. The active compound, which is more or less soluble in physiological fluids, is released over a period of time. It is possible to obtain a zero order kinetic in the active compound release. This allows the use of a smaller dose of active ingredient than with eye drops, thus reduces the occurrence of unwanted side effects [3].

These systems can be insoluble devices that need to be removed after a given period of time, or they can be designed to dissolve, erode or biodegrade at the ocular surface (Table 1).

Table 1. Some ocular insert devices.

Devices	Characteristics
Ocusert ®	Formulated with a EVA membrane (poly ethylene-vinyl acetate) and pilocarpine [28]. Used for the treatment of ocular hypertension, commercialized since 1974.
Ocufit SR®	Rod-shaped, it is made from silicone elastomers and comes in various sizes. Its advantages are a low risk of expulsion and good compatibility with cell membranes.
MiniDisc®	Ocular therapeutic system. It shaped contact lens and does not require great skill to insert it [29]. Produces non-erodible hydrophobic and hydrophilic systems and erodible devices based on hydroxypropyl cellulose.
NODS®	"New Ophthalmic Delivery System" That is a film of polyvinyl alcohol that is attached to the lower conjunctival sac and is slowly dissolved.
Mydriasert®	Insoluble device used to induce mydriasis during surgery or for examination of the fundus.

The polymers that are most employed in the formulation of soluble inserts are methylcellulose and its derivatives; namely, hydroxypropyl methylcellulose (HPMC), ethylcellulose, polyvinylpyrrolidone (PVP), polyvinyl alcohol, chitosan and its derivatives, such as carboxymethyl chitosan, gelatin, and various combinations of the aforementioned polymers [27]. All these components provide the formulation with favourable properties and are totally compatible with the eye surface.

The principal advantages and disadvantages of the ocular inserts are shown in Table 2.

Table 2. Advantages and disadvantages of ocular inserts [30].

Advantages
✓ Increased residence time/bioavailability.
✓ Precision dosing with controlled release, avoids pulsate drug delivery.
✓ Minimal systemic absorption.
✓ Low administration frequency.
✓ Conjunctiva/sclera route to internal target.
✓ Better shelf life and no preservatives.
✓ Combinational therapeutic approaches.
Disadvantages
✕ Physical and psychological obstacles of placing solid objects on the eye, foreign body sensation.
✕ Movement around the eye can interfere with vision.
✕ Potential accidental loss.
✕ Some devices difficult to insert or remove.
✕ Potential burst release upon insertion prior to controlled delivery.

2.4. Conjunctivitis

Conjunctivitis is the most common eye disease. The term conjunctivitis refers to inflammation of the conjunctiva, regardless of the cause. It is normally present as a bilateral infection and is benign and self-limiting, but proximity to the cornea can sometimes cause injuries to this tissue (keratoconjuntivitis). Depending on the duration of this condition it can be classified as acute or chronic [31].

There are two aetiologies; non-infectious and infectious (Table 3).

Table 3. Classification of conjunctivitis depending on the aetiology of the disease.

Aetiology	**Classification**
Non-infectious	Allergic (immunological reaction).
	No allergic (toxins, permanent irritation or other diseases).
Infectious	Bacterial, which can be acute, hyperacute and inclusion.
	Viral.

For treatment of bacterial conjunctivitis we have a large arsenal of antibiotics for topical use presented in the form of eye drops or ointment. As a general rule eye drops are used every 3 hours or 4 hours and ointments every 6 hours or 8 hours. Ointments

provide a higher concentration of the drug over a longer period of time, but their use during the day is limited, since they cause blurred vision. They can be used overnight to achive an effective drug concentration during sleep.

The most commonly used antibiotics are the following groups [32,33]:

Quinolones. Fluoroquinolones are used in ophthalmology, and include ciprofloxacin, ofloxacin, norflaxacin, lomefloxacin and moxifloxacin (Mox). They exert activity against gram negatives, gram positives and even mycobacteria. As a topical treatment they are indicated for the treatment of conjunctivitis, blepharitis and bacterial keratitis.

Aminoglycosides, as gentamicin and tobramycin, are actives against aerobic gram negative bacilli, staphylococci and mycobacterium. These compounds could present toxicity in the corneal epithelium.

Tetracycline, as oxytetracycline, doxycycline and tetracycline. They cover a wide spectrum, including gram positive, gram negative, some anaerobic bacteria and other germs including spirochete *Mycoplasma*, *Chlamydia* and *Rickettsia*.

Macrolides, such as erythromycin and zithromycin, exert potent antibacterial activity on most gram-positive cocci, many anaerobic bacteria and gram-positive bacilli. They are often used for blepharitis and blepharoconjunctivitis.

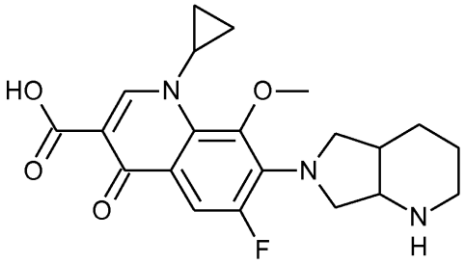
2.4.1. Moxifloxacin

Moxifloxacin is a fourth-generation fluoroquinolone with a methoxy group at the C-8 position and a bulky C-7 side chain that is used for the treatment of conjunctivitis [6] (Table 4). It is a hydrophilic molecule (log $P_{oct/wat}$ = 0.95) [7]. Its mechanisms of action are inhibition of the DNA gyrase and topoisomerase IV required for replication repair and recombination of bacterial DNA [34]. Mox exerts similar *in-vitro* activity against gram-negative bacteria such as ciprofloxacin and ofloxacin and an enhanced activity against gram-positive bacteria, including *S. Aureus* [6,34–36].

This drug is capable of penetrating deep into the ocular tissues (cornea and conjunctiva), with concentrations detected in the aqueous and vitreous humor. It does

not contain preservatives (benzalkonium chloride), which affords greater security at the ocular surface.

Table 4. Chemical information of moxifloxacin obtained from PubChem 152946.

	Moxifloxacin
Chemical name	1-Cyclopropyl-6-fluoro-7-((4aS,7aS)-hexahydro-1H-pyrrolo[3,4-b]pyridin-6(2H)-yl)-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid
Chemical structure	
Molecular Formula	$C_{21}H_{24}FN_3O_4$
Molecular weight	401.43 g/mol
Lipophilia	log $P_{oct/wat}$ = 0.95
Solubility	In water, 1146 mg/l at 25 °C

Currently, it is only marketed for topical ocular use as eye drops - Vigamox® (5 % mg/ml, eye drops solution, Alcon Cusí, S.A.) - with an administration regime (8 hours) that is disruptive for patients and sometimes associated with corneal epithelium toxicity [37]. To overcome this limitation, ocular inserts containing Mox could be a good alternative dosage form treatment.

2.5. Retinitis pigmentosa

The disease named retinitis pigmentosa (RP) is a genetically and phenotypically heterogeneous family of inherited blinding diseases that result in selective photoreceptor cell death. Inherited retinal degeneration is a broad term applied to dystrophies including RP.

The main symptoms of RP include progressive loss of visual functions with night-blindness or nyctalopia, which results from rod photoreceptor degeneration (typically, rod photoreceptor cells permit vision under dim light conditions). As rod cells die, cone photoreceptor viability (the source of high-resolution color vision in daylight), is compromised and the disease progresses towards loss of peripheral fields, tunnel vision

and finally blindness. Other characteristic features include pigmentary deposits in advanced RP and attenuation of the retinal blood vessels [38].

Despite the diversity of retinal degeneration disorders, apoptosis of photoreceptors seems to be a feature common to all [38]. The signaling pathways of apoptosis in photoreceptor cell death are still not fully understood, but it has been postulated that oxidant-antioxidant balance is involved in the apoptotic process.

Production of free radicals has been related to RP [39] and other diseases. These radicals can directly or indirectly affect several cellular and physiological mechanisms, and can lead to alterations of vial molecules.

An antioxidant is defined as any substance which, when present at low concentrations compared to the oxidizable substrate, significantly delays or prevents oxidation of that substrate [40]. The term “oxidizable substrate” includes almost any macromolecule found in living cells, such as proteins, lipids, carbohydrates and DNA.

Detoxification of oxygen free radicals is a prerequisite for aerobic life, so an important antioxidant defense system has been developed [41]. Antioxidant, such as glutathione, arginine, citrulline, taurine, creation, selenium, zinc, vitamin E, vitamin C (Vit C), vitamin A and tea polyphenols, help to regulate the reactive oxygen species thus generated. Antioxidant defense is further supported with antioxidant enzymes; e.g. superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase [42].

Antioxidants are classified as exogenous (natural or synthetic) or endogenous.

Natural antioxidant systems are sorted into two major groups: enzymatic and non-enzymatic. Enzymatic antioxidants are comprised of a limited number of proteins, such as catalase, glutathione peroxidase and superoxide dismutase, along with some supporting enzymes. Non-enzymatic antioxidants include direct acting antioxidants, which are extremely important in defense against oxygen free radicals.

Increasing evidence suggests that oxidative stress contributes to the pathogenesis of many retinal degenerative disorders, including age-related macular degeneration, glaucoma, diabetic retinopathy, and light damage [43–47]. This is further supported by reports which show that oxidative damage is present in cone

photoreceptor degeneration [48–50]. It has been shown that antioxidants decrease cone photoreceptor cell death in different mouse models of RP [51].

2.5.1. Therapeutic strategies in retinitis pigmentosa

Several strategies to preserve visual functions are being studied in different RP animal models. They comprise transplantation of stem cells, retinal pigmented epithelium or retina, delivery of a large number of neuroprotective cytokines, gene therapy including replacement of missing proteins or removal of harmful molecules, implantation of subretinal or epiretinal chips or cortically based [52–54].

At present, scientific research is currently focused on two ways to find a cure for inherited retinal diseases such as RP: replacement and rescue. Replacement approaches include different techniques to substitute degenerating or lost photoreceptors. On the other hand, the rescue strategy aims at preserving or prolonging the life-span of affected photoreceptor cells; this approach includes neuroprotection, some gene-therapies and the use of antioxidants.

2.5.1.1. Antioxidants

It has been reported in epidemiological studies that many antioxidant compounds possess anti-inflammatory, antitherosclerotic, antitumor, antimutagenic, antcarcinogenic, antibacterial and antiviral activities to greater or lesser extent [55].

Several studies have shown that the use of antioxidants *in-vitro* and *in-vivo* delays the photoreceptor degeneration process significantly in different RP animal models [50,51,56].

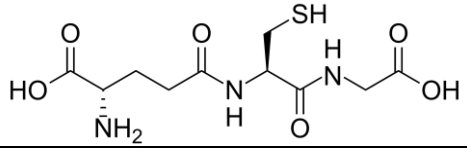
Komeima *et al* 2007 showed that antioxidants (α-tocopherol, ascorbic acid, Mn (III) tetrakis (4benzoic acid) porphyrin, and α-lipoic acid) decrease cone photoreceptor cell death in different mouse models of RP [57].

Other researchers have also reported significant protection when antioxidants are administered while rods are dying; specifically by using a combination of antioxidants (zeaxantin, lutein, α-lipic acid and glutathione) that drastically reduced the number of rod photoreceptors displaying oxidatively damaged DNA and delayed the

degeneration process significantly in rd1 mice [56]. A similar treatment [47] increased glutathione peroxidase activity and glutathione (GSH) levels and decreased cysteine concentrations in rd1 retinas.

GSH is an important antioxidant that protects against injury by reducing peroxides in a reaction catalyzed by glutathione peroxidase (Table 5). It can be used to detoxify reactive aldehydes generated from lipid peroxidation and can support detoxification of free radicals by Vit C and vitamin E dependent mechanisms.

Table 5. Chemical information of glutathione obtained from PubChem 124886.

	Glutathione
Chemical name	(2S)-2-amino-5-[[(2R)-1-(carboxymethylamino)-1-oxo-3-sulfanylpropan-2-yl]amino]-5-oxopentanoic acid
Chemical structure	
Molecular Formula	$C_{10}H_{17}N_3O_6S$
Molecular weight	307.32 g/mol
Lipophilia	log P = -6.4
Solubility	295.5 mg/ml

3. Transdermal administration

3.1. Skin structures

The skin is a complex multilayered organ, with a surface area of 1.6 m^2 [58], a weight of approximately 4 kg and an acidic pH surface (5.2) [59]. It forms a physical barrier to the environment and performs four essential body functions: retention of moisture; prevention of permeation or loss of other body molecules; protection from microbes and harmful external influence, temperature regulation and sensation [60].

The structural layers of the skin are the **hypodermis**, the **dermis** and the **epidermis** (Figure 5). Skin is considered a dynamic organ in a constant state of change, as cells of the outer layers are continuously shed and replaced by inner cells moving up to the surface [60].

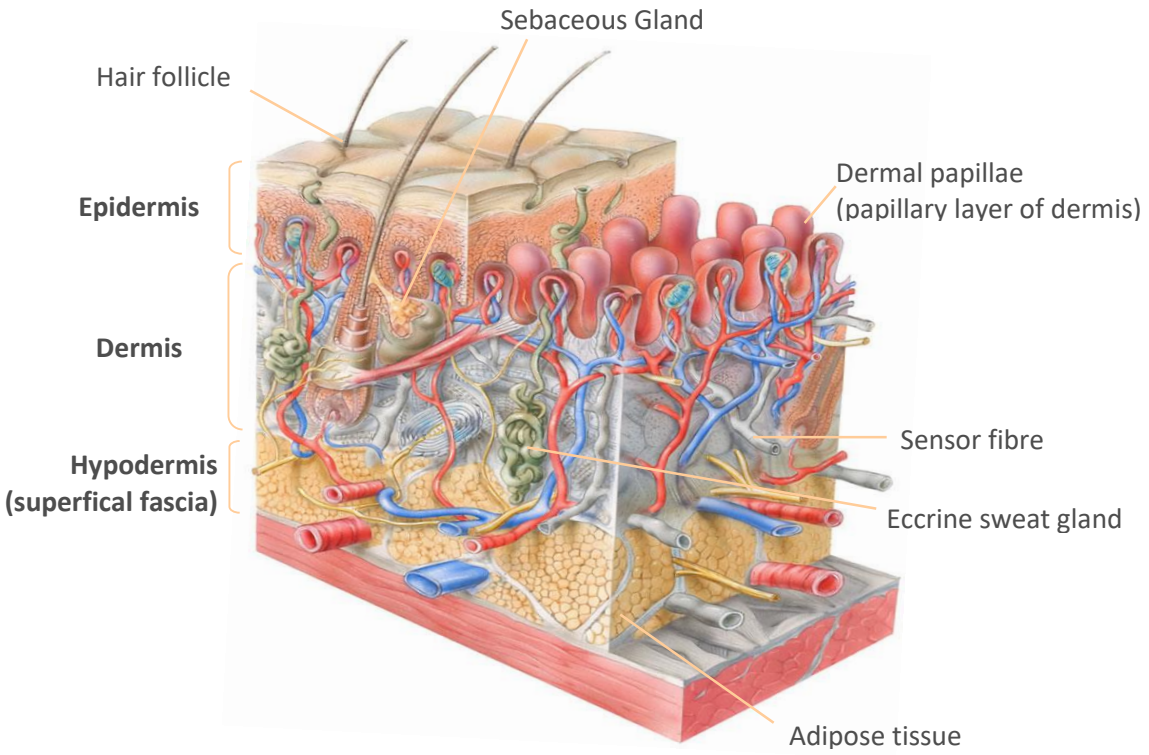


Figure 5. Schematic representation of human full skin obtained from reference *www.carecreations.basf.com.*

The **hypodermis**, located between the overlying dermis and the underlying body, is an important depot of fat. This layer of adipose tissue serves principally to insulate the body and to provide mechanical protection against physical shock.

The **dermis** is the deeper layer providing the structural support of the skin. Is made up of fibroblasts, which produce collagen (70 % of the dermis giving it strength and toughness [61]), elastin (maintains normal elasticity and flexibility) and structural proteoglycans (provide tensile strength, viscosity and hydration [61]), together with immunocompetent mast cells (T cells) and macrophages [61]. Embedded within the fibrous tissue of the dermis are the dermal vasculature, lymphatics, nervous cells and fibres, sweat glands, hair roots and small quantities of striated muscle. If a molecule is able to reach this vasculature it is absorbed and is incorporated into the general circulation of the body. It protects the body from mechanical injury, retains water, provides thermal regulation and includes receptors of sensory stimuli. The dermis has structures embedded within it: blood and lymphatic vessels, nerve endings,

pilosebaceous units (hair follicles and sebaceous glands) and sweat glands (eccrine and apocrine) (Figure 6).

Figure 6. Magnified detail of hair follicle.

The **epidermis** is the outer layers of the skin, and serves as the physical and chemical barrier between the interior body and exterior environment. It is considered a stratified squamous epithelium formed by five separate layers (*stratum basale, stratum spinosum, stratum granulosum, stratum lucidum* and *stratum corneum*) (Figure 7).

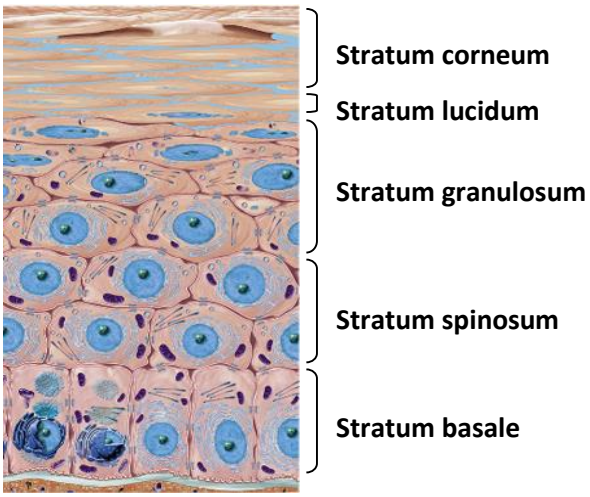


Figure 7. Structural microscopy of epidermis obtained from reference *www.eucerin.com* (Eucerin®).

The different layers of the epithelium are composed basically of four types of cells: keratinocytes (that will end forming the stratum corneum), melanocytes (pigment

forming cells), Merkel cells (responsible of light-touch response and immunoreactivity) and Langerhans cells (immune system) [62]. The epidermis presents a thickness of 75 µm - 150 µm in humans [63].

The stratum corneum is considered the main natural physical [64] and water-retaining barrier of the skin. This layer is formed by over 10 - 30 layers of keratinocytes that can absorb three times their weight in water [64]. The thickness of the stratum corneum is 10 µm - 20 µm in humans [63]. In order to maintain a constant stratum corneum thickness, the external part of the stratum corneum is continuously shed in a process called desquamation (Figure 8).

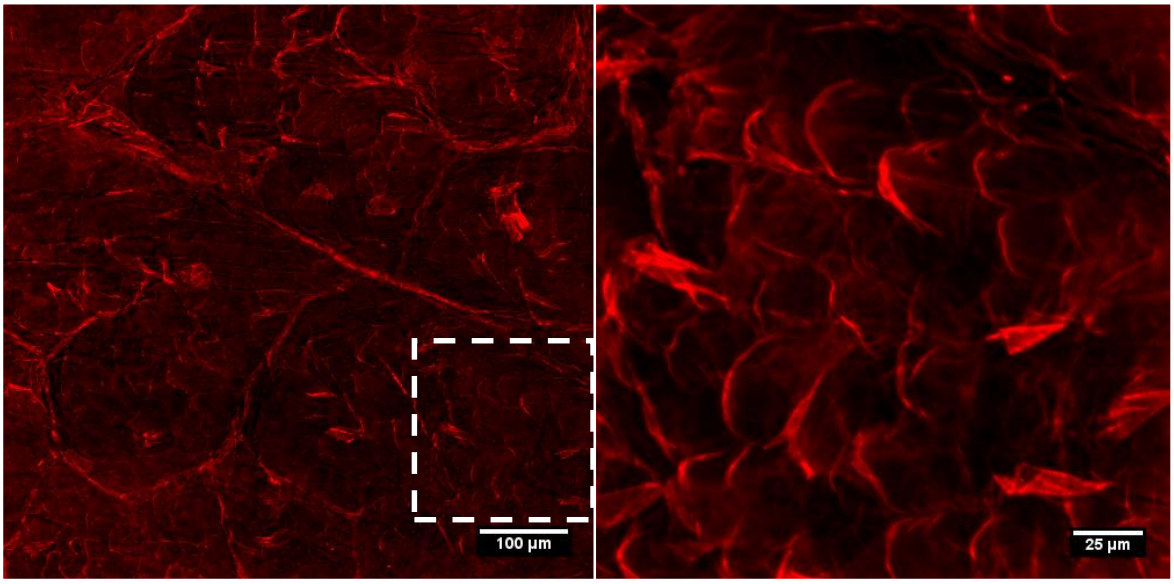


Figure 8. Magnified detail of the stratum corneum.

3.2. Routes of transdermal absorption

The administration of drugs via the transdermal pathway is becoming a regular option, ranking with oral treatments as the most successful innovate research area in the field of drug delivery [65]. Transdermal formulations are usually easy to use, increasing patient compliance with the treatment and therefore its efficacy, especially when the drug in question has a short half-life. Another advantage of this route of administration is a reduced first-pass metabolism with respect to oral administration, which makes this administration route especially useful for drugs that undergo high hepatic extraction when administered orally [65] (Table 6).

Table 6. Benefits and restrictions associated with transdermal dosage forms.

Advantages
✓ Avoids direct damage of the gastrointestinal mucosa and first pass metabolism linked to oral administration.
✓ Provides less fluctuation of drug levels and reduces the frequency of dosages.
✓ Reduces side effects associated with peak of drug concentrations.
✓ Improves patient acceptance and compliance.
✓ Useful if there is gastrointestinal distress, disease, or surgery.
✓ Great flexibility of dosage.
✓ Allows easy dose termination in the event of any adverse reactions, whether local or systemic.
✓ Does not cause physical or psychological pain.
✓ Provides an alternative in diseases and disorders of the central nervous system.
✓ May reduce overall healthcare treatment costs.
Disadvantages
✕ A low melting point (good solubility properties) with sufficient aqueous and lipid solubility is required for the permeant to traverse the stratum corneum.
✕ Skin is an excellent barrier against large, hydrophilic and polar compounds; thus, a log $P_{oct/wat}$ of approximately 2 is required.
✕ Drug diffusivity, in general, is size-dependent. A molecular weight of less than 500 Da is essential if there is to be diffusion across the stratum corneum.
✕ Some drugs can irritate or sensitize skin.
✕ Drugs may be metabolised by bacteria on the skin surface, and the presence of enzymes, such as peptidases and estereases, can metabolise the drug in the skin to an inactive product.
✕ Lack of equivalence of this administration method compared to other routes of administration.
✕ Lipophilic drugs can accumulate in a skin depot so that they need an extended period of time to be depleted (e.g. fentanyl transdermal dosage form).
✕ High intra- and inter-variability associated with the permeability of intact and diseased human skin.

Currently, very few drug candidates have been considered to have the appropriate physicochemical properties to be approved for transdermal delivery; namely a low melting point (< 150 °C; related with solubility), low molecular weight (< 500 Daltons) [66] and intermediate lipophilicity (octanol-water partition coefficient (log $P_{oct/wat}$ between -1 to 4) [67] as well as a high potency (total daily dose < 10 mg).

Transdermal drug administration is not free from disadvantages. Generally, the stratum corneum is considered to limit the rate of transdermal drug absorption. The natural structure of the stratum corneum controls the diffusion of outer molecules and water loss from inside the body. In addition, external substances can be degraded when

in contact with the skin because of the proteases expressed by its microflora [61]. Due to this natural body barrier, the passive diffusion of drugs is usually not sufficient to reach therapeutical plasma levels (Table 6).

There are multiple potential phases that occur between application of a molecule to the skin surface and its appearance in the systemic circulation. Based on the physiology of the skin, three possible pathways exist for passive transport of drug molecules through the skin to the vascular network: intercellular diffusion through the lipid lamellae; transcellular diffusion through both keratinocytes and lipid lamellae; or diffusion through the sweat ducts via the hair follicles and sebaceous glands (collectively called the shunt or appendageal route). The appendages route covers only 0.1 % of the whole skin surface area, and thus does not contribute significantly to absorption [68]. The choice of pathway is influenced mainly by the physicochemical properties of the compound (Figure 9).

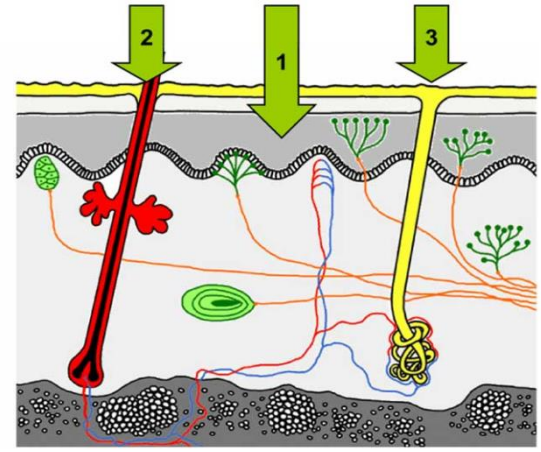


Figure 9. Major routes of penetration through the skin: transepidermal (1) and transapendicular through the pilosebaceous follicles (2) and sweat glands (3). Obtained from reference *www.skin-care-forum.basf.com.*

Because of the stratum corneum's structure, two penetration pathways are possible: the intercellular route and the transcellular route (Figure 10). In the case of intercellular absorption, substance transport occurs within the lipid bilayer structure of the stratum corneum and is currently the predominantly used pathway [68].

The selection of drug candidates for potential transdermal delivery is generally based on both the physicochemical and the pharmacokinetic characteristics of a given drug.

The evaluation of drug permeation across the stratum corneum in preformulation studies is necessary to develop transdermal drug dosage forms.

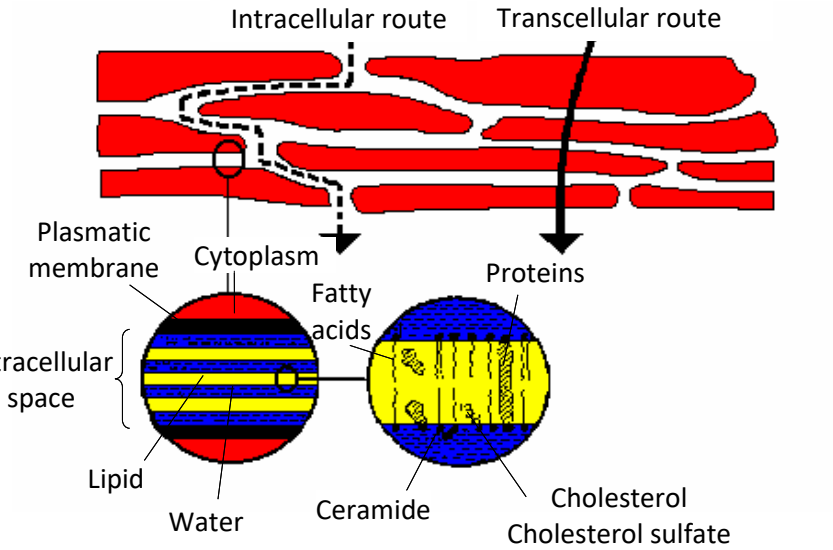


Figure 10. Intracellular pathways and transcellular penetration through the epidermis [69].

Drug permeation across the stratum corneum obeys Fick's first law, where the amount of a material passing through a unit area per unit time (its flux) is proportional to the concentration gradient measured across the section (Equation 1), where J is the flux of the drug, D is the diffusion coefficient of the drug in the stratum corneum, dC/dx is the concentration gradient and C is the concentration of diffusing substance and x the space co-ordinate.

Equation 1.

$$J = -D\frac{dC}{dx}$$

After a sufficient period of time, steady-state permeation is achieved. Under these conditions, Equation 1 can be simplified to Equation 2.

Equation 2.

$$J_{SS} = \frac{C\ P\ D}{H}$$

where J_{SS} is the steady-state flux of the drug, P is the partition coefficient between the stratum corneum and the vehicle and H is the diffusion path length or membrane thickness (Equation 2).

However, the Fick's first law does not consider non-steady state diffusion processes. Under such conditions, Fick's second law of diffusion (derived from Fick's first law) is applied (Equation 3).

Equation 3.

$$\frac{dC}{dt} = D\frac{d^2C}{dx^2}$$

3.3. Transdermal formulations

According to the US Food and Drug Administration (FDA), topical dosage forms can be divided into different groups (Table 7) [70].

Table 7. Some dosage forms according to the FDA, adapted from reference [70].

Semisolid and solid dosage forms	Liquid dosage forms	Other forms
Cream and ointment	Emulsion	Transdermal system
Gel	Lotion	
Paste	Solution	
Powder	Spray	
	Suspension	

Creams and ointments are generally used for topical application and are semisolid preparations in which solid or liquid substances are dispersed. The vehicle contains > 20 % water and volatiles and/or < 50 % hydrocarbons, polyols, or waxes. The consistency and rheological characteristics of creams depend on the emulsion type (oil in water or water in oil) and the physicochemical properties of the solids used [70].

A **gel** is a clear preparation consisting of a liquid phase (solution or colloidal dispersion) within a three-dimensional polymeric matrix caused by the addition of a gelling agent. It may contain suspended particles. Gels can be divided into hydrophobic and hydrophilic gels [70].

Pastes are generally intended for topical application. They are preparations with high concentrations of insoluble solids (20 % - 50 %) in a fatty vehicle [70].

Powders represent a mixture of drugs and/or chemicals, which are of a dry and finely divided nature [70].

Emulsions are characterized by at least two liquids that are immiscible so that one component is dispersed in droplets within the other component. Thus, emulsions are special two-phase dosage forms. Stability is given by the addition of at least one emulsifier [70].

Lotions are generally intended for topical application. They are emulsions in a liquid dosage form [70].

A **solution** is clear and homogeneous. It may contain more than one chemical dissolved in one solvent or a mixture of miscible solvents [70].

A **spray** is characterized by a liquid divided into small components by a jet of air or steam [70].

A **suspension** contains a liquid vehicle as the basis, in which solid particles are dispersed in [70].

In contrast with solutions and powders, which lack staying power (retention time) on the skin, semisolid formulations (ointments, creams, gels and pastes) are the preferred vehicles for dermatological therapy because they remain in contact with the skin for a longer period [71].

3.3.1. Transdermal drug delivery systems

A transdermal drug delivery system is a solid and flexible pharmaceutical preparation of varying sizes, containing one or more active substances. They are intended to be applied to unbroken skin in order to deliver the active substance(s) that, after passing through the skin, reach the systemic circulation over a controlled period of time. In addition, transdermal drug delivery systems can provide therapeutic concentrations of a drug for extended periods of time [65].

The primary difference between a topical liquid or a gel formulation and a transdermal system is that the rate of drug absorption of the former is controlled by diffusion through the stratum corneum, while that of the latter is controlled by release from the system itself [72].

All TS follow one of two basic design principles: the reservoir type and the drug-in-adhesive type (Figure 11).

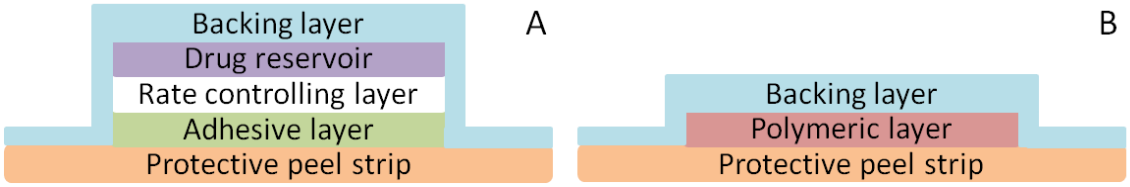


Figure 11. Transdermal delivery systems: the reservoir type (A) and the drug-in-adhesive type (B).

The reservoir type contains an occlusive backing layer that facilitates systemic efficacy by increasing skin hydration and temperature, a drug reservoir layer where the drug is stored, a rate-controlling membrane that limits the amount of drug released from the drug reservoir, a contact adhesive layer that ensures that the TS clings on to the skin while the drug is being delivered, and a protective peel strip that stops the drug from leaking out before it is applied the skin and is removed prior to applying the TS onto the skin.

In the case of the drug-in-adhesive type, the layers of the drug reservoir, the controlled release membrane and the adhesive are combined to create a single polymeric layer of drug/adhesive, hence reducing the thickness of the TS [73].

3.4. Enhancer strategies

Different skin penetration enhancement techniques have been developed to increase the range of drugs for which topical and transdermal delivery is a viable option [74]. The bioavailability improvement that these enhancement strategies aim to achieve differ in the way in which they interact with and influence the stratum corneum [74].

Passive enhancers include the use of saturated and supersaturated systems, prodrugs and metabolic approachs, ion-pairs, chemical enhancers, eutectic systems, and liposomes and other vesicles. Active enhancement methods, iontophoresis,

electroporation, ultrasounds, laser radiation and photomechanical waves and microneedles, among other physical techniques, involve the use of external energy as a driving force and/or to overcome the barrier of the stratum corneum [69].

This thesis will focus on iontophoresis and microporation.

3.4.1. Iontophoresis

Transdermal iontophoresis is defined as the application of an electrical potential that maintains a constant electric current across the skin (typically 0.5 mA/cm^2) and enhances the delivery of ionized and unionized molecules [12].

The drug is applied under an electrode of the same charge as the drug, while an indifferent counter electrode is positioned elsewhere on the body. Therefore, when the current is applied, and following the rule that like charges repel and opposite charges attract [75], the active electrode effectively repels the active substance and forces it across the skin [75] (Figure 12).

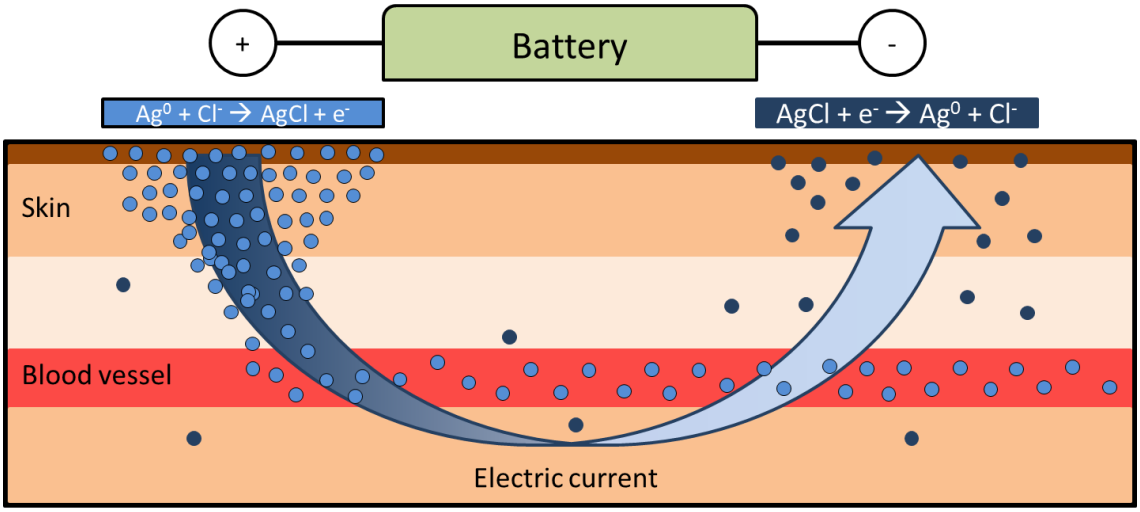


Figure 12. Transdermal iontophoresis scheme. Adapted from reference [76].

The two main mechanisms governing during iontophoretic delivery are electromigration (EM) (J_{EM}; EM flux) for charged molecules and electroosmosis (EO) (J_{EO}; EO flux) for neutral molecules. Therefore, the total flux (J_{TOT}) during iontophoretic delivery can be defined as Equation 4.

Equation 4.

$$J_{TOT} = J_{EM} + J_{EO}$$

EM describes the direct effect of the applied electric field on the charged species present in the formulation, and is considered the principal contribution to drug transport [77–79]. With the electromigration/electrorepulsion flow a charged ion is repelled from an electrode with the same charge. The following equation describes the dependency of the transdermal flux of each ion when the current is applied [80] (Equation 5).

Equation 5.

$$J_{EM} = \frac{tn\ I}{F\ z}$$

Where tn is the transport number (fraction of the total passing current carried by a specific ion), I is the current intensity, F is Faraday's constant (96484 colombs/mol) and z is the valence of the molecule. As can be referred from the above equation, flux is directly proportional to the applied current.

However, iontophoretic delivery is not dependent on current alone. Conservation of charge requires the sum of the electrical current carried by each ion to equal the total electrical current supplied by the power source. This means that the drug competes with all the other ions present in the system [80], implying that the iontophoretic transport of chemicals is influenced by the composition of the donor phase as well as of the acceptor phase.

EO has its origin in the fact that the skin is a negatively charged membrane at physicological pH and therefore acts as a permselective membrane for cations. This preferential passage of counterions induces an EO solvent flow that carries neutral molecules in the anode-to-cathode direction [80] (Equation 6).

Equation 6.

$$J_{EO} = C\ v$$

The drug flux due to the EO mechanism is proportional to the concentration of the solute and to the solvent flow (v), which is proportional to the potential gradient established by the electric field [81].

3.4.2. Electroporation

Stratum corneum ablation can be classified as laser, suction or thermal, the last of the three techniques being the most common. Laser ablation uses high-powered thermal pulses with wavelengths similar to the main absorption peak of water or tissue proteins; the matching wavelengths cause vibrational heating, which vaporizes the stratum corneum through microexplosions [82,83] (Figure 13).

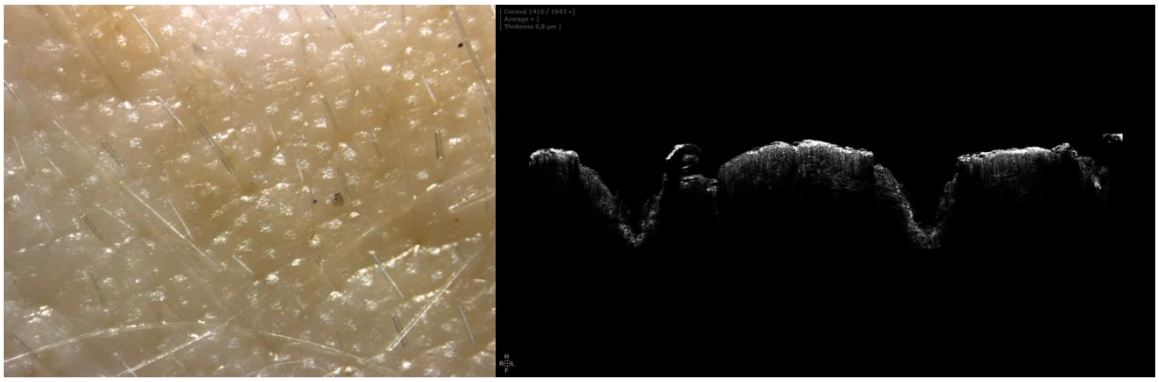


Figure 13. Detail of laser pattern and a transversal section of the skin.

Its application to the skin has been shown to increase transdermal drug delivery by several orders of magnitude. Moreover, electroporation, used alone or in combination with other enhancement methods, expands the range of drugs (from small to macromolecules, lipophilic or hydrophilic, charged or neutral molecules) which can be delivered transdermally. The efficacy of transport depends on the electrical parameters and the physicochemical properties of the drug. Under electroporation stratum corneum resistance drops immediately and the electric field distributes the drug deeper into the tissues. Although *in-vivo* application of high voltage pulses is well tolerated, muscle contractions are usually induced. Electroporation has been extensively studied in animals, but its effects in humans have received limited attention. The design of the electrode and patch is an important factor in reducing the discomfort caused by electrical treatment in humans [84].

3.5. Schizophrenia

Schizophrenia is a mental illness. The disease can occur as a schizophrenic or psychotic episode that lasts a short time and leaves no negative symptoms (i.e., loss of

normal functions/activities that characterize healthy people), or as chronic schizophrenia, in which case the manifestations of the illness are maintained over time, when reappear late as negative symptoms appear. Thus, the course of the disease can be divided into three phases:

The **first phase** or premorbid or prodromal phase. This is the period preceding the onset of the disease. Sometimes, sick children seem lonely, quiet, underachieving. Shortly before the onset of the first episode, excitability, anorexia, anhedonia, difficulty concentrating, insomnia, depression and isolation may appear, among other symptoms.

The **second phase** or active phase or progression. This phase develops when the disease begins, episode or crisis. Symptoms that do not appear in healthy people (positive symptoms), such as delusions, hallucinations. It is at this stage that the patient is usually diagnosed. The episodes can occur suddenly or slowly. The duration of episodes varies between patients, but tends to be of a similar length in the same person. The interval between episodes also varies with each patient.

The **third phase** or residual phase. In which the negative symptoms (inability to function like a healthy person) get worse and the patient's life deteriorates. This phase does not occur in all patients.

Symptoms may begin acutely or gradually, at any age, though their appearance is less common before the age of 10 and after the age of 50. Among the symptoms that schizophrenic patients may experience are the following:

- Hallucinations, false perceptions of the senses that the patient cannot identify and which can be auditory, visual, tactile, taste and smell.
- Delusions, unusual beliefs that are not based on reality and often contradict the evidence.
- Behavioral changes.
- Thought disorder; the patient cannot control the flow of ideas through their mind, eventually believing that their thoughts are controlled by some external "force".

- Other symptoms such as anxiety, irritability, insomnia, palpitations, sweating, dizziness.

Some of these symptoms may be more marked, depending on the phase of schizophrenia:

Paranoid schizophrenia. The typical symptoms are anxiety, irritability, anger, violence, paranoia and auditory hallucinations. The patient tends to try to hurt themself or other people in their immediate environment.

Disorganized schizophrenia. Patients suffer disorders that hinder the organization of thought, being manifested in childish behavior. This type usually starts early in life.

Catatonic schizophrenia. Patients have a severe psychomotor impairment which may include inactivity or excessive motor activity, lethargy or apathy, abnormal facial expressions, stereotyped movements, mutism, imitation of what others do or say and little reactivity to other people.

Hebephrenic schizophrenia. Usually begins at 12 or 13 years old. In the early years it is often confused with mental retardation. It is manifested as behavioral disturbances and delusions.

Undifferentiated schizophrenia. Thus, type is characterized by symptoms of various types of schizophrenia, which makes it difficult to classify.

Residual schizophrenia. Patients who suffer from this type do not express all the symptoms. It appears when the symptoms become chronic.

All these disorders of perception and thinking can make it difficult for patients to follow a normal routine [85].

Schizophrenia is often associated with consumption of substances such as alcohol, tobacco, cannabis or cocaine, as this helps individual to counteract some of the symptoms of the disease, such as depression and anxiety [85].

This causes an increase in the metabolism of some pharmacological treatments, such as neuroleptics, which in turn reduces plasma levels and the effect of the drug, resulting in the need to increase the average dose [86].

The treatment of schizophrenia, as with many mental illnesses, must act on several aspects of the disease. Thus, in addition to drug therapy, psychological therapy and family education are recommended. Pharmacological treatment can be given via a large number of drugs that include antipsychotics and benzodiazepines.

Antipsychotic drugs work by blocking D_2 receptors in the brain [87]. These in turn are divided into typical or atypical first generation or second generation antipsychotics. The first group includes haloperidol, chlorpromazine and fluphenazine, among others. These drugs produce a great number of mainly extrapyramidal side effects (such as dystonia, Parkinsonian movements…) [88]. Atypical antipsychotics include clozapine, olanzapine (OLZ) and risperidone. The latter group is usually the first-choice treatment for schizophrenia, because it provides a reduction of extrapyramidal side effects, although other side effects have been associated, such as weight gain, dyslipidemia or agranulocytosis.

Benzodiazepines: The most common drug used in schizophrenia is diazepam, which has a long half-life. Benzodiazepines facilitate transmission of GABA and inhibit dopamine. Benzodiazepine treatment is administered in very high doses to try to reduce arousal disorders of thinking, delusion and hallucinations. They can be used as the sole treatment when patients refuse treatment with antipsychotics or as combination treatment with them [89].

Electro-convulsive therapy may be recommended in cases where drug therapy is ineffective and there is recurrent psychosis. However, due to memory loss and the ability to produce musculoskeletal injuries, as well as the limited benefits it presents, its use has been reduced considerably [90].

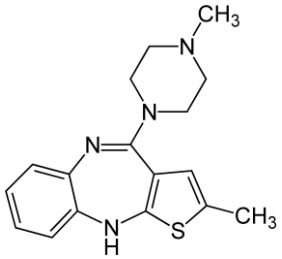
3.5.1. Olanzapine

Olanzapine is an atypical antipsychotic drug widely used for both treatment of positive and negative symptoms of schizophrenia in the acute stage and maintenance phase, and also when there is remission of the secondary emotional symptoms of mental disorder and related diseases [14,91] (Table 8).

OLZ was approved by the US-FDA in 1996 and is also used to treat maniacal or mixed episodes of bipolar disorder; however, drug non-compliance and oral side effects are always a problem.

This drug is available as a tablet in varied strengths and as an orally disintegrating wafer that dissolves on the tongue. It is also available as a rapid-acting intramuscular injection for short-term acute use. The latter two dosage forms overcome the poor bioavailablility problems associated with tablets (approximately 40 % of the dose is metabolized by the liver before reaching the systemic circulation) [92].

Table 8. Chemical information of olanzapine obtained from PubChem 4585.

	Olanzapine
Chemical name	2-methyl-4-(4-methylpiperazin-1-yl)-5H-thieno[3,2-c][1,5]benzodiazepine
Chemical structure	
Molecular Formula	$C_{17}H_{20}N_4S$
Molecular weight	312.43 g/mol
Lipophilia	log $P_{oct/wat}$ = 3
Solubility	In water 39.88 mg/ml at 25 °C
pKa	4.01; 7.24; 14.17

4. *Ex-vivo* absorption studies through membranes: ocular and transdermal

The most common technique for measuring *ex-vivo* permeation is the Franz cell system (Figure 14). Several guidelines have been documented by the pharmaceutical and cosmetic industries for its use in skin permeation. It has been used by some authors for ocular permeation.

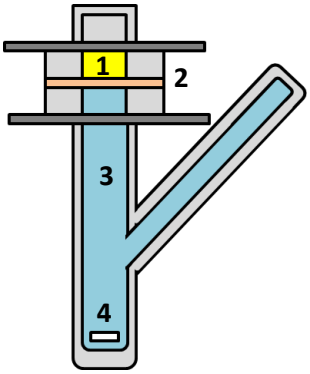


Figure 14. Franz cell system. **1:** Donor compartment; **2:** Biological membrane; **3:** Receptor compartment; **4:** Teflon-coated magnet.

The technique utilizes a sampling cell which contains a donor compartment and a receptor compartment with a sampling port; both portions of the Franz cell are separated by a biological membrane. Once the cell has been mounted, the formulation is added to the top of the cell and samples are taken periodically from the cell receptor. The amounts of drug quantified in the receptor are plotted *versus* time to develop a time-penetration profile.

Different animal skin models have been selected to mimic human skin diffusion behavior; these include primate, porcine, mouse, rat, guinea pig and snake models [93]. Of these, the pig model is the most relevant model of human skin, as its histological and biochemical properties are most similar to those of human skin [94].

In addition, different animal ocular tissue models have been selected to mimic human eye diffusional behavior, including cow, goat, pig and rabbit eyes, though the most common models are pig and rabbit. Rabbit eyes are physiologically very similar to human eyes, and have been used extensively to study corneal permeation [95–101]. Furthermore, it is the most common model for *in-vivo* ocular studies [102,103].

In this type of study there are two different application methods employed for evaluation the penetration through membranes according to the concentration of the drug in the donor compartment (Figure 15).

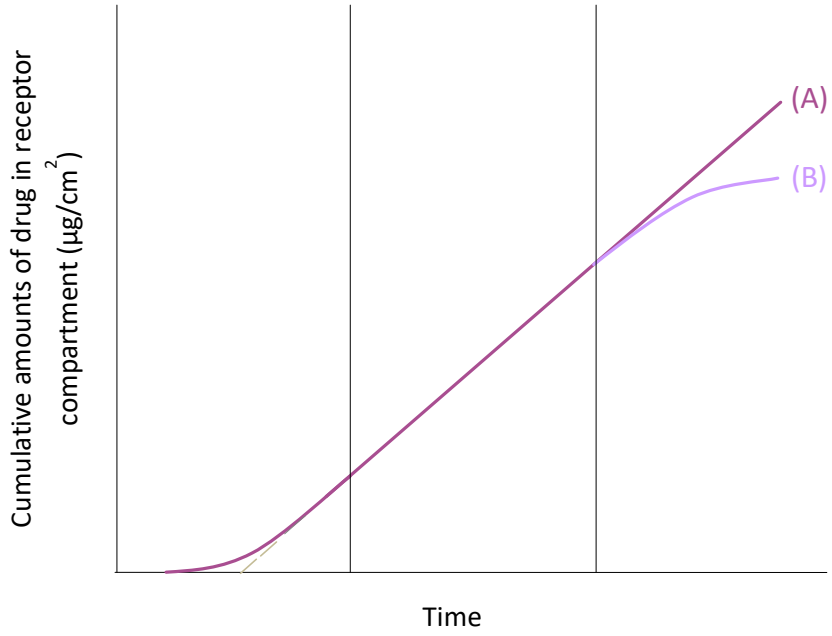


Figure 15. Infinite (A) and finite (B) dose representations.

In infinite-dose techniques (A), the donor concentration remains constant (≥ 90 % of the initial concentration). These studies are more suitable if the experimental objectives include the evaluation of the mechanism of permeation [104]. On the other hand, if a finite dose technique is employed (B), the donor concentration may show a marked depletion during an experiment, which permits closer stimulation of conditions commonly associated with topical drug use in a human subject [105].

Chapter 4

Ex-vivo rabbit cornea absorption studies with a soluble insert of moxifloxacin for the treatment of conjunctivitis

1. Introduction

Ease of application and patient compliance make topical administration of drugs to the eyes the most common route for the treatment of ocular diseases [2]. However, ocular absorption of topically applied drugs is poor because of inherent anatomical and physiological barriers of the eye [3]. The epithelium represents 90 % of all corneal cells and is a barrier for hydrophilic molecules due to their hydrophobic properties, while the stroma - the next layer - is composed of a highly hydrated extracellular matrix that limits the permeation of lipophilic substances [15].

Topical application of drugs activates the eyes defense mechanisms. One such mechanism is lacrimation, which dilutes and washes away the drug. This mechanism and the aforementioned anatomical barriers reduce drug bioavailability; less than 5 % of the drug administered as eye drops eventually penetrates the inner eye [3]. Ophthalmic inserts could be used to avoid these side effects of ocular administration and to increase drug efficacy. Ocular inserts are solid devices that deliver drugs to the anterior segment of the eye. They come in the form of thin disks or small cylinders made of polymeric material and whose size and shape are specially designed to fit the eye [103,106]. Inserts allow the reduction of systemic absorption and, in some cases, better patient compliance due to a reduced frequency of administration and lower incidence of visual and systemic side effects [4,5].

Conjunctivitis is the most common eye disease and refers to inflammation of the conjunctiva regardless of cause. Most often it involves a bilateral condition with a benign and self-limiting course, but proximity can sometimes produce corneal injury (keratoconjunctivitis). Depending on the course of infection, a distinction is made between acute and chronic conjunctivitis, which differ in duration [107].

Moxifloxacin (Mox) is a fourth generation fluoroquinolone with a methoxy group at the C-8 position and a bulky C-7 side chain that is used for the treatment of conjunctivitis [6]. It is a hydrophilic molecule (log $P_{oct/wat}$ = 0.95) [7]. This drug has similar *ex-vivo* activity against gram-negative bacteria such as ciprofloxacin and ofloxacin and an enhanced activity against gram-positive bacteria, including *S. Aureus* [6,34–36].

Mox is commercialized as eye drops with an administration regime (8 hours) that is disruptive for patients and sometimes associated with corneal epithelium toxicity [37]. To overcome this limitation, ocular inserts containing Mox could be a good treatment alternative.

Pawar *et al* (2012) [102] previously prepared an insert of Mox. This insert has been proposed to be good solution for the treatment of conjunctivitis. The biggest difference between this insert and the one proposed in this paper was the solubility of it. The Pawar insert needs to be removed after the treatment where as our soluble insert could be left in place without any additional manipulation after administration.

The aim of the present study is to evaluate the potential of an ocular soluble insert for the controlled administration of Mox, as a way of reduced the number of administration of the drug in a day, with better patient compliance, and increase the remaining time of the drug on the surface of the eye, to provide more effective treatment for conjunctivitis. Furthermore, we set out to analyze the viability of rabbit cornea preserved under different conditions to be used as a membrane for *ex-vivo* ocular absorption assays.

2. Material and methods

2.1. Reagents

Moxifloxacin (Vigamox®, 5 mg/ml, Alcon Cusí S.A) was composed of moxifloxacin hydrochloride (PubChem: 101526), sodium chloride (NaCl), boric acid, purified water and chloride acid or sodium hydroxide as pH regulators.

The bioadhesive polymers hydroxypropyl methylcellulose 4500 (HPMC), polyvinylpyrrolidone K30 (PVP-K30) and polyethyleneglycol (PEG) and the plasticizer agent glycerin were acquired from Guinama (Valencia, Spain).

High performance liquid chromatography (HPLC) grade acetonitrile, ammonium dihydrogen phosphate monobasic and water were obtained from Sigma-Aldrich (Madrid, Spain).

Phosphate buffered solution (PBS) was composed of disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride and bidestilled water, all of which were supplied by Sigma-Aldrich (Madrid, Spain). The pH of this solution was adjusted to 7.5 ± 0.1 by adding hydrochloric acid 5 N or sodium hydroxide 5 N as required.

Solutions and reagents for hematoxylin-eosin staining, Harris hematoxylin and eosin were acquired from Química Clínica Aplicada (Tarragona, España). Absolute ethanol for cosmetics was obtained from Guinama (La Pobla de Vallbona, España). Xylenol was purchased from WWR International (Fontenay sous Bois, Francia) and Diamount® (free assembly through xylenol) from Diapath (Martinengo, Italy).

2.2. Collection and preservation of the study membrane

Whole eyes from two-month-old hybrid albino rabbits of either sex were obtained after sacrifice. Eye balls were rinsed with saline to remove any trace of blood after which, adherent muscle was removed with scissors. Fresh excised corneas were obtained by cutting along the sclera-limbos junction and the individual excised tissues were used for absorption and histological studies [108] (Figure 16).

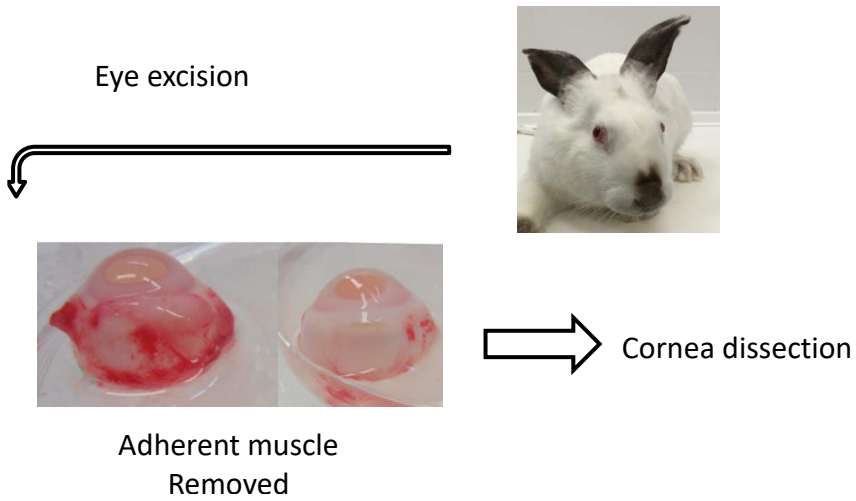


Figure 16. Outline of the process for obtaining the rabbit corneas.

In order to study their viability in *ex-vivo* absorption studies, the corneas were divided in three different groups according to the different conditions in which they were to be preserved: fresh, -20 °C and -80 °C. The group of fresh corneas was

maintained in PBS (pH 7.4) for up to one hour before initiating the study. The second and third groups of corneas were labeled accordingly and frozen for their future use. They were later defrosted by immersion in PBS and were kept in a buffer until studies began [98].

2.3. *Ex-vivo* ocular absorption studies

Transcorneal absorption of Mox was investigated using vertical standard diffusion cells (Franz type) with a diffusion area of 0.567 cm^2 (0.567 ± 0.008 cm^2) (DISA, Milan, Italy) [97] and fresh or preserved (at -20 °C or -80 °C) rabbit corneas.

To conduct the diffusion assays, the cornea was placed between the two compartments of the diffusion cell with the epithelium facing the donor compartment.

The receptor compartment of the diffusion cells – with a volume of 4.2 ± 0.1 ml – was filled with PBS (pH 7.4) at 37.0 ± 0.1 °C and stirred by a rotating Teflon-coated magnet in order to prevent possible boundary layer effects. When everything was prepared, the donor compartment was filled with 1 ml of Mox solution (Vigamox®).

During the 180 minutes assay, 200 µl samples were taken manually from the receptor chamber at predetermined time intervals; every 15 minutes during the first hour, and then every 30 minutes during the next 2 hours [96]. The volume of sample taken was immediately replaced with the same volume of fresh PBS, thus ensuring sink conditions in the receptor compartment [109,110].

2.4. Instrumentation and chromatographic conditions

A Waters system equipped with a Waters 1525 Binary HPLC Pump (including a Waters 2998 Photodiode Array Detector) and a Waters 2707 Autosampler were employed for HPLC analysis. Acquisition and treatment of computerized data were performed with Waters® Breeze 2 software.

Chromatographic separation of the components was achieved at room temperature (25 ± 2 °C) using a Kromasil® C18 (250 x 4.0 mm) reverse-phase column packed with 5 µm C18 silica particles.

A mixture of aqueous solution of ammonium dihydrogen phosphate monobasic (0.05 M, pH 2.5) - acetonitrile (80:20, v/v) pumped at a flow rate of 1 ml/min was used as a mobile phase. A volume of 50 µl was injected into the HPLC and absorbance was measured at 295 nm.

This analytical method has previously been validated for Mox determination by Srinivas *et al* (2008) [111].

2.5. Data analysis

Data were expressed as the mean ± SD. Results were evaluated statically using analysis of variance (one way - ANOVA). Post-hoc multiple comparison tests were performed with the Dunnett T3, since experimental data were found to be heteroscedastic ($p < 0.05$).

2.6. Histological studies

To carry out the histological examination the corneas were cut into 4 µm – thick slices that were subsequently dyed with hematoxylin – eosin staining. The slices were then covered with drops of hematoxylin for 3 minutes after which, they were rinsed with tap water for 5 minutes, covered with eosin for 1 minute, and then rinsed again with running tap water for a further 5 minutes. The slices were dehydrated by bathing them successively in ethanol of increasing concentrations: 70 % (1 minute), 96 % (2 minute), and 100 % (two times one minute washes). At the end of the dehydration process, the slices were immersed in two xylene baths for 5 minutes and 2 minutes respectively. Finally, the samples were mounted with Diamount® mounting medium. Following this, the morphology of the corneas was studied by microscopic observation, during which the thickness of the epithelium and stroma was measured.

2.7. Preparation and evaluation of the ocular inserts

Three ocular inserts were prepared. Each one was composed of Mox (active component), HPMC, PVP-K30 and PEG as bioadhesive polymers [112] and glycerin as a plasticizer agent (Table 9).

Table 9. Components and amounts used for each ocular insert of moxifloxacin.

	Insert-1	Insert-2	Insert-3
Vigamox®	20 ml (100 mg of Mox)	20 ml (100 mg of Mox)	20 ml (100 mg of Mox)
PVP-K30	600 mg	600 mg	1200 mg
HPMC	400 mg	400 mg	800 mg
PEG	0.5 ml	0.5 ml	0.5 ml
Glycerin	25 mg	25 mg	25 mg
Water	30 ml	---	---

PVP: polyvinylpyrrolidone. HPMC: hydroxypropyl methylcellulose. Mox: moxifloxacin. PEG: polyethyleneglycol.

All the inserts were prepared using a solvent casting method. For insert-1, the required quantity of polymers was weighed and dissolved in the required amount of water by gentle stirring. The required amount of film-forming agent was then added to the solution under stirring. Vigamox® was added at the end. For insert-2 and insert-3, the polymers were added directly to the required amount of drug solution (Vigamox®) and the required amount of plasticizer agent was added last, all under stirring conditions.

In the three formulations, the solution containing all the compounds was stirred for 24 hours at room temperature [102,113]. All the inserts were obtained via a lamination method [114].

Briefly the mixtures were laminated on an occlusive backing (Scotchpack™ 9733 backing layer) to a thickness of 600 µm. The insert was allowed to dry at room temperature in darkness.

For application of the insert, the first step was to remove the backing [115], after which the insert was applied directly to the wet ocular mucosa, at which moment the inserts became bioadhesive.

For insert *ex-vivo* absorption studies method described in point 2.3. of this chapter, was followed and fresh corneas were used as a membrane. An insert with 0.5 cm^2 surface was placed on the top of the cornea [116].

The external morphology of the ocular insert obtained after the drying process was analised by an optical microscope Leica DM 2000 (20X magnification) with polarized light and photografiated with a Nikon Digital shift Ds-H2 camera.

Moisture uptake was calculated also using 1 cm^2 pieces of the insert. Once completely dried, the inserts were weighed and then exposed to a relative humidity of 75 ± 5 % (100 ml of saturated solution of NaCl) under a dryer until their weight became constant [117].

The water absorption capacity of each insert was calculated as the increase of weight following the assay; Equation 7 [118].

Equation 7.

$$Water\ uptake\ (\%) = \frac{final\ weight - initial\ weight}{final\ weight} 100$$

To evaluated moisture loss, six 1 cm^2 fragments were obtained 24 hours after lamination, when it was possible to separate the lamination from the insert. These fragments were weighed at different times until they reached a constant weight Equation 8.

Equation 8.

$$Moisture\ loss\ (\%) = \frac{initial\ weight - final\ weight}{final\ weight} 100$$

The individual weights and thickness of six 1 cm^2 inserts were determined using an electronic scale (Sartorius ED124S) and a caliper.

To determinate the content of drug in the insert, three 1 cm^2 pieces of the formulation were solved in 1 ml of PBS in a beaker until they were totally dissolved. 200 µl of the solution was then analised and the amount of Mox of each was determinate.

3. Results and discussion

3.1. Viability studies

For ocular permeation studies of Mox, a rabbit cornea *ex-vivo* model was chosen. Rabbit eyes are physiologically very similar to human eyes, and have been extensively used to study corneal permeation [95–101]. Furthermore, it is the most common models for *in-vivo* ocular studies [102,103].

After selecting the membrane model based on the above mentioned criteria, we set out to evaluate if it could be possible to use the tissue after frizzing. For this aim there were performed transcorneal *ex-vivo* absorption studies with moxifloxacin in solution (Vigamox®) with the three groups of corneas (fresh, frozen at -20 °C and frozen at -80 °C). It was compared the permeated amounts of Mox in the three conditions. As well it was performed some histological studies of the corneas under the three different conditions.

Figure 17 shows accumulated amounts (µg/cm^2) of Mox in the receiver compartment *versus* time for the three assayed conditions (fresh, -20 °C and -80 °C), which represent the transcorneal kinetics of the absorption of Mox.

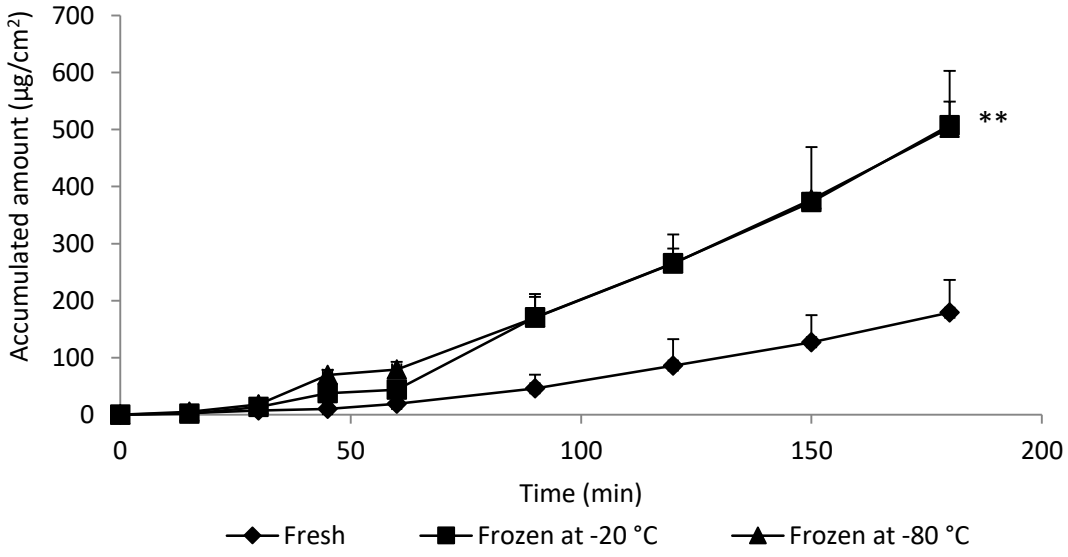


Figure 17. Accumulated amounts of moxifloxacin in the receptor compartment (µg/cm^2) as a function of time, obtained from samples of *ex-vivo* transcorneal diffusion of Mox solution with corneas preserved in different conditions (fresh, -20 °C, -80 °C). (Mean ± SD; n ≥ 3) *p < 0.05.

Statistical analysis of the data revealed statistically significant differences between the amounts of Mox accumulated in fresh *versus* frozen corneas at different temperatures ($p < 0.05$). In contrast, no significant differences were detected between the amounts of Mox accumulated in corneas preserved at -20 °C and -80 °C ($p > 0.05$).

Histological studies were also performed. Epithelial thickness was measured in the three groups of corneas; an average thickness of 29.72 ± 4.76 µm was obtained for the fresh corneas, while in the case of the frozen corneas preserved at -20 °C and -80 °C, a thickness of 23.63 ± 4.31 µm and 22.69 ± 2.99 µm were obtained, respectively (Figure 18).

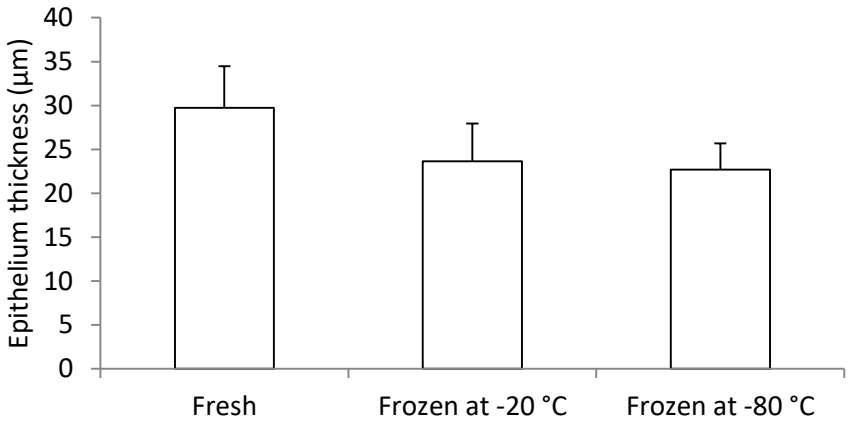


Figure 18. Graphical representation of the thickness (µm) of the **epithelium** of the three different groups: Fresh, frozen at -20 °C and frozen at -80 °C (Mean ± SD; n ≥ 3).

Statistical analysis of the data showed no significant differences ($p > 0.05$) in epithelial thickness among the three groups.

Stroma thickness was measured in the three cornea groups; average values of 205.70 ± 8.73 µm in fresh corneas, 297.40 ± 10.15 µm in corneas frozen at -20 °C and 253.32 ± 15.68 µm in those frozen at -80 °C were obtained (Figure 19).

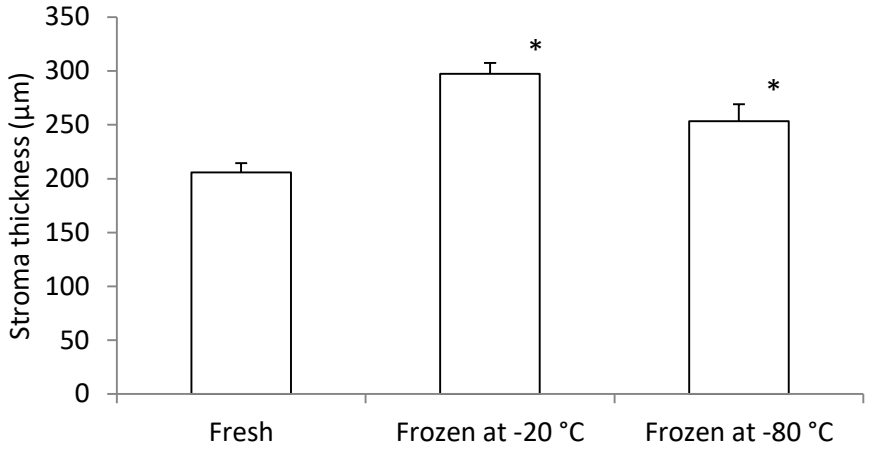


Figure 19. Graphical representation of **stroma** thickness (µm) in the three different groups; fresh, frozen at -20 °C and frozen at -80 °C (Mean ± SD; n ≥ 3) *p < 0.05.

Statistical analysis of the data revealed statistically significant differences ($p < 0.05$) in the thickness of the corneal stroma between fresh and frozen corneas. However, no significant differences between the two groups of frozen corneas were detected.

Figure 20 illustrates clear morphological differences between fresh and frozen corneas. For example, the difference in the size of the stroma is evident, and the epithelium seems to be degraded in the latter case.

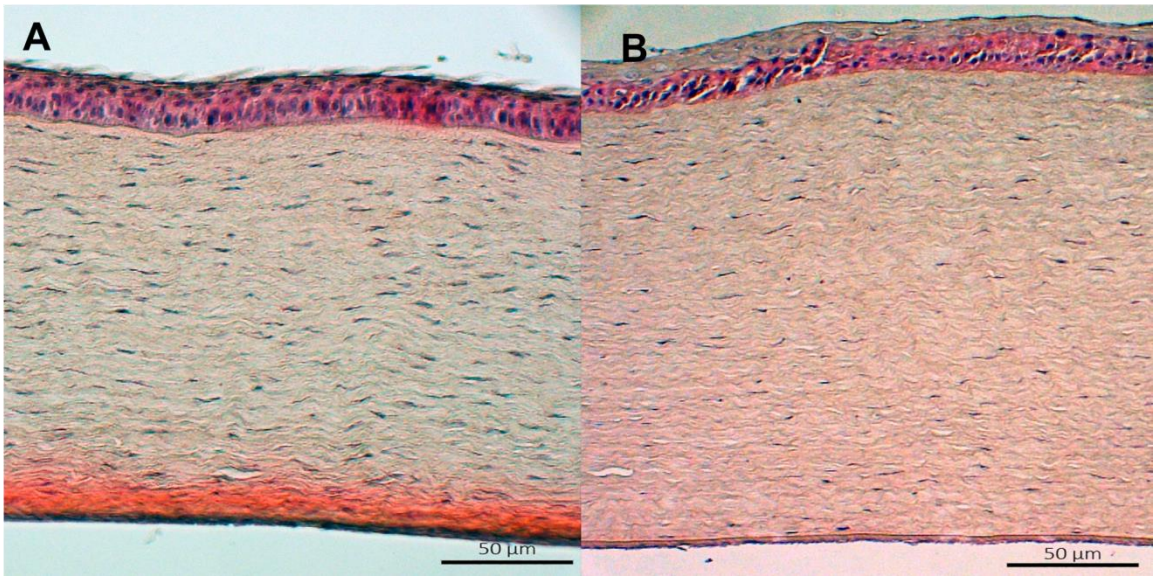


Figure 20. A: Picture at 20X of a fresh cornea. B: Picture at 20X of a cornea frozen at -80 °C.

Results of *ex-vivo* absorption studies revealed that accumulated permeated amount of Mox at the end of the experiment were much higher in frozen corneas (Figure 17). This could be because Mox is a hydrophilic molecule and epithelium is the main barrier for hydrophilic molecules. This could have been due to degradation of the cornea's structure after freezing and thawing, as previously described in (Figure 19). Histological studies showed that the epithelium was damaged after freezing, so the preservation processes may have affected the absorption of Mox and the results obtained.

Van Der Bijl *et al*. performed 24 hours studies with fresh and frozen (at -80 °C) rabbit and human corneas. They concluded that there was a correlation between rabbit and human corneas, in terms of substance permeation, and they also observed non-significant differences between frozen and fresh corneas of both species. But they think that this process could affect small and hydrophilic molecules. This is in line with our results, and is to be expected given that Mox is a small and hydrophilic drug. Furthermore, our experiments lasted 3 hours, while the aforementioned group recorded data after 2 hours [101]. The duration of the experiment and the sampling time could be factors that give our differences in the final results of the study.

Based on our viability study, we recommend the use of fresh corneas for ophthalmic *ex-vivo* absorption studies, as frozen tissue would seem to present some problems for this type of experiment, depending on the characteristics of the drug.

3.2. Preparation and evaluation of the ocular inserts

Evaluation of the three formulations containing different concentrations of bioadhesive polymers showed that insert-1 and insert-2 were inconsistent and thus invalid for future application, as they could not be laminated. Furthermore, insert-1 provided a lower concentration of drug than insert-2 and insert-3, as shown in Table 9. Because insert-1 had more water apart of the one provide by Vigamox® in the formulation.

We concluded that, of the three soluble ocular inserts prepared, insert-3 showed the most potential, as it provided the highest concentration of bioadhesive polymers and the best consistence for lamination (Table 9).

Once prepared, insert-3 was very thin and almost transparent, with a yellowish tinge. Insert-3 was evaluated as described in materials and methods. Other authors used similar procedures to analyze their formulated inserts [102,119–121].

The analysis revealed a completely homogeneous polymer matrix in which the drug was distributed without crystallization.

Once the ocular insert was prepared, and after the drying process, the hygroscopicity of the formulation was determined.

As in other studies of ocular inserts [102], the ability of formulations to capture water was high (29.26 ± 1.45 %), which is beneficial, because, in this way, the insert adheres better to the ocular surface. The adhesiveness of these ocular inserts depends on the moisture they allow. In addition, this ensures that the insert is dissolved when applied to the eye, thereby releasing the active substance it contains.

Following this study it was determined that 24 hours after lamination, the average weight of the inserts was 0.0058 ± 0.0001 g, while a constant weight (0.0053 ± 0.0001 g) was obtained 6 days after lamination. Therefore, the percentage of moisture loss at this time point was 9.88 ± 3.83 %. These results show that the minimum drying time for application is 6 days.

The average weight of the ocular insert after drying was 0.0053 ± 0.0001 g, and its average thickness was 0.050 ± 0.002 mm.

There was little variation between the different areas of the laminated insert. Moreover, our insert is much thinner than those produced by other authors [102,103,122], so its application to the eye will be easier, as it will be less noticeable by the patient. In addition, it weighs less than those formulated by other authors [123].

There were determinated the content of Mox in the insert. Three pieces of 1 cm^2 of the insert were solved in buffer solution and analised by HPLC. It was found that the average amount of Mox present was 172.86 ± 9.47 $\mu g/cm^2$.

3.2.1. *Ex-vivo* ocular absorption studies with the selected insert

Figure 21 shows the amounts accumulated in the receiver compartment *versus* time in the *ex-vivo* absorption assays of the formulated ocular insert using fresh cornea.

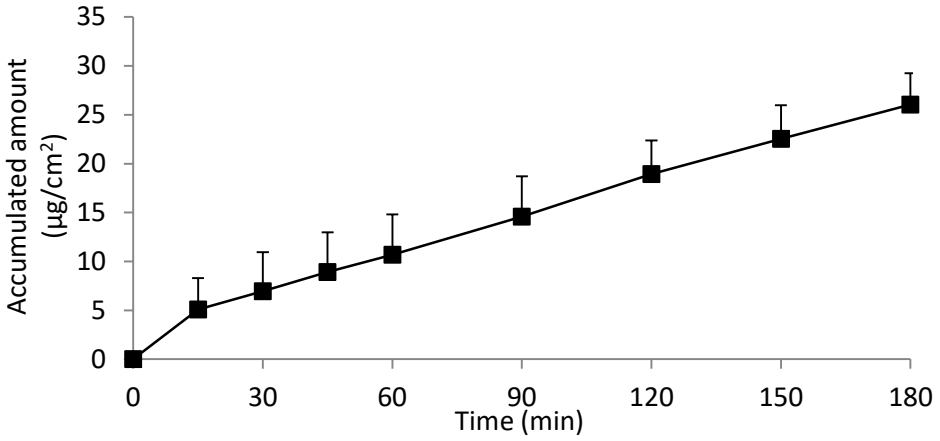


Figure 21. Accumulated amounts of moxifloxacin in the receptor compartment (µg/cm^2) as a function of time, obtained after *ex-vivo* transcorneal diffusion of samples with the selected insert. (Mean ± SD; n ≥ 3).

At the end of the experiment the amount of Mox accumulated in the receptor compartment was 26.04 ± 3.21 µg/cm^2. Other authors have reported that the concentration of Mox in the aqueous humor in the human eye after 60 minutes of ocular administration of a drop is 0.6681 ± 0.4980 µg/ml [124]. In our experiments, we observed that 10.68 ± 4.13 µg/cm^2 of the drug had permeated after 60 minutes. Thus, using an insert of 0.5 cm^2, and assuming an ocular volume of 6.5 ml. The permeating amount of drug, would be approximately 5.34 µg, a concentration of 0.82 µg/ml. Therefore, with the soluble ocular insert developed in this study higher concentrations could be achived when compared to current commercial pharmaceutical forms such as drops. Furthermore, the dosage of Mox could be easy modified by simply changing the size of the insert.

Chapter 5

Ex-vivo ocular absorption studies with glutathione formulations as antioxidant treatment

1. Introduction

It is suggested that oxidative stress contributes to the pathogenesis of many neurodegenerative disorders, including Retinitis Pigmentosa (RP) and other ocular pathologies [43–46].

RP is a heterogeneous group of inherited retinal degenerative diseases that lead to blindness, and typically involves the progressive loss of rod photoreceptors and, subsequently, cones. In the early stages of the disease there is night blindness followed by a loss of peripheral vision, with a progression over many years that can be very rapid or slow. At present, no effective treatment is available to prevent or cure the disease. It is, therefore, of great importance to find a means to slow down the pathological process in order to delay photoreceptor cell death and total blindness [56].

Oxidative damage is reported to be present in cone photoreceptor degeneration [48,49]. In his context, there are different compounds with variable antioxidant activity that may provide additional protection against increased oxidative stress [125].

Glutathione (GSH) metabolism is implicated in the oxidative stress involved in this disease This drug is an important antioxidant that protects against injury by acting as a reductant of peroxides in a reaction catalyzed by glutathione peroxidase. It can be used to detoxify reactive aldehydes generated from lipid peroxidation and can support detoxification of free radicals by vitamine C (Vit C) and vitamin E dependent mechanisms [126].

Komeima *et al* (2006) showed that injecting a combination of antioxidants (α-tocopherol, ascorbic acid, α-lipoic acid) in a mice model with RP decreased cones photoreceptor cell death [51].

M.M. Sanz *et al* (2007) performed *in-vivo* studies with a mixture of antioxidants administered orally to rd1 mice and containing GSH. Their results showed that the combination of antioxidants slowed down rd1 rod photoreceptor degeneration, indicating an additive or synergistic effect [56].

The findings of the aforementioned studies suggest that GSH is an effective option for delaying or preventing photoreceptor cell death in RP. In addition, GSH is

water soluble, is distributed well throughout the tissue, including nervous tissue, and has the ability to maintain a normal redox status [45].

The most common route for the treatment of ocular diseases is topical administration, as application is easy, patient compliance is high and dosage and secondary effects are minimized [2]. However, the absorption of drugs applied topically to the eye is very poor because of the eye's inherent anatomical and physiological barriers [3]. The main problem is drug bioavailability, which is less than 5% of the drug administrated by drop instillation [3].

Ophthalmic inserts could be used to avoid the side effects of ocular administration and to increase drug efficacy. Ocular inserts are solid or semisolid devices that deliver drugs to the eye [103,106]. Inserts allow for accurate dosing, reduced systemic absorption and, in some cases, better patient compliance due to a reduced frequency of administration and lower incidence of visual and systemic side effects [4,5].

The main aim of this study was to evaluate the possibility of administrating GSH directly to the eye. The secondary objectives were (1) to study the stability of this compound in aqueous solution, (2) to determinate the absorption of GSH by the cornea and sclera, and (3) to develop and evaluate ocular formulations whose composition includes GSH.

2. Material and methods

2.1. Reagents

GSH and Vit C were purchased from Sigma-Aldrich (Madrid, España).

The iodoacetic acid, potassium hydroxide (KOH), potassium bicarbonate ($KHCO_3$) and 1-fluoro-2,4-dinitrobenceno (DNFB) were obtained from Sigma-Aldrich (Madrid, España). The m-cresol was purchased from Acros Organics (New Jersey, EE. UU.). The perchloric acid (PCA) was acquainted form Panreac (Barcelona, Spain).

Phosphate buffered solution (PBS) was composed of disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride and bidestilled water, all of which were supplied by Sigma-Aldrich (Madrid, Spain). The pH of this solution was

adjusted to 7.5 ± 0.1 by adding hydrochloric acid (HCl) 5 N or sodium hydroxide (NaOH) 5 N as required.

Methanol, water and glacial acetic acid for high performance liquid chromatography (HPLC) analysis were obtained from J.T. Baker® (Deventer, Holanda) and sodium acetate was purchased from Sigma-Aldrich (Madrid, España). Cellulose membrane filters (pore diameter of 0.2 µm) were obtained from Sartorius Stedim Biotech (Madrid, España) and Kromasil® Amino 5 µm, 250 x 4.6 mm column was purchased from Análisis Vínicos (Barcelona, España).

The bioadhesive polymers hydroxypropyl methylcellulose 4500 (HPMC), polyvinylpyrrolidone K30 (PVP-K30) and polyethyleneglycol (PEG) and the plasticizer agent glycerin were acquired from Guinama (Valencia, Spain).

2.2. Stability studies

A GSH solution (concentration 5 mg/ml) was stored in different conditions to determine the stability of the compound in aqueous solution. The possibility of adding vitamin C as an antioxidant to inhibit degradation of GSH was also analised. For this purpose the same GSH solution (5 mg/ml) including 3 mg/ml of vitamin C was prepared.

Different temperatures for long-term preservation and the influence of light exposition were studied. Drug stability was evaluated at 25 °C and at 4 °C, in darkness and under light exposition. Samples were withdrawn at days 0 – 2 – 7 – 14 – 30.

2.3. Ocular formulations with glutathione

Two different liquid formulations containing different concentrations of GSH were prepared. For this aim, we used PBS and added different amounts of GSH to obtain 10 mg/ml and 5 mg/ml solutions. The final pH of this solution was adjusted to 6.5 by adding HCl 5 N or NaOH 5 N as required.

Using a solvent casting method we prepared a semisolid ocular insert composed of GSH in a matrix of bioadhesive polymers (HPMC, PVP and PEG) [112], and glycerin as a plasticizer.

The required quantity of polymers was weighed and dissolved in the required amount of water by gentle stirring (Table 10). The corresponding amount of plasticizing agent was then added to the solution and was stirred for 24 h at room temperature [102,113]. Finally, GSH was added.

Table 10. GSH ocular insert composition.

Component	Amount
Glutathione	100 mg
Polyvinylpyrrolidone K30	600 mg
Hydroxypropyl mehylcellulose 4500	400 mg
Polyehyleneglycol	0.5 ml
Glycerin	25 mg
Water	10 ml

2.4. Study membranes source

Whole eyes from two-month-old hybrid albino rabbits of either sex were obtained after sacrifice [99,100]. Eye balls were rinsed with saline to remove any trace of blood, after which adherent muscle was removed with scissors. Freshly excised corneas and scleras were obtained by cutting along the sclera-limbos junction and the individual excised tissues were used for absorption studies [108] (Figure 16, Chapter 4).

2.5. *Ex-vivo* ocular absorption studies

Transcorneal and transsclera absorption of GSH was investigated using vertical standard diffusion cells (Franz type) with a diffusion area of 0.567 ± 0.008 cm^2 (DISA, Milan, Italy) [97]. Fresh rabbit cornea or sclera were used as a membrane. Both membranes were used in the study because the target tissue was the retina, which is directly in contact with the sclera.

To conduct the diffusion assays, the membrane was placed between the two compartments of the diffusion cell with the external part facing the donor compartment.

The receptor compartment of the diffusion cells – with a volume of 4.2 ± 0.1 ml – was filled with PBS (pH 7.4) at 37.0 ± 0.1 °C and stirred by a rotating Teflon-coated

magnet in order to prevent possible boundary layer effects. The donor compartment was filled with 0.5 ml of GSH solution (5 mg/ml or 10 mg/ml) or 0.5 ml of the previously obtained semisolid GSH (10 mg/ml) insert.

During the 180 minutes assay, 180 µl samples were taken manually from the receptor chamber at predetermined time intervals; every 15 minutes during the first hour, and then every 30 minutes during the next 2 hours [96]. The volume of sample removed was immediately replaced with the same volume of fresh PBS, thus ensuring sink conditions in the receptor compartment [95,99,100]. The samples were preserved by adding 20 µl of PCA in an eppendorf tube for future analysis.

2.6. Determination of glutathione

GSH concentrations in the samples obtained from experiments were determined by the procedure described by Reed *et al* [127]. The method is based on the iodoacetic acid reaction with thiol groups to form carboxymethyl derivatives followed by chromophore derivatization of amino groups with Sanger's reagent (2,4-dinitrofluoro-bencene). These derivatives are readily separated by HPLC, which allows the quantification of nanomolar levels of GSH.

A Gilson system equipped with Gilson 322 pump, Gilson 150 UV/VIS detector, Gilson 864 degasser and Gilson 234 automatic injector was employed for HPLC analysis. Computerized data were acquired and treated with the Unipoint TM System. The instrumentation components were purchased from Gilson Inc. For the chromatographic separation we used a Kromasil Amino 5 µm, 250 x 4.6 mm column.

The mobile phase A was prepared with 80:20 methanol:water (v/v) and the mobile phase B was prepared with 20 % of sodium acetate 5 M, glacial acetic acid and water and 80 % of phase A. Subsequently, both phases were filtered through a cellulose membrane filter with a pore diameter of 0.2 µm.

The initial conditions (1.0 ml/min, 80 % mobile phase A and 20 % mobile phase B) were maintained for 10 minutes and followed by a linear gradient to 95 % mobile phase B for 40 minutes. Finally we rebalanced with the initial conditions for 10 minutes before starting with the next sample analysis. The wavelength used was 365 nm. The range of the UV detector used was 0.005 and the response time was 5 seconds.

The calibration stock solution – 10 mM of GSH in ultra-pure water - was prepared daily and stored in a refrigerator at 4 °C. The solutions used in the derivation process are described in Table 11.

Table 11. Components of the derivatization solutions.

Solution-1	Iodoacetic acid 100 mM m-cresol 0.2 mM
Solution-2	KOH 10 M $KHCO_3$ 3 M
Solution-3	DNFB (1-fluoro-2,4-dinitrobenzene) 15 % (v/v) Ethanol absolute

KOH: potassium hydroxide. $KHCO_3$: potassium bicarbonate.
DNFB: 1-fluoro-2,4-dinitrobenceno.

Samples and standards were prepared in 1.5 ml tubes. The calibration curve concentrations of GSH were 0 µM, 20 µM, 40 µM, 60 µM, 80 µM and 100 µM. For these standard preparations, 180 µl were taken and 20 µl of perchloric acid 20 % was added to acidify the solution.

The derivatization protocol is shown in Figure 22. Firstly, 40 µl of the solution-1 was added to the standards and samples. The pH's were then adjusted to 8.5 - 9 with 60 µl of solution-2 and the tubes were subsequently incubated at room temperature in darkness for 30 minutes. 200 µl of solution-3 were then added and the tubes were vortexed. Following this, 20 µl of solution-2 were affixed. Finally, the samples were incubated for at least 4 hours at 4 °C in darkness, after which the tubes were centrifuged at 13000 rpm for 10 minutes. The supernatant was analised with the HPLC equipment. Samples not analised within 24 hours were stored at -20 °C.

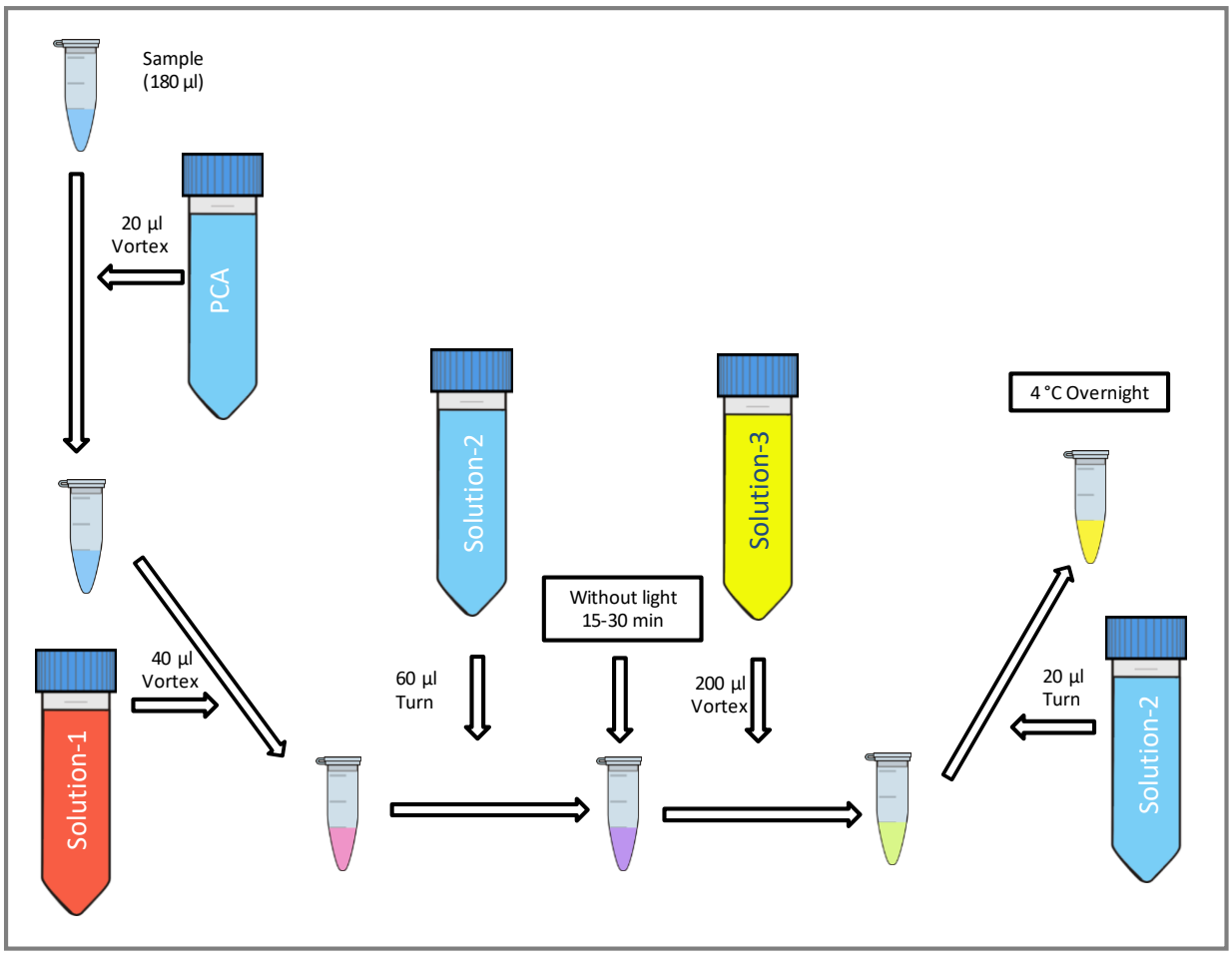


Figure 22. Schema of GSH derivatization protocol.
PCA: perchloric acid. Solution-1, Solution-2, Solution-3 were described in Table 11.

2.7. Data analysis

Data were expressed as mean ± SD. Results were evaluated statistically using analysis of variance (one way - ANOVA) and post-hoc multiple comparison tests (Holm-Sidak test). The level of significance was fixed at $p < 0.05$.

3. Results and discussion

To perform stability studies of the GSH solutions, different conditions were selected, as explained in section 2.2 of this chapter.

The variation in the percentage of GSH concentration with respect to the initial concentration for the different storage conditions can be observed in Table 12.

Table 12. Stability results obtained for GSH solution (5 mg/ml) and GSH (5 mg/ml) + Vit C (3 mg/ml) solution. Concentration mean in percentage ± SD (n = 3).

	Day	25 °C Light	25 °C Darkness	4 °C Darkness
GSH	2	97.56 ± 3.27	97.30 ± 3.95	99.44 ± 2.35
	7	88.88 ± 1.21	89.37 ± 2.26	99.19 ± 7.78
	14	88.87 ± 1.21	87.44 ± 0.45	98.78 ± 7.29
	30	71.14 ± 0.35	72.92 ± 2.71	92.27 ± 5.78
GSH + Vit C	2	86.81 ± 5.09	95.86 ± 2.54	96.93 ± 8.28
	7	77.17 ± 10.16	86.99 ± 0.39	94.72 ± 6.25
	14	73.87 ± 4.81	78.40 ± 3.00	91.47 ± 4.57
	30	58.71 ± 0.25	64.64 ± 3.20	89.97 ± 1.21

GSH: glutathione. Vit C: vitamin C.

These results show that the concentration at 30 days was higher than 90 % when the solution was stored at 4 °C without light exposure. However, when we compared this result with that of the GSH + Vit C solution under the same circumstances no significant differences were found ($p > 0.05$).

In the case of the GSH solutions without Vit C, the final concentration of the samples stored at 25 °C was 90 % after 30 days, with significant differences between day 0 and day 30 ($p < 0.05$). These results demonstrated that storing the GSH solution at 25 °C in any light condition did not allow adequate preservation.

In the case of the solutions of GSH with Vit C, GSH concentration was below 90 % after 30 days for all the conditions studied. Furthermore, significant differences were found when day 0 and 30 at 25 °C were compared ($p < 0.05$). These results showed that a temperature of 25 °C with or without light exposition was not a viable option for preservation of a GSH + Vit C solution.

When we compared the two types of solution (GSH and GSH + Vit C) under the same condition significant differences were seen between solutions preserved at 25 °C but not between those stored at 4 °C in darkness ($p < 0.05$).

We also concluded that vitamin C did not help to preserve the GSH in any of the studied conditions. In the case of the solution preserved at 25 °C with light exposure or darkness the degradation of GSH increased when vitamin C was added. This may have been due to the fact that GSH and Vit C are two antioxidants that work together

physiologically [56], and so the GSH could have preserved the Vit C, though we would need to carry out further studies to test this hypothesis.

Regarding the absorption experiments, Figure 23 and Figure 24 show the absorption profiles revealed by experiments performed with cornea and sclera, respectively.

In Figure 23 we can see that the accumulative amount of GSH in the receptor compartment obtained from the 10 mg/ml and 5 mg/ml solutions were 191.89 ± 53.44 μg/cm^2 and 91.29 ± 7.97 μg/cm^2, respectively, which were significantly different ($p < 0.05$). A direct relation between the permeated amount and the concentration of the solution can also be seen.

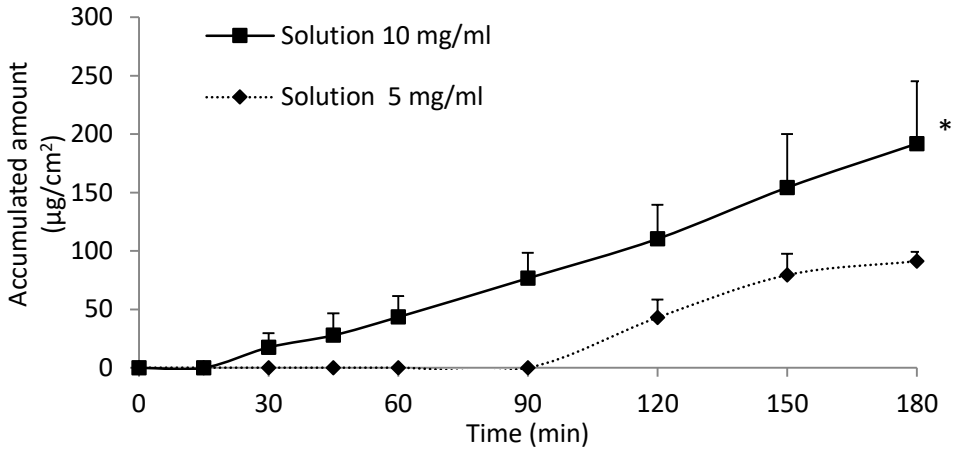


Figure 23. Accumulated amounts of GSH in the receptor compartment (μg/cm^2) as a function of time, obtained from samples employed in ***ex-vivo*** **transcorneal** diffusion studies of different **solutions**. (Mean ± SD; n ≥ 3) *p < 0.05.

The latency period of permeation for the 5 mg/ml solution of GSH was 90 minutes and only 15 minutes with the 10 mg/ml solution. Doubling the concentration reduced the latency period of more than 60 minutes. These results supported the decision to prepare an ocular insert with a concentration of 10 mg/ml.

In Figure 24 we can see that the accumulative amounts of GSH in the receptor compartment obtained from the 10 mg/ml and 5 mg/ml solutions were 596.74 ± 78.05 μg/cm^2 and 251.29 ± 57.69 μg/cm^2 respectively. These were significant differences ($p < 0.05$). The figure also shows a direct relation between the two concentration solutions, as occurred in the studies performed with cornea.

The latency period for the 5 mg/ml solution was 45 minutes, while there was no latency period for the 10 mg/ml solution. This result was of great interest, as the layer under the sclera is the retina, which was our target. These results are supported by Rotman *et al* 2015 and Rip *et al* 2014, who prepared different glutathione PEGylated liposomal formulations for targeting the neuronal tissue [128,129]. As stated by F. Paquet-Durand in ARVO'16, retinal tissue is similar to neuronal tissue, which points to the possibility of GSH being used to target other substances to the retina [130].

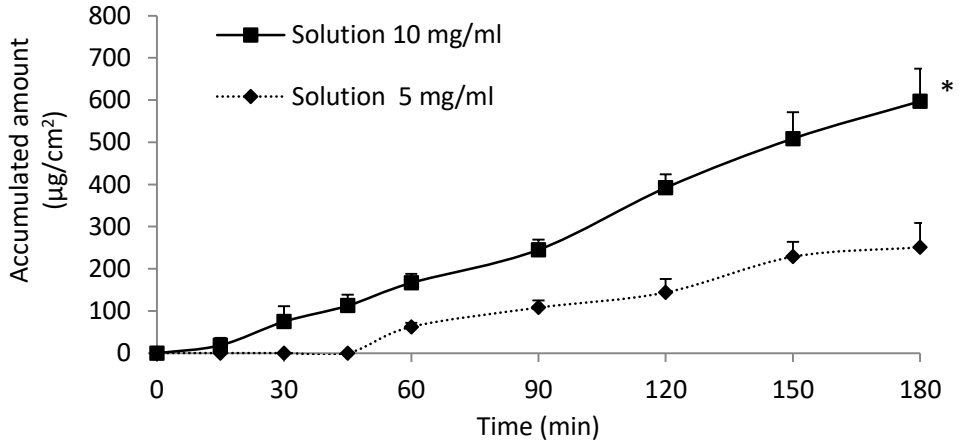


Figure 24. Accumulated amounts of GSH in the receptor compartment (µg/cm^2) as a function of time, obtained from samples of ***ex-vivo*** **transscleral** diffusion with different **solutions**. (Mean ± SD; n ≥ 3) *p < 0.05.

The results obtained with the GSH semisolid insert are shown in Figure 25. The accumulative amount of GSH in the receptor compartment in the cornea and sclera were 109.46 ± 17.61 µg/cm^2 and 249.09 ± 51.17 µg/cm^2, respectively. The figure shows that the amount of GSH permeating through the sclera was significantly higher than that permeating through the cornea ($p < 0.05$).

The insert with a 10 mg/ml GSH concentration provided permeation results similar to those obtained with the 5 mg/ml solution, however the insert was in contact with the ocular surface for longer than the eye drops [4,5], which endorses its potential as a continuous treatment.

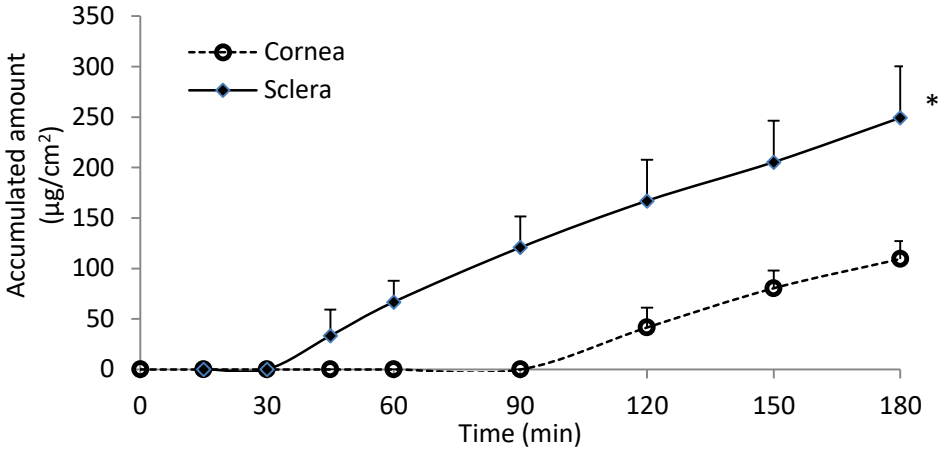


Figure 25. Accumulated amounts of GSH in the receptor compartment (µg/cm^2) as a function of time, obtained from samples of ***ex-vivo*** **transcorneal and transscleral** diffusion studies with the formulated **insert**. (Mean ± SD; n ≥ 3) *p < 0.05.

In Figure 26 we can see the accumulative amounts of GSH in the receptor compartment after 3 hours for all the studied conditions. If we compare the 10 mg/ml solution with the 5 mg/ml solution and the semisolid insert (10 mg/ml) significant differences are apparent (p < 0.05).

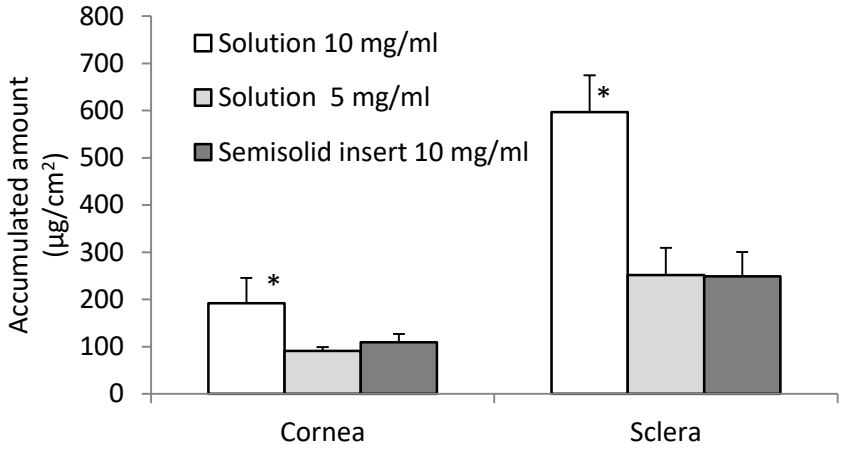


Figure 26. Accumulated amount of GSH in the receptor compartment after 3 hours of experiment (µg/cm^2) obtained from samples of *ex-vivo* transcorneal and transscleral diffusion studies performed with different formulations. (Mean ± SD; n ≥ 3) *p < 0.05.

Moreover, we observed that the amount permeating through the sclera was greater than that permeating through the cornea. This could have been a consequence of the anatomy and physiology of the two tissues. The cornea is a complex tissue with different layers [16] - for example the epithelium and the stroma - which have a different affinity to hydrophilic and lipophilic molecules [15]. This makes it more difficult

for the molecule to permeate through this tissue into the internal part of the eye. On the other hand, sclera is composed of a simple layer of collagen fibers [23] and is easily permeate by hydrophilic molecules such as GSH. This could explain the higher latency of penetration with the 5 mg/ml solution and with the insert.

Chapter 6

Transdermal permeation of olanzapine after fractional laser ablation

1. Introduction

Olanzapine (OLZ) is an atypical antipsychotic drug widely used for the treatment of schizophrenia, bipolar disorder, and the remission of secondary emotional symptoms of mental disorders and related diseases [14,91].

Considering the non-compliance associated with OLZ oral administration and the inconveniences associated with parenteral administration in patients with mental illnesses, it is necessary to exploit alternative routes of administration, such as transdermal delivery. However, this route has several difficulties, mainly because the stratum corneum limits the cutaneous delivery of molecules [76,131]. Different strategies can be used to improve transdermal delivery, some of which aim to compromise the integrity of the stratum corneum to facilitate the absorption of the molecules. Controlled laser microporation can be used to eliminate the integrity of this barrier [13]. Also available is the painless laser epidermal system (P.L.E.A.S.E.), a novel laser microporation technology [83]. This fractionally ablative Er:YAG laser emits µs pulses at a wavelength of 2936 nm that excite water molecules in the epidermis and dermis, and their explosive evaporation results in the formation of micropores. The absorption of small molecules can be enhanced with this technique [132].

Before performing a permeation study with olanzapine, a specific analytical method must be developed to quantify the amounts of OLZ permeated across and retained by the skin. The samples obtained in the transdermal experiments included endogenous substances released from the skin that may have interfered with subsequent analyses. A review of the literature reveals that different analytical methods have been used to detect OLZ in different situations. Several of the methods and infrastructures used were not available to smaller laboratories [133–149] and others relied on complex sample preparation [150–154].

The aims of this research were (1) to develop and to validate a specific high performance liquid chromatography (HPLC) method that can be used for the quantitative determination of OLZ after skin permeation studies; and (2) to study the transdermal delivery of olanzapine to intact skin and after fractional laser ablation as a strategy to increase the permeability of skin to drugs.

2. Materials and methods

2.1. Reagents

Olanzapine was purchased from Watsonnoke Scientific. The buffer 2-morpholino-ethanesulfonic acid monohydrate (MES) was obtained from Fluka (Buchs, Switzerland). The phosphate buffer saline (PBS) was prepared with disodium hydrogen phosphate, sodium dihydrogen phosphate and sodium chloride, all of which were supplied by Sigma-Aldrich (Buschs, Switzerland). The pH of this solution was adjusted to 7.5 ± 0.1 by adding hydrochloric acid 5 N or sodium hydroxide 5 N as required.

The polytetrafluoroehylene (PTFE) and nylon filters were obtained from VWR, (Switzerland).

UPLC/MS grade acetonitrile (ACN) was purchased from VWR International AG (Dietikon, Switzerland). All aqueous solutions were prepared using Milli-Q water (resistivity >18 MΩ.cm). Ammonium acetate, acetic acid, triethylamine and, Tween 80® (T80) were purchased from Sigma-Aldrich (Buschs Switzerland). All other chemicals were of at least analytical grade.

2.2. Preparation of standard solutions

A 2 mg/ml concentration stock solution of olanzapine was prepared in ACN. Five standard solutions (1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml, and 100 µg/ml) were obtained by further dissolution of drug stock solution with PBS. These standard fresh solutions were used for the preparation of calibration curves.

2.3. Instrumentation and chromatographic conditions

An ASI-100 auto-sample equipped with a P680A LPG-4 pump and UVD 170U detector (Formerly Dionex AG, now Thermo Fisher Scientific AG; Reinach, Switzerland) was used for HPLC analysis. The system was controlled by Chromeleon® Chromatography Management Software.

Chromatographic separation of the analytes was achieved using a LiChrospher® (BGB Analytik AG; Boeckten, Switzerland) C8 Reverse-phase analytical column (250 x 4.0 mm, 5 µm). A LiChrospher® guard column (10 x 4.0 mm, 5 µm) with the same packing material was mounted upstream from the analytical column. The column temperature was kept at 45 ± 1 °C, the flow rate was maintained at 1.2 ml/min, and the UV absorbance wavelength was set at 260 nm. The injection volume was 50 µl. The mobile phase was composed of phase A, $C_2H_7NO_2$ buffer (30 mM, pH 6.5 with acetic acid) 0.05 % triethylamine and phase B, ACN (40:60, v/v).

2.4. Olanzapine analytical method validation

The method was validated according to ICH guidelines Q2 (R1) [155] with respect to linearity, detection limit (DL) and quantification limit (QL), specificity, accuracy and precision.

The linearity of the analytical method was validated using the five standard solutions previously described. Calibration curves were obtained by least squares linear regression analysis of the peak area obtained as a function of drug concentration. Each concentration was assayed six times to determine intra-day reproducibility. In order to detect inter-day validation, the procedure described above was repeated on six different days.

The detection limit is the lowest amount of a substance that can be detected but not necessarily quantified as an exact value. The quantification limit is the lowest amount of the drug that can be quantitatively determined with defined precision under the experimental conditions. Assuming a normal distribution of measured concentration values, DL and QL were calculated from the standard deviation of the regression data (SD) and from the slop of the linear regression (b), following Equation 9 and Equation 10:

Equation 9.

$$\mathrm{DL} = 3.3\frac{\mathrm{SD}}{b}$$

Equation 10.

$$\mathrm{QL} = 10\frac{\mathrm{SD}}{b}$$

Specificity was the absence of interference from endogenous compounds present in the skin and was investigated by injecting 10 samples of porcine skin extract.

Accuracy expresses the closeness between the calculated value and the accepted reference value. It was defined as the relative error of the nominal solution concentration. Measurements had to be within ± 10 % for all concentrations to be considered acceptable. The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous drug solution under fixed conditions and provides information regarding random error. The variance of the repeatability and intermediate precision, and the corresponding relative standard deviation (RSD), were calculated from the estimated concentrations. To be considered acceptable, the RSD had to be lower than 10 % at all of the concentrations analysed.

The stability of OLZ in contact with the skin was studied. An aliquot of OLZ was kept in contact with skin, for 24 hours (n = 3; mean ± SD), after it was centrifuged, filtered and analised by HPLC.

The effect of filtering was also evaluated to demonstrate that there were no significant differences between results after filtering. For this propose an olanzapine solution (50 µg/ml; n = 3) was filtered using either a PTFE filter (0.45 µm) or a nylon filter (0.45 µm). The concentration of the solution post-filtration and pre-filtration was measured and results were compared.

In order to determine the experimental solubility of olanzapine in buffer solution (MES 60 mM, pH 5.5) at 25 °C, the drug was added to Eppendorfs with 0.5 ml of these isotonic buffer solutions in a quantity that exceeded the saturation limit. This was carried out at room temperature (~25 °C) and the solutions were stirred for 12 hours to ensure saturation. Then the Eppendorfs were centrifuged for 20 minutes at 12000 rpm. Finally, samples were taken and diluted to be quantificated by HPLC.

To evaluate the procedure of extraction of olanzapine from the skin after the diffusion experiment, portions of skin (2 cm^2) were cut into small pieces and placed in contact with 100 µl of a 1 mg/ml solution of olanzapine that was stirred for 30 minutes. Afterwards, the skin was shaken for 12 hours with 5 ml of a mix of acetonitrile-water (50:50, 75:25, 25:75) in order to extract the drug. Samples of 1.5 ml were centrifuged for 20 minutes at 12000 rpm. Finally, samples of 1 ml of supernatant were filtered and analysed by HPLC.

2.5. Transdermal delivery of olanzapine

2.5.1. Skin source

Porcine ears were obtained from an abattoir (CARRE; Rolle, Switzerland). The skin was excised (thickness 1.1 mm) with a surgical blade, wrapped in Parafilm™ and stored at -20 °C for a maximum period of two months.

2.5.2. Passive permeation experiments

The validated HPLC method was used to quantify olanzapine in the samples obtained from *ex-vivo* transdermal diffusion studies. These experiments were performed employing vertical Franz diffusion cells (Glass Technology; Geneva, Switzerland) with a diffusion area of ~2 cm^2 and skin from pig ear as the membrane.

One ml of a solution of olanzapine (3 mg/ml) prepared in an isotonic buffer (MES 60 mM, pH 5.5) was placed in the donor compartment. The receptor compartment (12 ml volume) was filled with isotonic buffer (PBS-T80 1 %, pH 7.4), thermostated at 36.9 ± 0.1 °C (n = 30) and stirred by a rotating Teflon-coated super magnet placed inside the cell to prevent boundary layer effects. One ml samples were removed manually from the receptor chamber at predetermined time intervals (every hour starting at hour 4 of the experiment) during the 8 hours that the experiment lasted. The volume of sample removed was replaced with the same volume of buffer. The olanzapine contained in each sample was recorded in order to calculate the accumulative amount (Q) in the receptor compartment. At the end of the *ex-vivo* transdermal diffusion experiments the amount of drug retained in the skin was also determined by the previously validated extraction procedure. The transdermal flux was estimated from the slope of the linear

region (steady-state portion) of the plot of the cumulative amount of olanzapine (μg/cm^2) against time (hours).

2.5.3. P.L.E.A.S.E.® Microporation permeation experiments

The skin surface was dried and samples were then mounted on a custom-designed assembly [83]. Afterwards, laser treatment was performed to determine the effect of pore density (fractional ablated area) and pore derp, and microporation parameters were set to provide 10 % pore density at a fluence of 89.9 J/cm^2 (Figure 27). OLZ experiments were performed on the same skin samples, following the method described previously.

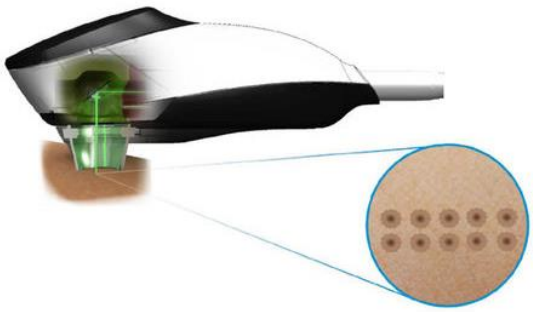


Figure 27. "P.L.E.A.S.E.® private". Obtained from reference *www.pantec-biosolutions.com.*

2.6. Data analysis

Data were expressed as mean ± SD. Results were evaluated statically using analysis of variance (one way - ANOVA), and the Holm-Sidak test was performed for post-hoc multiple comparison. The level of significance was fixed at $p < 0.05$.

3. Results and discussion

We describe a specific and sensitive HPLC method to determine OLZ. This method enables us to determine OLZ in samples obtained from *ex-vivo* transdermal permeation experiments. The methods used for this purpose need to be highly specific, as these kinds of samples usually contain endogenous compounds present in the skin.

Furthermore, the method needs to be sufficiently sensitive due to the frequently low concentration of drug in the samples collected in these experiments.

Since the OLZ absorbance spectra showed a maximum peak of absorbance at 260 nm (Figure 28), the wavelength selected was sensitive and specific enough to analyze OLZ in the range of concentrations of the samples collected in the transdermal diffusion experiments. Under these conditions, we obtained a good separation between OLZ and endogenous compounds from skin in our samples.

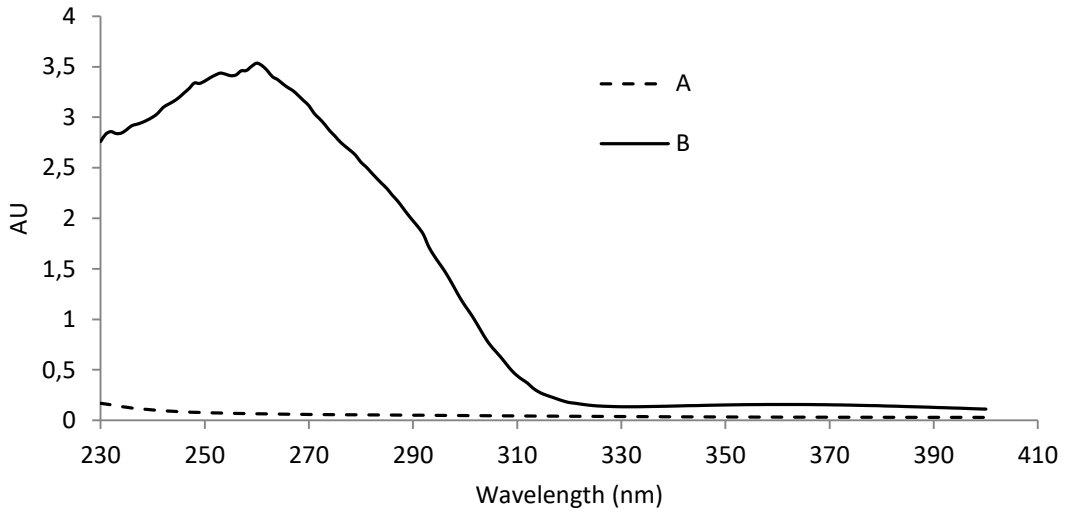


Figure 28. Olanzapine UV spectra obtained with a BioTek Synergy Mx Multi-Mode Reader showing the peak at 260 nm. **A:** water. **B:** water – olanzapine.

Representative chromatograms under the chromatographic conditions selected for PBS-skin extract and an OLZ standard solution prepared with PBS-skin extract are shown in Figure 29. The olanzapine retention time was 5.22 ± 0.07 minutes and the total running time was 7 minutes. The method was considered to be specific, as there was no interference from endogenous compounds from the skin and the olanzapine peak was separated from the solvent front.

The method was validated using the following criteria: specificity, linearity, precision, accuracy, DL, QL, stability and applicability to *ex-vivo* diffusion studies [155].

Ten blank samples of skin extracts in PBS were analysed to investigate the specificity of the method. No interference was found at the olanzapine retention time (Figure 29).

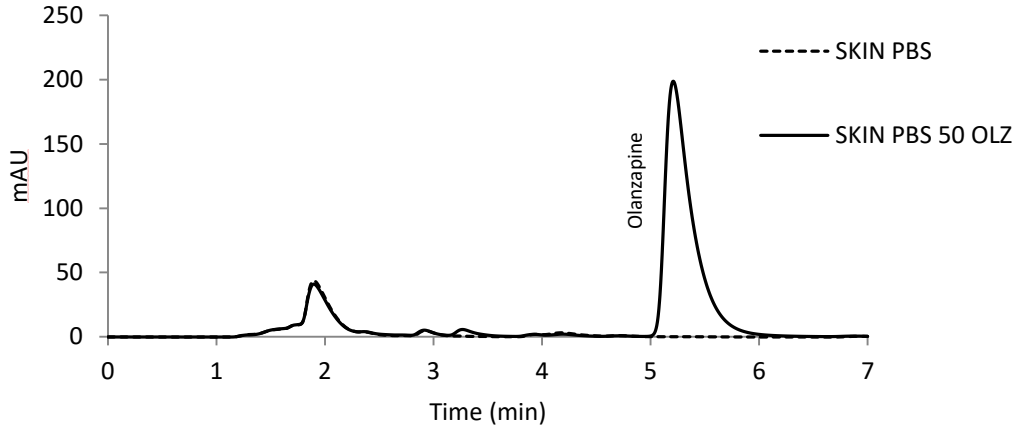


Figure 29. Chromatogram of olanzapine 50 µg/ml with skin extract with PBS and with skin extract with PBS without olanzapine.
PBS: phosphate buffer saline. OLZ: olanzapine.

The method exhibited linearity between the response and the corresponding concentration of olanzapine over the 1 µg/ml – 100 µg/ml concentration range. The least square linear regression analysis showed a correlation coefficient of 0.999. The slope of the calibration curve was statistically different from zero, and the intercept was not statistically different from zero.

The relative error value was computed for each concentration of calibration standard (Table 13). Accuracy was within acceptable limits, the values obtained for all concentrations were below 10 %, and the results of the intra-day and inter-day precision were below 10 % in all cases (Table 13).

Table 13. Intra and inter-day precision and accuracy of the HPLC method.

Nominal concentration (µg/ml)	Intra-day variability (n = 6)			Inter-day variability (n = 6)		
	Concentration measured (µg/ml)	Accuracy (%)	RSD (%)	Concentration measured (µg/ml)	Accuracy (%)	RSD (%)
1	1.02 ± 0.09	1.5	8.69	0.997 ± 0.086	0.3	8.64
5	4.75 ± 0.25	5.0	5.26	4.78 ± 0.14	4.4	2.84
10	9.30 ± 0.15	7.0	1.63	9.15 ± 0.24	8.6	2.67
50	50.0 ± 1.9	0.0	3.70	50.1 ± 1.7	0.3	3.40
100	99.6 ± 3.04	0.3	3.05	99.2 ± 1.4	0.8	1.45

RSD: relative standard deviation.

The DL and QL of olanzapine were 0.28 µg/ml and 0.84 µg/ml, respectively. The QL reported by other authors were smaller due to the detector employed; for example, a MS/MS detector [133,135,136,139,141,142,144,145,148], electrochemical detector

[137,146,147], electrospray ionization mass spectrometry [149], amperometic detector [138] or voltammetry [140]. Other groups have employed a complicated method to prepare the sample before analysing it [150–154] or they have used longer running time for the HPLC analysis [156].

Other methods similar to ours have obtained lower DL and QL but also had a small linearity range (25 μg/ml - 75 μg/ml) [157]. We have compromised between a lower sensitivity and a wider linearity range, which we consider important for our studies.

Olanzapine was considered to be stable in the presence of skin, since the concentration measured after 24 hours of exposure was 94.4 ± 0.4 % (n = 3; mean ± SD) of the initial value.

The solubility of olanzapine in the tested isotonic buffers is shown in Table 14.

Table 14. The solubility of olanzapine in different solvents.

Isotonic Solution	Mean ± SD (μg/ml)
Water	131.78 ± 26.87
MES (60 mM) pH 5.5	3478.54 ± 566.47
PBS-T80 1 % pH 7.6	267 ± 16.81

MES: 2-morpholino-ethanesulfonic acid monohydrate. PBS-T80: phosphate buffer saline Tween 80®. SD: standard deviation.

Ex-vivo methods are designed to measure the penetration of skin by chemicals and their subsequent diffusion across the skin into a fluid reservoir [158]. To perform these *ex-vivo* permeation studies, diffusion Franz cells were employed. Finally, MES buffer (60 mM, pH 5.5) was selected to prepare the donor solution, as it had the highest solubility of the tested solvents [159,160]. PBS–T80 1 % (pH 7.6) was selected for the receptor compartment to mimic real *in-vivo* conditions. Other authors use a mix of PBS and methanol in the receptor compartment [150] or a mix of organic solvents [161]. OLZ has better solubility in this media, but the solutions obtained do not resemble *in-vivo* skin diffusion conditions. For this reason we selected PBS–T80 1 % as a receptor solution.

The concentration of the drug measured after filtration through a PTFE and nylon filter was 78.7 ± 2.4 % and 94.4 ± 0.4 % (n ≥ 3; mean ± SD), respectively, of the initial value. Based on this study a nylon filter was selected for use in the rest of studies.

The skin extraction procedure efficiency was 90.08 ± 18.5 %, 91.08 ± 20.4 % and 76.27 ± 19.2 %, respectively, when using ACN:water (75:25, 50:50, 25:75) (n = 3). Following this evaluation, a 50:50 ratios was selected.

3.1. Olanzapine transdermal *ex-vivo* experiments

OLZ was not detected in the receptor compartment after 8 hours of passive permeation.

P.L.E.A.S.E.® Microporation was chosen as the enhancer technique. OLZ permeation was detected after absorption *ex-vivo* experiments using a laser and a OLZ 3 mg/ml solution. Figure 30 shows the permeation profile of OLZ after laser ablation. The accumulative amount of OLZ after 8 hours was 178.46 ± 31.69 µg/cm^2.

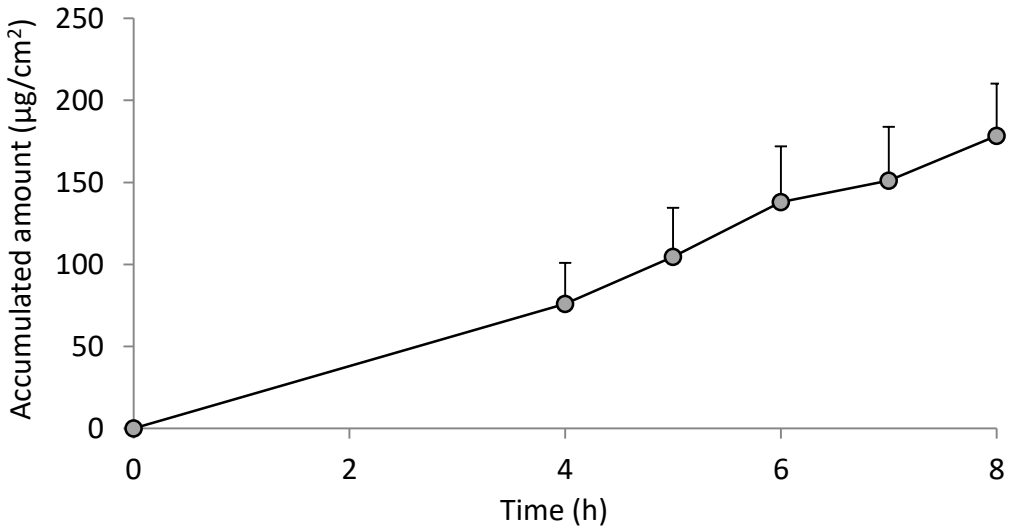


Figure 30. Quantification of the cumulated permeation amount of OLZ after an absorption study using laser ablated skin. (Mean ± SD; n ≥ 3).

The total accumulative amount of OLZ in the skin (Figure 31) was 51.26 ± 1.98 µg/cm^2 after the passive study and 194.30 ± 36.78 µg/cm^2 after microporation. The amount of OLZ deposited in the skin was significantly higher in the microporated samples ($p < 0.05$). This could have been due to the integrity of the

stratum corneum being affected by the laser and because the OLZ bypassed the first layers of the skin easily [83].

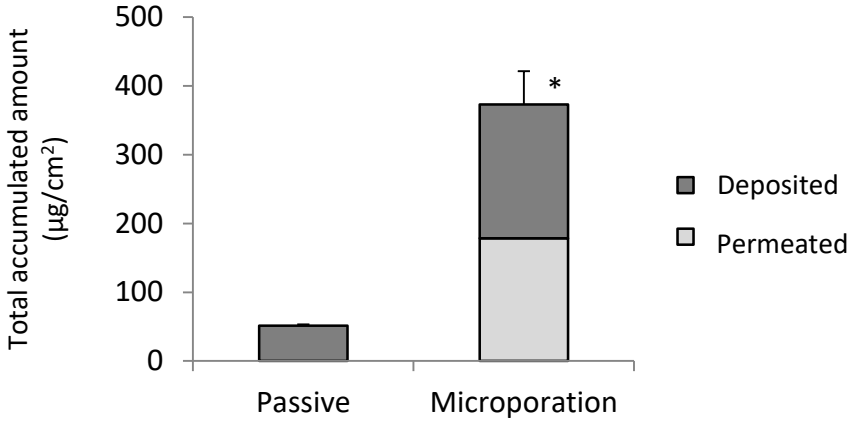


Figure 31. Total accumulated amount of OLZ – the sum of the amount permeated across and deposited within the membrane – after 8 hours of transdermal permeation using intact skin (passive) or microporated skin (Mean ± SD; n ≥ 3).

Finally, when we compared the sum of the total accumulative amount of OLZ, this quantity was found to be more than 7 times higher in the microporated skin, which was a statistically significant difference ($p < 0.05$).

Our results show that laser ablation, which compromises the integrity of the stratum corneum and increases the absorption of OLZ across the skin, is a potential strategy to bypass the stratum corneum.

Chapter 7

Controlled iontophoretic delivery of olanzapine: development of transdermal formulations

1. Introduction

Atypical antipsychotic drugs are used for the treatment of mental disorders such as schizophrenia. This therapeutic group includes olanzapine (OLZ), a thienobenzodiazepine derivative, which has proven to be effective against the positive and negative symptoms of this illness [92].

Currently, OLZ is commercially available as orally disintegrating tablets, which should be dissolved in the mouth and swallowed with or without liquid, as well as in the form of intramuscular injections. Although OLZ is an effective antipsychotic agent, considering patient noncompliance with oral administration and the inconveniences associated with parenteral administration, a pharmaceutical form of transdermally-administrated OLZ could have obvious benefits for the treatment of schizophrenia.

Transdermal administration is advantageous in that it avoids gastrointestinal incompatibility and the first-pass metabolism and consequent reduction of the bioavailability of the drug. In the case of OLZ, approximately 40 % of the dose is metabolized by the liver before reaching the systemic circulation [162]. Moreover, percutaneous administration reduces the frequency of dosing and is comfortable and non-invasive for the patient, which could improve patient compliance [76,163].

The skin is a natural barrier against the penetration of most drugs, and, consequently, different methodologies have been applied in order to enhance the transdermal absorption of pharmacological products.

Recently, microporation has been introduced as a physical technique to increase the permeation OLZ (see Chapter 6). This physical enhancer undermines the integrity of the skin [83] and requires trained healthcare personnel to administer it. In contrast, iontophoresis by-passes the stratum corneum without compromising the integrity of the skin, while there are devices available for personal administration [164].

Iontophoresis aids drug transport across the skin by means of the application of a low-level electric current (≤ 0.5 mA/cm^2) [78]. The mechanisms by which iontophoresis enhances molecular transport across the skin are: (a) electromigration (EM), in which a charged ion is repelled from an electrode with the same charge; (b) electroosmosis (EO), the convective flow of solvent that occurs through a charged pore in response to the

preferential passage of counter ions when the electric field is applied; and (c) a current-induced increase in skin permeability.

The application conditions of the iontophoretic field determine molecular transport across the skin; for example, iontophoretic transdermal transport is more efficient, as the background electrolyte concentration is lowered and is directly dependent on the current density applied [165,166].

Of the different formulations that can be applied with iontophoresis, transdermal drug delivery systems are one of the most common. There are different types of said systems; for example the reservoir transdermal system (reservoir-TS) and matrix transdermal systems (TS). A semisolid formulation is required in the case of reservoir-TS, and some of the most frequent semisolid polymers are hydrophilic polymers, like polyvinyl pyrrolidone (PVP), hydroxypropyl metilcellulose (HPMC) or hydroxyethyl cellulose (HEC). A matrix-TS relies on other options, and one of the most used polymers is PVP [114,167,168].

The aim of the present work was (1) to prepare different transdermal formulations with OLZ; (2) to characterize drug transdermal absorption across pig ear skin; (3) to assay the effect of iontophoresis current density on the transdermal absorption of OLZ; and (4) to evaluate the effect of iontophoresis on the permeation of OLZ from these formulations.

2. Materials and methods

2.1. Reagents

Olanzapine was purchased from Watsonnoke Scientific.

2-morpholino-ethanesulfonic acid monohydrate (MES) was obtained from Fluka (Buchs, Switzerland). Phosphate buffer saline (PBS) was prepared with disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride (NaCl) and bidestilled water, all of which were supplied by Sigma-Aldrich (Buschs, Switzerland). The pH of this solution was adjusted to 7.5 ± 0.1 by adding hydrochloric acid 5 N or sodium hydroxide 5 N when required.

PVP, HPMC, HEC, sorbitol, acetaminophen (ACM), carbopol and agarose were purchased from Sigma-Aldrich (Buschs, Switzerland).

Scotchpack™ Backing 9733 layer, used to laminate the TS, was obtained from 3M™ (USA).

The PVC tubing (ID 3.17 mm; OD 4.97 mm) used to prepare the saline bridges in the iontophoresis experiments was bought from VWR International AG (Dietikon, Switzerland). Silver (Ag) wire and silver chloride (AgCl) used for making electrodes were supplied by Sigma-Aldrich (St. Louis, US).

UPLC/MS grade acetonitrile was bought from VWR International AG (Dietikon, Switzerland). All aqueous solutions were prepared using Milli-Q water (resistivity >18 MΩ.cm). All other chemicals were of analytical or high performance liquid chromatography (HPLC) grade.

2.2. Preparation of olanzapine transdermal formulations

Different hydrophilic gels with OLZ were formulated to be used in reservoir-TS. A matrix-TS containing OLZ was also developed.

The polymers most applied to skin belong to different groups, such as cellulose derivatives, chitosan, carrageenan, polyacrylates, polyvinylalcohol, polyvinylpyrrolidone and silicones [169]. They are used as gelatin agents and matrixes in transdermal systems.

For gel preparation, the following polymers were selected based on their compatibility with the drug: PVP (15 %, w/w), HPMC (2 %, w/w) or HEC (2 %, w/w). These polymers provide a hydrophilic matrix that improves the delivery of OLZ and are suitable for topical formulations [169].

To elaborate an OLZ matrix, TS PVP (20 %, w/w) was used as a polymer. This polymer has been employed previously to prepare different TS of different drugs, including sumatriptan succinate [114,167], pizotifen malate [170] and memantine hydrochloride [168].

For the creation of the transdermal formulations, a solution of MES 60 mM with OLZ was prepared and the required amount of PVP, HPMC or HEC slowly added. These

solutions were left overnight to allow the elimination of air bubbles from the gel. The composition of the gels is set out in Table 15.

For preparation of the OLZ-TS we followed the same procedure as that used to prepare the PVP gel, but including sorbitol as a filmogen agent (Table 15). The final mixture was laminated (1 mm) on an occlusive layer (Scotchpack™ 9733 backing) and dried using a Mathis Labcoater LTE-SM, a preindustrial machine employed to prepare TS. The optimal drying conditions were as follows: surface temperature 50 °C; dwell time 20 minutes; and time model mode B.

Table 15. Composition (%, w/w) of transdermal formulations prepared with olanzapine.

Composition	OLZ Gel			OLZ TS
PVP	15	-	-	20
HPMC	-	2	-	-
HEC	-	-	2	-
Sorbitol	-	-	-	5
MES Solution	55	68	68	45
Olanzapine	30	30	30	30

OLZ: olanzapine; TS: transdermal system; PVP: polyvinylpyrrolidone; HPMC: hydroxylpropyl methylcellulose; HEC: hydroxyethyl cellulose; MES: 2-morpholino-ethanesulfonic acid monohydrate buffer.

2.3. Olanzapine transdermal absorption assays

2.3.1. *Ex-vivo* diffusion studies

For *ex-vivo* transdermal diffusion experiments porcine ear skin was used as a membrane, as it is considered a good research model of human skin [171,172]. The ears were obtained from a local abattoir (CARRE; Rolle, Switzerland) within a few hours of sacrifice and were cleaned under cold running water. The skin was removed carefully from the inner region of the ear and separated from the underlying cartilage with a scalpel. The tissue was wrapped in Parafilm™ and stored at -20 °C for a maximum period of one month. Before beginning the experiment, the skin was defrosted and equilibrated by dipping in isotonic buffer solution at room temperature for one hour.

Permeation experiments were performed following the previously explained procedure (2.5.2. Passive permeation experiment, Chapter 6). The transdermal flux was

estimated from the slope of the linear region (steady-state portion) of the plot of the accumulative amount of OLZ (μg/cm^2) against time (hours).

The procedure described in chapter 6 (2.5.2. Passive permeation experiment) was employed for transdermal absorption studies of the formulations (PVP, HPMC, HEC gels with OLZ, and PVP TS with OLZ). 1 ml of gel or a ~2 cm^2 piece of TS was placed in the donor compartment. TS are not adhesive in the dry state, but become bioadhesive when applied to wet skin. For this reason, before application of the system, the skin was moistened with 15 μl/cm^2 of water to allow its adhesion [114].

2.3.2. Iontophoresis transdermal experiments

Iontophoresis experiments were performed following the method previously described for the *ex-vivo* diffusion studies. In this case, electrodes were added to apply the current and a solution containing 15 mM of acetominophene was incorporated into the donor compartment to determine the contribution of electroosmosis [173].

The anodal and cathodal compartments were regardless of the diffusion cells (Figure 32). Both compartments were filled with 10 ml of saline solution with NaCl (133 mM). The anodal compartment was assessed separately take apart to avoid ionic competition for diffusion between the drug and other electrolytes that might have been present in the donor compartment [174], and the cathodal compartment was assessed separately take separately to minimize the interaction between the drug and the cathode. Saline bridges (3 % agarose + 0.1 M NaCl) were employed to electrically connect the physically isolated anodal compartment with the donor compartment, and the isolated cathodal compartment with the receptor compartment.

A reversible pair of Ag^+ and AgCl electrodes were placed in the anodal and cathodal compartment, respectively. Electrodes were made in-house by covering an Ag wire with melted AgCl. The melted AgCl solidifies on cooling and fixes to the Ag^+ wire. The rest of the wire was covered with plastic heat shrink tubing to protect it from corrosion.

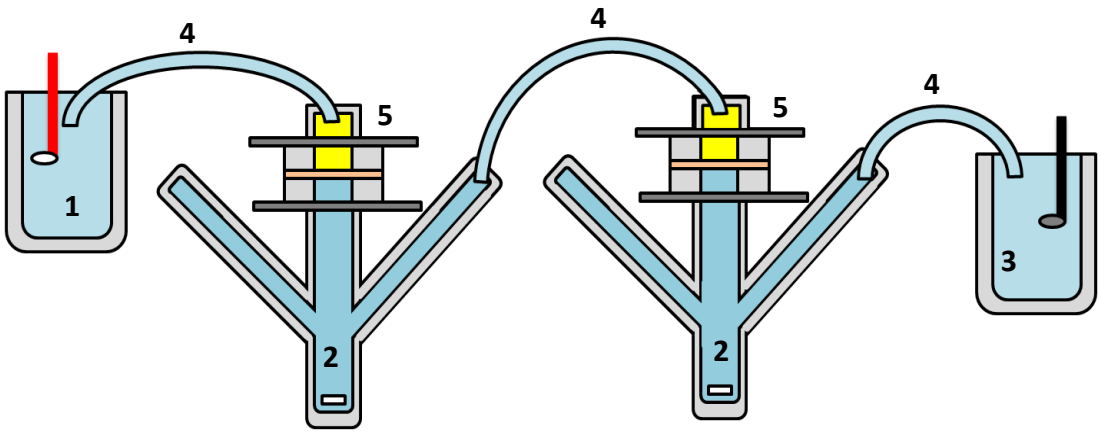


Figure 32. Iontophoresis set-up used for *ex-vivo* permeation experiments.
1: Anodal compartment; 2: Receptor compartment; 3: Cathodal compartment; 4: Saline bridge; 5: Donor compartment.

The effect of current on molecular transport of OLZ was studied using three constant current densities - 0.25 mA/cm^2, 0.4 mA/cm^2, and 0.5 mA/cm^2 - applied for 8 hours, using a APH 1000 M power generator (Kepco Inc; Flushing, US).

Co-iontophoresis of acetaminophen (a neutral hydrophilic molecule whose iontophoretic transport is due exclusively to electrically-induced convective solvent flow, EO) was used to study the contribution of electromigration and electoosmosis in the total iontophoretic flux of OLZ [175–177]. Experiments were performed without OLZ to determine the flux of ACM under a current density of 0.5 mA/cm^2, to determine the inhibition factor, and to report the effect of OLZ transport on skin permselectivity [175–177]. In brief, the equations (11, 12 and 13) used were as follows:

Equation 11.

$$J_{EM} = J_{tot} - J_{EO}$$

Equation 12.

$$V_W = \frac{J_{ACM}}{C_{ACM}}$$

Equation 13.

$$IF = \frac{Q_{ACM}}{Q_{ACM.OLZ}}$$

Where,

J_{tot} = total flux

J_{EM} = flux due to electromigration

$J_{EO} = V_W C_{OLZ}$

J_{EO} = flux due to electroosmotic flow

V_W = linear velocity of solvent flow

C_{OLZ} = concentration of OLZ

J_{ACM} = flux of the marker molecule (ACM)

C_{ACM} = donor concentration of the marker molecule (ACM)

IF = inhibition factor

Q_{ACM} = cumulative permeation of ACM in 8 hours in the absence of OLZ

$Q_{ACM.OLZ}$ = cumulative permeation of ACM in 8 hours in the presence of OLZ

It was used to perform permeation assays in which iontophoresis was applied to the developed formulations.

The same procedure as that employed for the experiments with the OLZ solutions was followed for the assays of the OLZ gels, with the exception that 1 ml of the formulation was applied to the donor compartment and a current density of 0.5 mA/cm^2 was applied.

The previous methodology was adapted for iontophoresis with the TS. In this case, a small ~2 cm^2 disc of agarose with NaCl was placed in the donor compartment, in contact with the TS. On top of this agarose, 1 ml of carbopol gel with NaCl was added, and was at the same time in contact with the saline bridge. This set-up allowed the current to be conducted through the TS.

2.4. Instrumentation and chromatographic conditions

An ASI-100 auto-sample equipped with a P680A LPG-4 pump and UVD 170U detector (Formerly Dionex AG, now Thermo Fisher Scientific AG; Reinach, Switzerland) was used for HPLC analysis. The system was controlled by Chromeleon® Chromatography Management Software.

Chromatographic separation of the analytes was achieved on a LiChrospher® (BGB Analytik AG; Boeckten, Switzerland) C8 Reverse-phase analytical column (250 x 4.0 mm, 5 µm). A LiChrospher® guard column (10 x 4.0 mm, 5 µm) with the same packing material was mounted upstream from the analytical column. The column temperature was maintained at 45 ± 1 °C, the flow rate was maintained at 1.2 ml/min, and the UV absorbance wavelength was set at 260 nm. The injection volume was 50 µl. The mobile phase was comprised of phase A, $C_2H_7NO_2$ buffer (30 mM, pH 6.5 with acetic acid) 0.05 % triethylamine, and phase B, acetonitrile (40:60 v/v).

ACM from iontophoresis assays was analysed by HPLC-UV/VIS, modifying the mobile phase composition with respect to the OLZ analysis. In this case, the mobile phase consisted of a mixture of ammonium di-hydrogen phosphate solution (pH 4.6) and acetonitrile (90:10 v/v).

2.5. Data analysis

The samples obtained in the transdermal absorption experiments were analysed using the previously mentioned HPLC-UV/VIS method.

Once the samples of OLZ and ACM were quantified, the accumulated amounts of OLZ and ACM in the receptor compartment were calculated. The absorption profiles of OLZ and ACM were obtained by plotting the accumulated amount in the receptor chamber (µg/cm^2) *versus* time (h). The transdermal flux observed for the iontophoresis study was estimated in the steady state and obtained from the slope of the linear region of the accumulated amount of OLZ and ACM in the receptor chamber (µg/cm^2) *versus* time (h).

Fluxes, permeated amount and efficiency of OLZ delivery were compared by one-way ANOVA. Post-hoc multiple comparison was performed by means of Holm-Sidak tests (level of significance for statistical differences was $p < 0.05$).

3. Results and discussion

Control passive diffusion experiments with OLZ lasting 8 hours resulted in very poor permeation, with OLZ levels below the detection limit. In contrast, OLZ anodal

iontophoresis of a drug solution (3 mg/ml) with the highest density assayed (0.5 mA/cm^2) resulted in accumulative permeation of 212.51 ± 66.26 µg/cm^2 after 8 hours.

The accumulative anodal iontophoretic permeation, skin deposition and efficiency of OLZ as a function of current densities obtained in the experiments we performed are shown in Figure 33. The efficiency is defined as the ratio of the amount of drug permeated to the amount contained in the formulation in the donor compartment. The permeation amount, the total accumulative amount and the efficiency of OLZ increased linearly when the current density rose (r = 0.999, 0.992 and 0.992 respectively). This information may be of interest to physicians, as it may help to determine dosage options for each patient.

In reference to the inhibition factor (Equation 13) obtained after comparison of the accumulative permeation amount of ACM in the presence and absence of OLZ with 0.5 mA/cm^2 current density, a value of 0.91 ± 0.09 was obtained. This IF value indicated that skin permselectivity was not altered by OLZ.

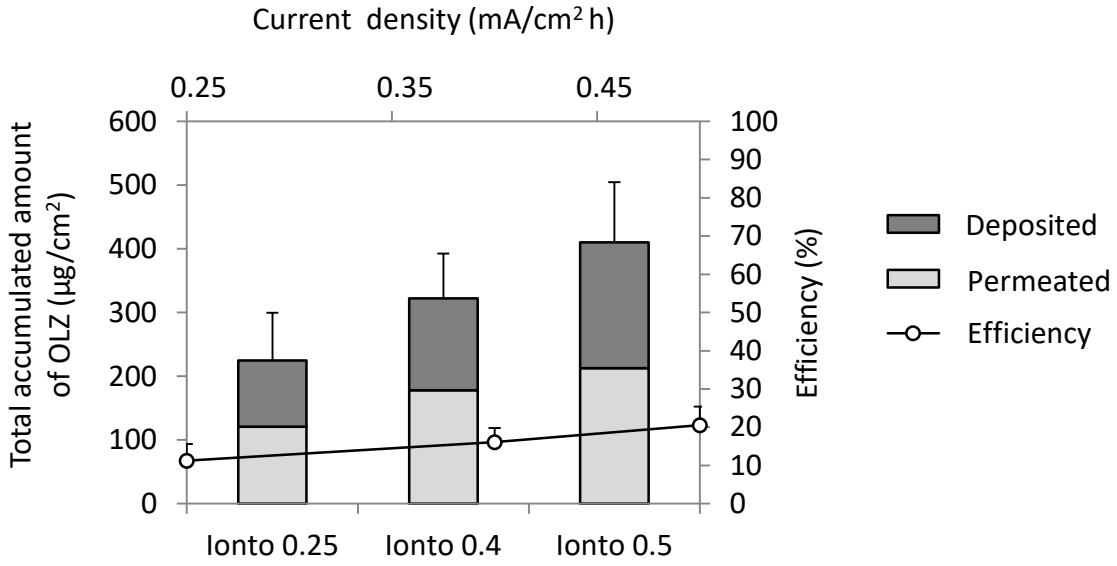


Figure 33. Effect of current density (0.25 mA/cm^2, 0.40 mA/cm^2 and 0.50 mA/cm^2) on total accumulated amount of OLZ – the sum of the amount permeated across and deposited within the membrane – and efficiency after 8 hours transdermal iontophoresis using a 3 mg/ml solution (Mean ± SD; n ≥ 3).

With respect to principal iontophoretic transport mechanism in OLZ electromigration (Table 16), the contribution % of EM obtained with the current densities of 0.25 mA/cm^2, 0.4 mA/cm^2 and 0.5 mA/cm^2 was 98.03 %, 97.61 % and 96.61 % respectively.

Table 16. Data of EO flux, EM flux and total flux (mean ± SD; n ≥3) obtained after permeation studies with ACM and OLZ under different current densities. Correlation coefficients obtained in different conditions.

Current Density (mA/cm^2)	Total Flux (μg/cm^2/h)	EM flux (μg/cm^2/h)	EO flux (μg/cm^2/h)
0.25	16.1 ± 4.1	15.8 ± 3.9	0.3± 0.3
0.40	22.0 ± 7.5	21.5 ± 7.6	0.5 ± 0.2
0.50	28.0 ± 4.6	27.0 ± 4.5	0.9 ± 0.4
r^2	0.98	0.99	0.91

EO: electroosmosis. EM: electromigration.

Moreover, there was linearity between the results obtained for the different current intensities and EO, EM and total fluxes, as can be observed in Table 16. There were significant differences between the flux of EM and EO ($p < 0.05$). As the results show, EM flux was more than 30 times higher than EO flux. These results show that OLZ transport across the skin was governed by EM; thus, it was dependent mainly on the charge of the molecule. These were logical results, as OLZ had two positive charges at pH 5.5, and these charges made the drug an excellent candidate for the iontophoresis enhancer technique.

The feasibility studies described above were performed using aqueous buffer solutions. However, transdermal iontophoresis devices for clinical use are microprocessor systems containing a drug reservoir in a polymeric matrix or a TS matrix. PVP, HPMC and HEC gels formulated with olanzapine were developed in order to be easily used as a drug reservoir within a TS. The PVP matrix TS we developed were thin, transparent and non-adhesive in a dry state, only becoming bioadhesive in the presence of water, and remained adhered to the skin throughout the assay.

After formulating the different gels and TS, the amount of drug in the formulation was determined. In the PVP, HEC and HPMC these concentrations were

2.980 ± 0.046 mg/ml, 3.030 ± 0.055 mg/ml and 2.964 ± 0.063 mg/ml, respectively. The concentration of OLZ in the TS was lower than in the gels: 0.422 ± 0.009 mg/cm^2.

In the passive permeation studies of the formulations, OLZ was not detected in the receiving compartment, but small quantities were deposited in the skin (Table 17), There were not significant differences between the deposited amount in the skin obtained with the OLZ solution and with the formulations ($p > 0.05$).

Table 17. Accumulative amount of olanzapine in receptor compartment at the end of the experiments (μg/cm^2), olanzapine amount deposited in the skin (μg/cm^2), delivery efficiency (%) and transdermal flux of olanzapine across skin (μg/cm^2/h). (Mean ± SD; n = 3).

		Current (mA/cm^2)	Cumulative amount (μg/cm^2)	Deposited in skin (μg/cm^2)	Efficiency (%)	Flux (μg/cm^2/h)
Solution		-	-	47.91 ± 1.78	2.40 ± 0.09	-
		0.2	120.51 ± 71.82	104.02 ± 20.94	11.23 ± 4.35	16 ± 4
		0.4	178.12 ± 55.88	143.93 ± 42.49	16.10 ± 3.65	22 ± 8
		0.5	212.51 ± 66.26	197.55 ± 85.62	20.50 ± 4.85	28 ± 5
Gel	PVP	-	-	21.66 ± 4.61	1.44 ± 0.31	-
	PVP	0.5	156.65 ± 47.68	58.65 ± 31.80	10.77 ± 1.10	20 ± 5
	HPMC	-	-	28.48 ± 15.91	1.41 ± 0.65	-
	HPMC	0.5	181.02 ± 120.79	155.75 ± 64.88	17.71 ± 4.84	24 ± 4
	HEC	-	-	14.59 ± 7.90	0.90 ± 0.42	-
	HEC	0.5	128.82 ± 51.15	361.54 ± 198.95	17.76 ± 6.74	28 ± 8
TS	PVP	-	-	2.31 ± 3.28	0.55 ± 0.78	-
	PVP	0.5	23.66 ± 8.22	19.33 ± 8.52	10.77 ± 1.10	1.93 ± 0.6

TS: transdermal system; PVP: polyvinylpirrolidone; HPMC: hydroxypropyl methylcellulose; HEC: hydroxyethyl cellulose.

We did not observe statistically significant differences between the content of OLZ in the solution and in the formulated gels ($p > 0.05$). In contrast, the TS contained less amounts of OLZ, though differences with respect to efficiency when a 0.5 mA/cm^2 current density was applied were not statistically significant ($p > 0.05$). In addition, when we compared the flux obtained with the different formulations, there were statistically significant differences between PVP-TS and the rest of the formulations in the same conditions ($p < 0.05$).

Lastly, when we compared the amounts of OLZ accumulated in the receptor compartment with the amounts deposited in the skin after the *ex-vivo* studies performed with the different gels, no statistically significant differences were detected. between the permeated amounts or the deposited amounts of OLZ on the skin.

In 2013 Aggarwal *et al* developed a matrix transdermal system for the administration of OLZ. This transdermal system contained Eudragit® as a TS and natural oils as chemical enhancers [150]. In *ex-vivo* conditions, the fluxes obtained were similar to the fluxes that we have obtained with the different gels plus iontophoresis. After these studies Aggarwal *et al* performed some *in-vivo* pharmacological and pharmacokinetic studies with mices and rabbits, respectively.

Chapter 8: Discussion

1. Ocular administration

The most frequent pharmaceutical form for ocular administration of drugs is eye drops, but this dosage formulation normally has poor drug ocular absorption due to low permanence time in contact with the eye [3]. This problem is resolved by increasing the frequency of administration, but this can lead to poor patient adherence to the treatment. Ocular inserts are a non-invasive alternative to this problem [15]. These pharmaceutical delivery systems increase the permanence time of the drug in contact to the ocular surface, reducing the frequency of administration and resulting in better patient compliance [31].

One of the most common eye diseases is conjunctivitis, and **Moxifloxacin** is one of the drugs used for its treatment. Currently, it is commercialized as eye drops (Vigamox®). This thesis studies the possibility of developing a soluble ocular insert with moxifloxacin for its ocular administration. To characterise this insert, *ex-vivo* permeation studies were performed. Rabbit corneas were selected as a model, because they are easy to obtain and are the most commonly used animal model for *in-vivo* ocular studies. It can be difficult to obtain rabbit eyes daily, so some authors have used frozen rabbit corneas in their studies [101]. Furthermore, it is extend demonstrated in *ex-vivo* experiment performed with other membranes, like skin [171]. We analysed the viability of rabbit corneas preserved under different conditions as a membrane for use in *ex-vivo* permeation experiments.

Figure 34 shows statistical differences in the permeation of moxifloxacin through corneas preserved under three different conditions. As described previously, in Chapter 4, there were statistically significant differences between fresh corneas and the two groups of frozen corneas ($p < 0.05$).

Other authors have performed ocular experiments with fresh and frozen corneas, and obtained no significant differences between them [101]. However, they affirmed that, for small and hydrophilic molecules, such as moxifloxacin, the freezing procedure could affect permeation of the drug. Furthermore, our experiments lasted 3 hours, while the aforementioned group recorded data after 2 hours and their experiments lasted

24 hours. The duration of the experiment could be an important factor underlying differences between the final results of the two studies.

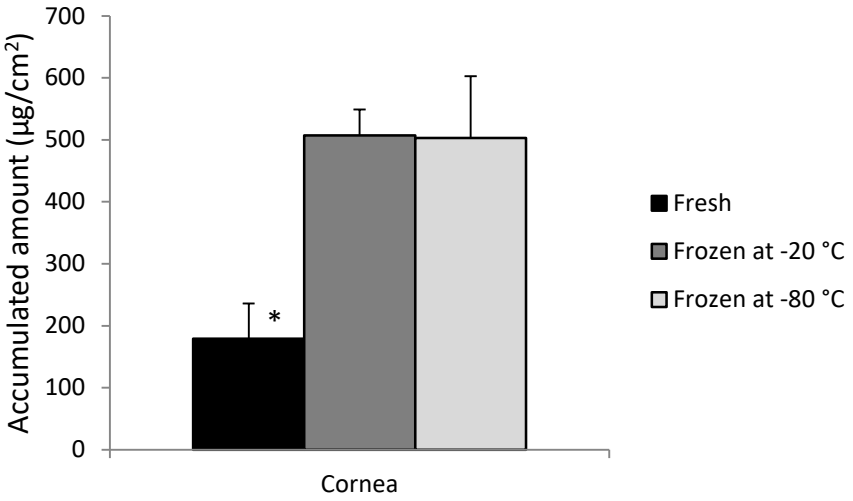


Figure 34. Accumulated amount of moxifloxacin obtained in the receptor compartment at the end of the experiment (µg/cm^2) with corneas preserved under different conditions. (Mean ± SD; n ≥ 3) *p < 0.05.

The results obtained in this work are consistent with the structure of the principal barrier of the cornea - the epithelium - which is entirely composed of live cells [16] and is likely to be affected considerably during the freezing and thawing processes (Figure 20). This structure of the cornea is radically different to the skin barrier, the stratum corneum, which is composed of dead cells [60]. This is probably why freezing and thawing do not induce any significant structural change in the skin. In light of this previous data, fresh corneas were selected to perform the *ex-vivo* permeation ocular studies with moxifloxacin.

Three soluble ocular inserts containing moxifloxacin were formulated with hyroxypropyl methylcellulose (HPMC), polyvinylpyrrolidone K30 (PVP-K30) and polyethylenglycol (PEG) as bioadhesive polymers and glycerin as a plasticizing agent (Table 9). After physical evaluation, insert-3, which had the highest concentration of the three bioadhesive polymers used, was selected for the *ex-vivo* permeation experiments. This insert contained 172.86 ± 9.47 µg/cm^2 of moxifloxacin, was practically transparent, adhered easily to the moist ocular mucosa and was thinner than similar inserts developed by other authors [102,103,122].

At the end of the *ex-vivo* permeation experiment performed with an insert of 0.5 cm^2, the amount of moxifloxacin accumulated in the receptor compartment was 26.04 ± 3.21 µg/cm^2. Other authors have reported a concentration of 0.6681 ± 0.4980 µg/ml moxifloxacin in the aqueous humour of the human eye 60 minutes after ocular administration of a drop [124]. In our experiment the permeated amount after 60 minutes was 10.68 ± 4.13 µg/cm^2. Assuming an ocular volume of 6.5 ml, the permeated amount of the drug would be approximately 5.34 µg and this would give an approximate concentration of 0.82 µg/ml. Therefore, the soluble ocular insert developed in this study could achieve higher concentrations than those referred to in the pharmacokinetic information of current commercial eye drops.

Gluthatione (GSH) is an antioxidant that has potential to be used as a treatment for retinal degenerative diseases such as retinitis pigmentosa [56]. One of the aims of this thesis was to evaluate the possibility of administering GSH through the eye and to develop a formulation for its possible use.

First, the stability of GSH was studied with and without vitamin C and under different conditions. After comparing the stability of the two solutions in the different conditions assayed, the optimal preservation conditions proved to be 4 °C, at which temperature the solution was stable for one month. Vitamin C did not have a bearing on the preservation of GSH in any of the conditions studied. When vitamin C was added to the solutions preserved at 25 °C in light or darkness the degradation of GSH increased. This could be because GSH and vitamin C are both antioxidants that physiologically work together [56] so that, in this case, GSH preserved the vitamin C, but more studies would be needed to test this hypothesis.

Two different liquid formulations (eye drops) and one semisolid insert containing GSH were prepared. The eye drops were formulated in phosphate saline buffer at pH 6.5 with two different concentrations of GSH: 5 mg/ml and 10 mg/ml. The semisolid insert was prepared with HPMC, PVP-K30 and PEG as bioadhesive polymers and glycerin as a plasticizer, and with a GSH concentration of 10 mg/ml.

We performed permeation studies for our formulations with cornea and sclera. We used both membranes in these *ex-vivo* experiments because the target tissue was

the retina, which is separated from the sclera by choroid [50], and so it was important to analyse permeation through this latter membrane.

Figure 35 shows the accumulative permeated amount of GSH through cornea and sclera for our three formulations and shows statistically significant differences between the two membranes for all three formulations ($p < 0.05$). The amount of GSH permeating through sclera was higher than that permeating through cornea. This could be a consequence of the different structure of the two tissues [16]. The cornea is a complex tissue with different layers that have different affinity for hydrophilic and lipophilic molecules [23]. This increases the difficulty of the molecule to permeate through this tissue to the internal part of the eye. On the other hand, the sclera is composed of a simple layer of collagen fibres [23], and hydrophilic molecules, like those of GSH, penetrate easily through this tissue.

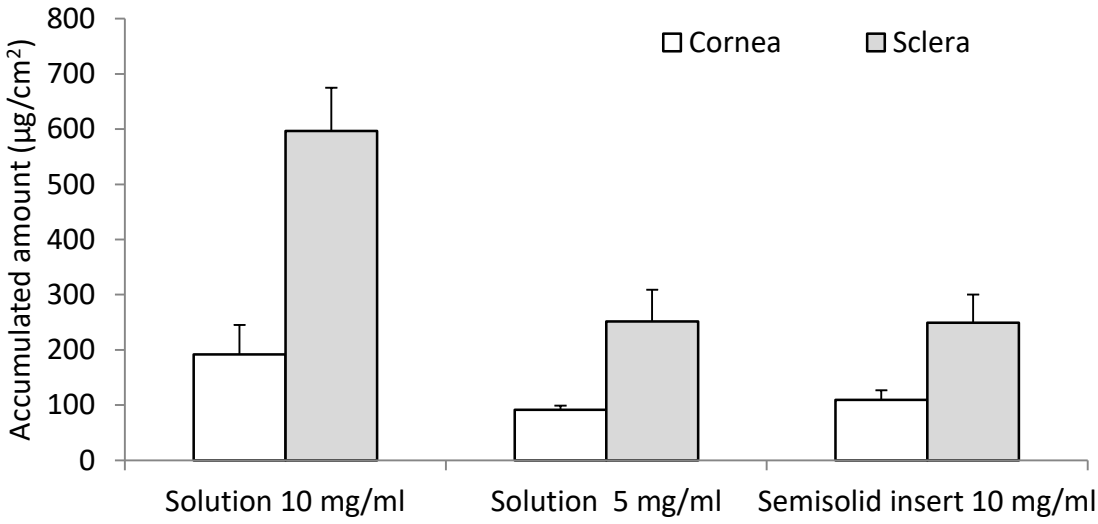


Figure 35. GSH accumulated amounts in the receptor compartment after 3 hours (µg/cm^2) in samples undergoing *ex-vivo* transcorneal and transsclera permeation studies performed with the different formulations. (Mean ± SD; $n \geq 3$).

In Figure 35 it is obvious that the highest permeated amount of GSH in the receptor compartment was obtained with the 10 mg/ml solution of GSH. No statistically significant differences were found in the amount of GSH accumulated in the receptor compartment between the 5 mg/ml solution and the semisolid insert with 10 mg/ml of GSH ($p > 0.05$). This could have been a result of the delivery process of GSH from the semisolid insert, before being absorbed by the eye.

Other research group in our university uses rd10 mice, as a model of retinitis pigmentosa, to study possible antioxidant treatments [178]. Collaboration with them could be possible to evaluate the efficacy of topical administration of GSH in this disease.

2. Transdermal administration

Transdermal drug delivery is used to administer drugs through the skin for a systemic therapeutic effect, and is employed as an alternative to oral, intravascular, subcutaneous and transmucosal routes [179]. Transdermal systems permit a transdermal systemic administration of drugs [1].

Olanzapine (OLZ) is an atypical antipsychotic drug used to treat schizophrenia and other mental disorders. The bioavailability of the drug after its first pass metabolism is approximately 60 % of the administrated dose [162]. This hepatic reduction of the drug could be avoided by a transdermal system (TS), and the reduction of administration frequency could improve patient compliance. Furthermore, this route is comfortable and non-invasive for the patient [76,163].

One of the objectives of this thesis was to analyse the feasibility of transdermal administration of OLZ as an alternative treatment for schizophrenia.

A high performed liquid chromatography method was validated to, for used in permeation studies. The method was specific and the response was accurate, precise and linear from 1 μg/ml to 100 μg/ml. The validated concentration range was suitable for quantification of olanzapine in samples from transdermal delivery studies.

Several passive absorption experiments were performed with porcine skin as the membrane and with a donor solution of 3 mg/ml of OLZ. After these experiments no permeated amount was observed in the receptor compartment (Figure 36).

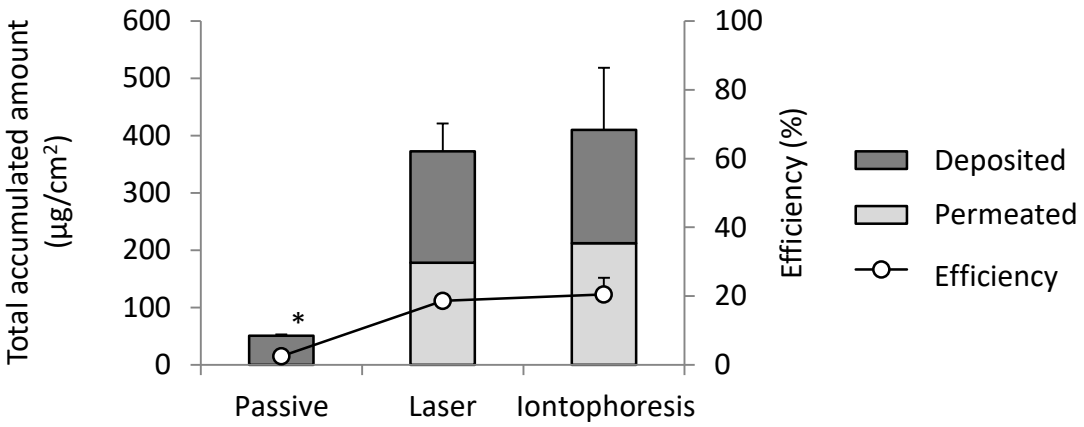


Figure 36. Total accumulated amount of olanzapine – the sum of the amount permeated across and deposited within the membrane- at the end of the experiment (µg/cm^2) asseessomg a solution of 3 mg/ml of OLZ, passively and using microporation and iontophoresis as enhancer techniques. (Mean ± SD; n ≥ 3) *p < 0.05.

Two different enhancer techniques were tested to increase this permeation of OLZ through the skin. Figure 37 shows microscopy pictures of intact skin, skin after microporation and skin after iontophoresis (0.5 mA/cm^2). The different effects of these enhancer techniques on the skin can be observed. The intact barrier of the skin (A), the hole made by microporation with the interruption of the integrity of the stratum corneum (B), and the effect of iontophoresis, with disruption of the stratum corneum after its application (C), can be seen.

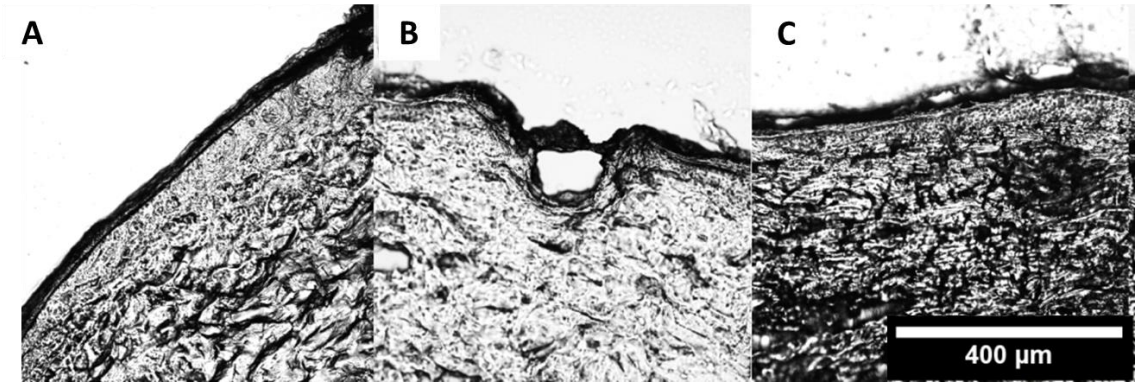


Figure 37. Skin photographs after use of different physical enhancer techniques. A: Intact skin. B: Microporated skin. C: Skin after 8 hours of iontophoretic treatment 0.5 mA/cm^2.

The results obtained after *ex-vivo* permeation experiments with an OLZ solution applied passively or under the effect of laser ablation (10 % pore density, fluency of

89.9 J/cm^2) or iontophoresis (0.5 mA/cm^2) are shown in Figure 36. No significant differences were observed in deposited amount, permeated amount or efficiency between the olanzapine permeation studies in which iontophoresis 0.5 mA/cm^2 was applied and those in which microporation was employed ($p > 0.05$). However, there were significant differences between the results obtained after passive assays and the ones performed with enhancers ($p < 0.05$). Moreover, both iontophoresis and microporation increased the total accumulated amount of OLZ 8- and 7-fold respectively. These results confirmed the appropriateness of these two enhancer techniques for increasing the transdermal administration of OLZ.

Other studies have compared the use of iontophoresis and that of different enhancer techniques whose aim is to overcome the barrier of the stratum corneum. For example, Abla *et al*. [174] compared the use of iontophoresis and tape-stripping for peptide administration and also found no differences between the two techniques.

Iontophoresis was selected as an enhancer technique for our assays because devices are available for personal administration [163] and because microporation needs to be performed by trained health workers, which could be annoying for the patient.

Different current densities were tested (0.5 mA/cm^2, 0.4 mA/cm^2 and 0.25 mA/cm^2). The total accumulative amount and efficiency of OLZ increased linearly as the current density rose ($r^2 = 0.999$; 0.992; 0.992). The inhibition factor obtained after comparison of the permeated amount of acetaminophen in the presence and absence of OLZ either 0.5 mA/cm^2 current density was 0.91. The electromigration flux was more than 30 times higher than the electroosmosis flux. This showed that iontophoretic transport is regulated principally by electromigration.

In addition, different formulations of olanzapine were prepared: three gels, each with a different polymer (PVP-K90, hydroxyethyl cellulose (HEC) and HPMC), and a TS of PVP-K90 (Table 15).

Ex-vivo permeation experiments were performed using these preparations alone or in conjunction with iontophoresis. Figure 38 shows that iontophoresis 0.5 mA/cm^2

significantly increased the permeated amount, deposited amount and efficiency of OLZ ($p < 0.05$).

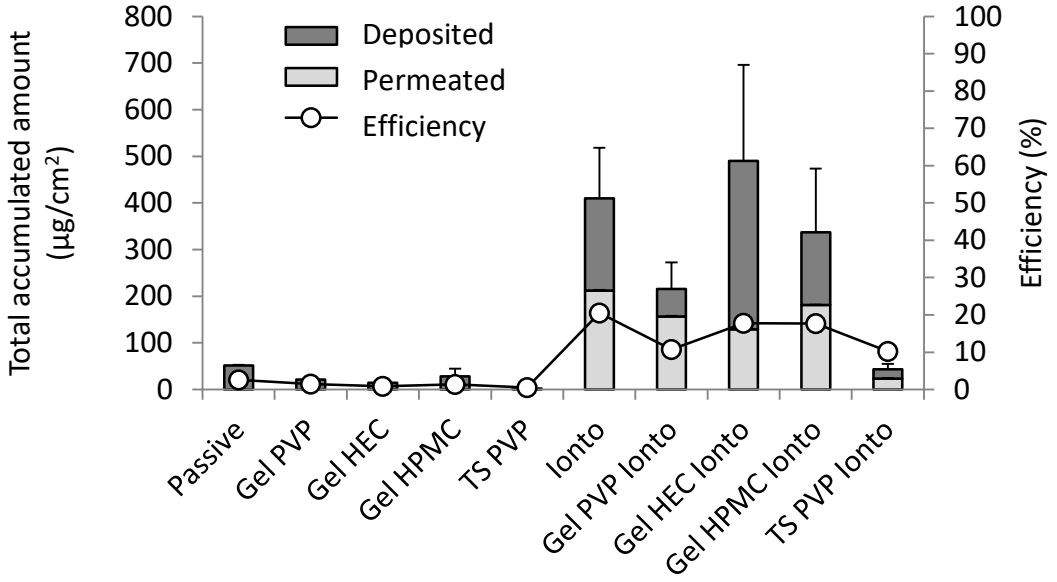


Figure 38. Total accumulated amount of olanzapine – the sum of the amount permeated across and deposited within the membrane- at the end of the experiment (µg/cm^2) assessing a solution of OLZ or the formulation containing OLZ, passively and using iontophoresis as an enhancer technique. (Mean ± SD; n ≥ 3).
TS: transdermal system; PVP: polyvinylpirrolidone; HPMC: hydroxypropyl methylcellulose; HEC: hydroxyethyl cellulose; Ionto: iontophoresis 0.5 mA/cm^2.

Although the solution and formulated gels of OLZ contained approximately the same amount of the drug and the TS contained a lower amount, no statistically significant differences between efficiency were found under the same administration conditions ($p > 0.05$).

In addition, statistically significant differences were not detected between the permeated amount and deposited amount of OLZ in the skin after the *ex-vivo* studies performed with the different gels and when iontophoresis was applied ($p > 0.05$).

The efficiency of OLZ increased approximately 7-fold in the case of the PVP-K90 gel, 12-fold in rather case of the HPMC gel, and more than 19-fold in that of the HEC gel and the PVP-K90 TS when applied with iontophoresis 0.5 mA/cm^2.

Other studies of transdermal administration of OLZ have been published by Aggarwal *et al*. In 2013, they developed a transdermal matrix system for the administration of OLZ that included Eudragit® as a polymeric film and natural oils as

chemical enhancers [150]. In *ex-vivo* conditions, the fluxes obtained were similar to those observed in the present study when the different gels were administered with iontophoresis. Aggarwal *et al*. performed subsequent pharmacological and pharmacokinetic *in-vivo* studies with this formulation, achieving promising data.

It can be concluded that the gels formulated in this study have produced promising results and encourage further investigation of the possibility of developing a reservoir TS for their administration in *in-vivo* studies applying iontophoresis to reach therapeutically plasmatic levels.

This thesis has studied three different drugs: moxifloxacin for the treatment of conjunctivitis, glutathione as an antioxidant treatment for retinal degenerative diseases, and olanzapine for the treatment of schizophrenia. Different drug delivery systems were developed for administration of the three drugs. The results obtained after ex-*vivo* permeation studies with the formulations were promising in the case of all three drugs. However, further *in-vivo* studies will be needed in order to ensure the efficacy of these formulations.

Chapter 9: Conclusions / Conclusiones

Conclusions

From the results obtained we can highlight the following conclusions:

1. It is possible to prepare a soluble ocular insert containing moxifloxacin for the treatment of conjunctivitis. The insert contains hydroxypropyl methylcellulose, polyvinylpyrrolidone K30 and polyethylenglycol as bioadhesive polymers and glycerin as a plasticizing agent. The insert is practically transparent, thin and adheres easily to the moist ocular mucosa.

2. In accordance with the pharmacokinetic information provided with the commercial formulation Vigamox® (eye drops of moxifloxacin), *ex-vivo* experiments performed with our ocular insert show that higher ocular concentrations of moxifloxacin can, theoretically, be achieved.

3. A glutathione solution (pH 6.5) can be preserved at 4 °C in darkness for one month. The incorporation of vitamin C into this solution does not lengthen its preservation.

4. Two solutions of glutathione (pH 6.5) and a semisolid ocular insert of glutathione containing hydroxypropyl methylcellulose, polyvinylpyrrolidone K30 and polyethylenglycol as bioadhesive polymers and glycerin as a plasticizing agent were prepared.

5. *Ex-vivo* permeation studies of the developed formulation showed that glutathione permeated through the cornea and sclera. Therefore, this formulation can be used as an antioxidant treatment in retinal degenerative diseases such as retinitis pigmentosa.

6. We have validated a HPLC method for determining olanzapine, a drug used in the treatment of schizophrenia, in samples obtained from *ex-vivo* permeation studies with pig skin. The method is specific and the response accurate, precise and linear from 1 µg/ml to 100 µg/ml.

7. In *ex-vivo* assays performed with pig skin, olanzapine is not detected in the receptor compartment. However, the application of iontophoresis and microporation as enhancer techniques favours the absorption of olanzapine accross the skin.

8. Iontophoresis was selected as an enhancer technique because it is easy for patients to apply. The iontophoresis transport of olanzapine is regularised by electromigration.

9. Different transdermal delivery systems with olanzapine were developed: gels with polyvinylpyrrolidone, hydroxypropyl methylcellulose, and hydroxyethyl cellulose, and a transdermal sytem with polyvinylpyrrolidone. When iontophoresis 0.5 mA/cm^2 was applied the best results were obtained with the hydroxyethyl cellulose gel.

Conclusiones

A partir de los resultados obtenidos, se pueden extraer las siguientes conclusiones:

1. Es posible preparar un inserto ocular soluble que contenga moxifloxacino para el tratamiento de la conjuntivitis. Este inserto contiene hidroxipropil metilcelulosa, polivinilpirrolidona K30 y polietlenglicol, como polímeros bioadhesivos y glicerina como agente plastificante. El inserto es prácticamente transparente, tiene un espesor reducido y se adhiere fácilmente a la mucosa ocular húmeda.

2. De acuerdo con la información farmacocinética de la formulación comercial Vigamox ® (colirio de moxifloxacino), y los resultados obtenidos en los estudios *ex-vivo* realizados con el inserto de moxifloxacino desarrollado, se podrían obtener teóricamente concentraciones oculares del fármaco superiores.

3. El glutatión en solución (pH 6.5) puede conservarse durante un mes a 4 °C y en oscuridad. La incorporación de vitamina C a esta solución no mejora su tiempo de validez.

4. Es posible elaborar dos soluciones de glutatión a pH 6.5 y un inserto ocular semisólido de glutatión compuesto por hidroxipropil metilcelulosa, polivinilpirrolidona K30 y polietilenglicol, como polímeros bioadhesivos, y glicerina, como agente plastificante.

5. Los ensayos *ex-vivo* de permeación realizados con las formulaciones desarrolladas muestran que el GSH permea a través de la esclera y la córnea y podrían

ser usadas como posible tratamiento antioxidante para enfermedades degenerativas de la retina, como la retinitis pigmentosa.

6. Se ha validado un método de HPLC para determinar la olanzapina, fármaco utilizado para el tratamiento de la esquizofrenia, en muestras obtenidas de experimentos *ex-vivo* de absorción a través de piel de cerdo. El método desarrollado es específico, exacto, preciso y lineal entre 1 µg/ml y 100 µg/ml.

7. En los ensayos *ex-vivo* realizados con olanzapina en piel de cerdo, no se detectó el fármaco en el compartimento receptor. Sin embargo, la aplicación de la iontoforesis y la microporación como técnicas promotoras favorece la permeación de la olanzapina a través de la piel.

8. De las técnicas estudiadas, se ha seleccionado la iontoforesis porque es más fácil su aplicación por parte del paciente El transporte de la olanzapina a través de la piel aplicando iontoforesis, está fundamentalmente regido por la electromigración.

9. Se han desarrollado diferentes sistemas de liberación transdérmica de olanzapina, geles de polivinilpirrolidona, hidroxipropil metilcelulosa y hidroxietil celulosa así como un sistema transdérmico de polivinilpirrolidona. Al aplicar estas formulaciones con iontophoresis 0.5 mA/cm^2, los mejores resultados se obtuvieron con el gel de hidroxietil celulosa.

Chapter 10: References

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