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EXPERIMENTAL AND COMPUTATIONAL OCULAR PHARMACOKINETICS WITH COCKTAIL ADMINISTRATION FOR OCULAR DRUG DEVELOPMENT

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Experimental and computational ocular pharmacokinetics with cocktail administration for ocular drug development
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ABSTRACT

Anterior eye diseases such as glaucoma, dry eye disease, inflammations, and infections can lead to visual impairment and blindness. Topical drug administration is the most common and convenient way of treating such diseases. However, the bioavailability of drugs after topical administration is low, typically less than 4% due to restrictions imposed by anatomical and physiological barriers. Understanding these barriers and the impact of the drug physicochemical properties on ocular pharmacokinetics is required to benefit drug development.

In this thesis, we aim to gain insight into ocular pharmacokinetics in albino rabbits after intracameral and topical administration of a drug mix (atenolol, timolol, betaxolol) and LC/MS-MS analytics. Our first aim was to quantitate the clearance of the intracamerally injected beta-blocking agents from aqueous humour (via iris-ciliary body blood flow and aqueous humour flow). We also wanted to analyse the impact of drug lipophilicity on clearance and volume of distribution after the intracameral administration. Our second aim was to quantitate the ocular bioavailability of three beta-blockers into aqueous humour after topical administration and see the impact of lipophilicity on ocular bioavailability. Our third aim was to quantitate the disposition of atenolol, timolol and betaxolol in tear fluid,

corneal epithelium, corneal stroma-endothelium, conjunctiva, sclera, vitreous humour and lens after topical administration. Also, the distribution of intracamerally injected drugs in aqueous humour and iris-ciliary body were measured. We wanted to quantitate the overall exposure of the three beta-blockers within the tissues and drug partitioning between different neighbouring tissues in the eye. We also wanted to investigate the impact of lipophilicity on drug dispositioning.

Cassette dosing of several compounds in the same dose was a useful approach that enables obtaining data with a smaller number of animals. This approach also reduces the inter-individual variation of pharmacokinetic data. Increasing lipophilicity enhanced drug clearance from aqueous humour over a 5-fold range (betaxolol > timolol > atenolol) and the relative impact of aqueous humour flow-mediated elimination decreased from 50% to 10% with increasing lipophilicity of the drug. The volume of drug distribution tends to increase at higher lipophilicity. Lipophilicity favours the corneal route of topical drug absorption, whereas more hydrophilicity favours the non-corneal route of absorption. Overall, ocular bioavailability increased substantially with increasing lipophilicity (0.07-3.82%). Increased lipophilicity may lead to increased drug exposure in all tissues except tear fluid. Partitioning of the drugs between neighbouring ocular tissues was logically dependent on the lipophilicity of the drug and the lipid content of the tissues.

The methodology and the data obtained from this approach can be useful in building predictive *in silico* models, favouring the 3R principles in animal testing and speeding up ocular drug development.

Medical Subject Headings: Administration, Ophthalmic; Aqueous Humor; Atenolol; Betaxolol; Biological Availability; Drug Development; Drug Elimination Routes; Eye; Eye Diseases; Gas Chromatography-Mass Spectrometry; Pharmacokinetics; Rabbits; Timolol

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Silmälääkekoktailin kokeellinen ja laskennallinen farmakokinetiikka silmälääkkeiden kehityksessä
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TIIVISTELMÄ

Silmän etuosan sairaudet, kuten glaukooma, kuivasilmäsairaus, tulehdukset ja infektiot, voivat johtaa näön heikkenemiseen ja sokeuteen. Paikallinen lääkkeiden antaminen silmän pintaan tippoina on yleisin tapa hoitaa tällaisia sairauksia. Silmätippoina annettujen lääkeaineiden biologinen hyötyosuus silmän etuosan kudoksiin on alle 4 % anatomisten ja fysiologisten esteiden asettamien rajoitusten takia. Silmän farmakokinetiikan ymmärtäminen auttaa uusien silmälääkkeiden kehitystä.

Tässä väitöskirjassa tutkimme silmän farmakokinetiikkaa albiinokanissa käyttäen lääkeaineiden seosta (atenololi, timololi ja betaksololi). Lääkeaineet annettiin kanien silmiin intrakameraalisesti ja silmätappoina. Lääkeaineiden pitoisuudet analysoitiin LC/MS-MS-analytiikan avulla. Ensimmäinen tavoite oli määrittää intrakameraalisesti injektoitujen lääkeaineiden puhdistuma kammionesteestä värikalvon ja sädekehän verenkierron sekä etukammionesteen virtauksen mukana. Toinen tavoite oli määrittää tutkittavien lääkeaineiden biologinen hyötyosuus silmän kammionesteessä silmätipan annon jälkeen. Kolmas tavoite oli tutkia atenololin, timololin ja betaksololin jakautuminen kyynelnesteeseen, sarveiskalvon epiteeliin, stroomaan ja endoteeliin, sidekalvoon, kovakalvoon, lasiaiseen ja linssiin silmätipan annon jälkeen. Myös intrakameraalisesti ruiskutettujen lääkeaineiden pitoisuudet kammionesteessä ja värikalvo/sädekehässä mitattiin. Lääkeaineiden

kokonaisaltistus kudoksissa ja jakautuminen vierekkäisten kudosten kesken määritettiin.

Useiden lääkeaineiden anto samassa liuoksessa silmään oli hyvä menetelmä, joka mahdollisti tiedon saamiseen pienemmällä joukolla kaneja, samalla vähentäen tulosten vaihtelua, kun samoista eläimistä määritetään monen lääkeaineen pitoisuus. Yhdisteiden lipofiilisyys paransi lääkeaineiden puhdistumaa kammionesteestä yli 5-kertaisesti (betaksololi > timololi > atenololi). Samalla kammionesteen virtauksen osuus eliminaatiosta pieneni 50 %:sta 10 %: in. Lipofiilisyys suurensi myös lääkeaineen jakautumistilavuutta silmän etuosassa. Lipofiilisyys parantaa lääkeaineen imeytymistä sarveiskalvon läpi tipan annon jälkeen, kun taas hydrofiilisyys korostaa sidekalvon ja kovakalvon kautta imeytymistä. Lääkeaineiden biologinen hyötyosuus etukammioon tippojen annon jälkeen parani lipofiilisyyden myötä (0,07–3,82 %). Lipofiilisyys paransi lääkealtistusta kaikissa silmän kudoksissa paitsi kyynelnesteessä. Lääkeaineiden jakautuminen vierekkäisten kudosten välillä riippui loogisesti yhdisteen lipofiilisyydestä ja kudosten lipidipitoisuudesta.

Tutkimuksessa kehitetyt menetelmät ja saadut tulokset auttavat laskennallisten mallien kehittämistä silmän farmakokinetiikkaan. Näin ollen menetelmät vähentävät koe-eläinten tarvetta ja nopeuttavat silmälääkkeiden kehitystä.

Yleinen suomalainen ontologia: biologinen aktiivisuus; farmakokinetiikka; kani; lääkeaineet; lääkehoito; lääkesuunnittelu; lääkkeen vapautuminen; silmät; silmätaudit

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Bonn, Germany 2022
Anam Fayyaz

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Fayyaz A, Ranta VP, Toropainen E, Vellonen KS, Ricci GD, Reinisalo M, Heikkinen EM, Gardner I, Urtti A, Jamei M, Del Amo EM. Ocular Intracameral Pharmacokinetics for a Cocktail of Timolol, Betaxolol, and Atenolol in Rabbits. Mol Pharm. 2020 Feb 3;17(2):588-594.

II Fayyaz A, Ranta VP, Toropainen E, Vellonen KS, Valtari A, Puranen J, Ruponen M, Gardner I, Urtti A, Jamei M, Del Amo EM. Topical ocular pharmacokinetics and bioavailability for a cocktail of atenolol, timolol and betaxolol in rabbits. Eur J Pharm Sci. 2020 Dec 1;155:105553.

III Fayyaz A, Vellonen KS, Ranta VP, Toropainen E, Reinisalo M, Valtari A, Puranen J, Ricci GD, Heikkinen EM, Gardner I, Ruponen M, Urtti A, Jamei M, Del Amo EM. Ocular pharmacokinetics of atenolol, timolol and betaxolol cocktail: Tissue exposures in the rabbit eye. Eur J Pharm Biopharm. 2021 Sep;166:155-162.

CONTENTS

ABBREVIATIONS

AUC_{inf}	Area under the curve from 0 to infinity
AUC_{last}	Area under the curve from 0 to last sampling point
AH	Aqueous humour
BA	Ocular bioavailability
C_{max}	Maximum concentration peak
CL	Clearance
HBD	Hydrogen bond donor
IC	Intracameral administration
ICB	Iris-ciliary body
$k_{1/2}$	Half-life
Log $D_{7.4}$	Logarithm of octanol-water distribution coefficient 7.4
Log P	Octanol water partition (unionized molecule)
MRT	Mean residence time
PSA	Polar surface area
Pk	Pharmacokinetics
T_{max}	Time to Peak concentration
Top	Topical administration
TF	Tear fluid
Vd	Volume of distribution
VH	Vitreous humour
DME	Drug metabolizing enzyme

1 INTRODUCTION

Visual impairment is a condition resulting in loss of visual performance of the eye. In 2020, an estimate showed that 43.3 million people are blind and 295 million have moderate to severe visual impairment. The prevalence of moderate to severe impairment has increased by 2.5% between the periods 1990-2020. Moreover, by 2050, an estimate of 61 million people will be blind and 474 million will develop moderate to severe vision impairment (Bourne et al., 2021). Cataract is the main cause of blindness worldwide, but glaucoma, diabetic retinopathy, age-related macular degeneration and degenerative myopia are becoming increasingly important causes of blindness (Adamsons and Taylor, 1990; Klaver et al., 1998; Pascolini and Mariotti, 2012).

Glaucoma is one of the major causes of eye disease with an estimated 79.6 million cases in 2020 that will increase to 111.8 million in 2040 (Quigley and Broman, 2006; Tham et al., 2014). Glaucoma is an irreversible process characterized by progressive degeneration of retinal ganglion cells resulting in a cupping of the optic disc leading to visual loss (Tham et al., 2014; Weinreb and Khaw, 2004). The underlying risk factors are old age, family history, African ethnicity, systemic or topical corticosteroid use and high intraocular pressure (Weinreb et al., 2014). Even though raised intraocular pressure is not the main cause of glaucoma, eye pressure reduction pharmacologically and surgically (i.e., shunt placement in the trabecular meshwork) are the main treatment methods of glaucoma (Boland et al., 2013; Spiegel and Kobuch, 2002). The initial target is to reduce intraocular pressure by 20-50% (Weinreb et al., 2014). Several classes of drugs are used for the treatment of glaucoma, including prostaglandins by increasing trabecular meshwork outflow facility (Bahler et al., 2008), whereas beta-adrenergic blockers (Trope and Clark, 1982), alpha-adrenergic agonists (Arthur and Cantor, 2011) and carbonic anhydrase inhibitors increase the outflow by acting on ciliary body and decreasing aqueous humour production (Becker, 1954).

Other anterior eye diseases include keratitis or corneal scarring caused by corneal infections. Viral infection is the leading cause of corneal ulcers in developing countries, but a fungus, bacteria, and parasites such as acanthamoeba can be causative agents as well (Garg and Rao, 1999). Treatments include different classes of drugs including fluoroquinolones and anti-fungal agents (Agarwal et al., 2001; Gangopadhyay et al., 2000). In addition, there are diseases, such as dry eye disease or keratoconjunctivitis sicca where approximately 20% of the adults above the age of 45 are enduring symptoms of dry eye (Brewitt and Sistani, 2001). Dry eye disease is an anterior eye disease defined by a disorder of the tear film, due to either tear deficiency or excessive tear evaporation. Dry eye disease results in damage to the ocular surface leading to symptoms of discomfort (Gayton, 2009; Lemp and Foulks, 2007).

Topical administration is the most common route of administration, and it is practiced at home. Instillation of eye drops is difficult for some patients and patient compliance in pharmacological glaucoma treatment is only about 50 % (Nordmann et al., 2010). Understanding the ocular absorption of topically administered drugs from eye drops is important for the development of novel therapies and the optimal use of existing medications. The anatomical and physiological barriers in the eye limit drug delivery to their ocular target sites. After topical administration drug bioavailability is only a few percent in the aqueous humour (Yamamura et al., 1999). In contrast, higher ocular exposure can be achieved with intraocular injection as it bypasses absorption barriers through the eye (Agrahari et al., 2016; Yamamura et al., 1999).

An improved understanding of the ocular barriers and their interaction with drugs with different physicochemical properties is important for achieving optimal drug efficacy after topical administration. Such knowledge may lead to improved ocular drugs and delivery systems, and it will facilitate the development of predictive pharmacokinetic models for topical ocular drug administration, thereby accelerating the development of novel therapeutics. Nevertheless, due to the complexity of ocular pharmacokinetics, further research is required to understand overall ocular pharmacokinetics. This work focuses on generating pharmacokinetic data

and building up an understanding of the factors affecting the ocular pharmacokinetics of eye drops.

2 REVIEW OF LITERATURE

2.1 OCULAR DRUG ADMINISTRATION

Anatomically the eye can be divided into anterior and posterior segments (*Figure 1*). The preferred route of drug administration depends on the target tissue of drug treatment. The diseases of anterior eye tissues (e.g. glaucoma, keratitis, corneal ulcer, conjunctivitis) are treated with topical drug administration (Crampton, 2003; Hyndiuk et al., 1996; Junejo et al., 2013). However, direct injection of antibiotics into the aqueous humour is used for conditions such as postoperative endophthalmitis (POE) which otherwise can lead to severe visual impairment (Daien et al., 2016).

Posterior segment eye diseases cannot be treated with topical eye drops, because only sub-therapeutic drug levels are reached in the posterior tissues. Therefore, treatment of the retina and choroid requires more invasive techniques for drug delivery. Intravitreal injections are widely used for retinal drug delivery in the treatment of diseases such as age-related macular degeneration (AMD) (Cohen et al., 2013; Holz et al., 2015).

Subconjunctival injections are mainly used to treat anterior eye tissues (i.e., cornea, conjunctiva, anterior uvea). However, they have been investigated for retinal and choroidal drug delivery, with sub-optimal results since most of the injected drug is absorbed into the systemic circulation (Maddison et al., 2008; Nomoto et al., 2009). The different routes of drug administration are presented in *Figure 1*.

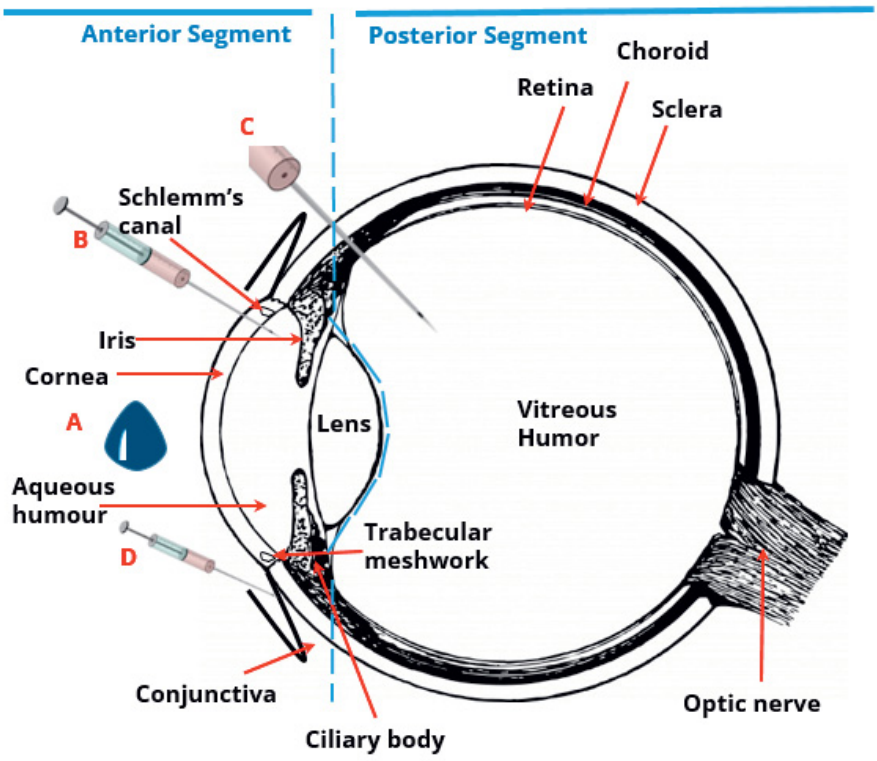


Figure 1. Ocular routes of administration. A: Topical; B: Intracameral; C: Intravitreal; D: Subconjunctival (the base figure was taken and modified from:https://www.publicdomainpictures.net/en/viewimage.php?image=130389&picture= medical-eye).

2.1.1 Topical administration

Topical administration is the most common route of ocular drug administration. It is convenient, non-invasive, and can be performed by patients without the need for medical assistance. The bioavailability of topically instilled drugs to the anterior chamber is only a few percentages of the dose. Anyway, the local administration results in ineffective drug concentrations in the anterior eye tissues with typical doses that are less than a milligram, whereas several orders of magnitude higher doses would be needed to achieve such concentrations after systemic drug administration. Such doses would lead to systemic adverse effects and, therefore, local administration is preferred. The onset of pharmacological

effects after topical administration is also faster than the one for systemic drugs (Group, 2000; Patel et al., 2013).

Drawbacks with topical administration include poor bioavailability in the posterior segment and low anterior bioavailability, especially for hydrophilic and high molecular weight drugs. The retention of eye drops after application to the ocular surface is often short and the drug is eliminated from the tear fluid typically within a few minutes (Chrai et al., 1973). Furthermore, frequent topical dosing is needed to maintain the therapeutic drug levels in the target tissues (Alvarez-Trabado et al., 2017; Yavuz and Kompella, 2016). To address some of these limitations different formulations have been developed to increase residence on the ocular surface to improve ocular bioavailability (e.g., ointments, suspensions, emulsions) (Patel et al., 2013).

Topical administration is used to treat mostly anterior eye segment targets as listed in *Table 1*. Target sites of action for this route of administration include different layers of the cornea, conjunctiva, sclera, and iris and ciliary body depending on the illness. A schematic representation of the command topical target tissues are presented in *Figure 2*.

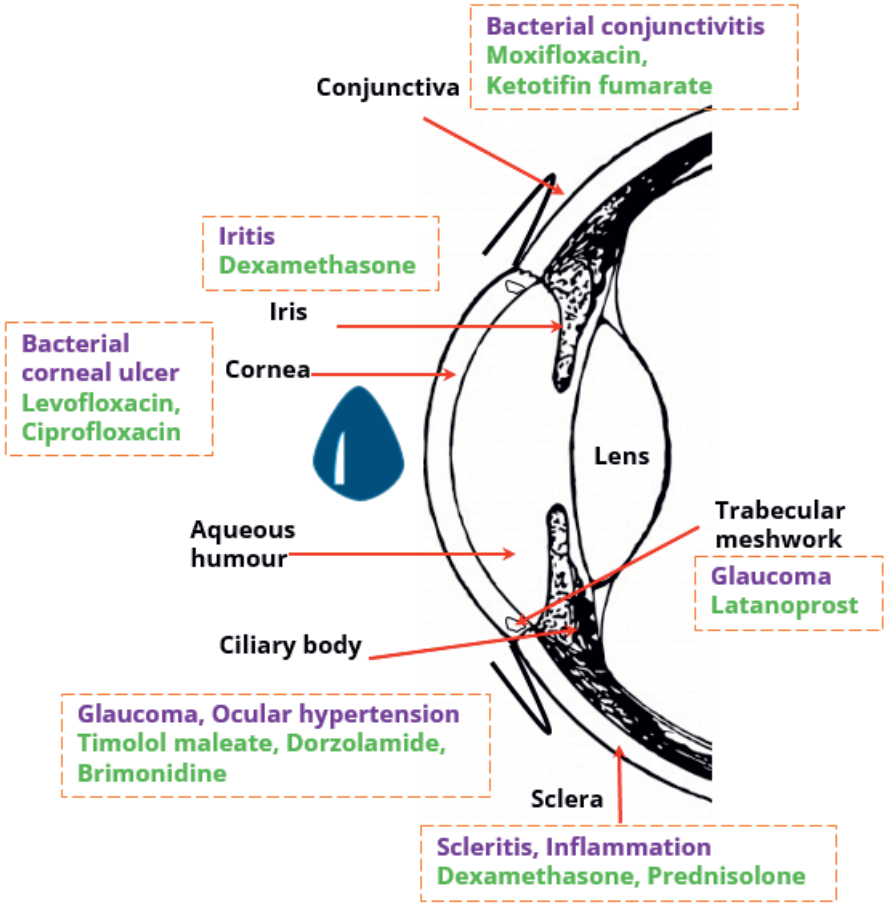


Figure 2. Schematic representation of the target sites of ophthalmic drugs (in green) to treat various illnesses (in violet) of the anterior segment of the eye. (The Base figure was taken and modified from: https://www.publicdomainpictures.net/en/viewimage.php?image=130389&picture= medical-eye).

Table 1. Clinical application of topical ophthalmic products.

Classification	*Active ingredient*	*Formulation and Concentration (%)*	*Indication*	*Reference*
Anti-bacterial	Besifloxacin	Suspension 0.5	Bacterial conjunctivitis	(Karpecki et al., 2009)
	Moxifloxacin	Solution 0.5	Bacterial conjunctivitis	(McDonald et al., 2009)
	Ciprofloxacin	Solution 0.3	Bacterial conjunctivitis, bacterial corneal ulcer, bacterial keratitis	(Hyndiuk et al., 1996)
	Levofloxacin	Solution 0.5-1.5	Bacterial corneal ulcer	(Hwang et al., 2003; Jensen et al., 2005; Suzuki et al., 2013)
	Gatifloxacin	Solution 0.3	Bacterial corneal ulcer	(Junejo et al., 2013)
	Gentamycin	Solution 0.3	Blepharitis, purulent dacryocystitis	(Huber et al., 1991)
	Azithromycin	Solution 1	Blepharitis/ Blepharoconjunctivitis, Dry eye	(Luchs, 2010; Nichols et al., 2012)
Anti-viral	Ganciclovir	Ointment 0.15	Herpes simplex dendritic keratitis	(Croxtall, 2011; Hoh et al., 1996)
	Acyclovir	Ointment 3	Herpes simplex dendritic keratitis, keratitis	(Hoh et al., 1996; Tsatsos et al., 2016)
	Cyclosporin	Emulsion 0.05	Dry eye disease	(Perry et al., 2008)
Beta-blocking agents	Timolol maleate	Solution 0.5	Glaucoma	(Barnebey et al., 2005)

Table 1. Clinical application of ophthalmic products.

Classification	*Active ingredient*	*Formulation and Concentration %*	*Indication*	*Reference*
	Carteolol hydrochloride	Solution 1-2	Glaucoma	(Baudouin and de Lunardo, 1998; Yamamoto, 2007)
	Levobunolol	Solution 0.5	Glaucoma	(Yablonski et al., 1987)
	Betaxolol	Solution 0.25-0.5	Glaucoma	(Allen et al., 1986)
Corticosteroid	Dexamethasone	Solution, suspension 0.1	Anterior Uveitis, Scleritis, inflammation	(Babu and Mahendradas, 2013; Cunningham Jr and Wender, 2010; Skjelbred and Løkken, 1982)
	Prednisolone acetate	Suspension 1	Anterior Uveitis, Scleritis, inflammation, dry eye disease	(Babu and Mahendradas, 2013; Beyazyıldız et al., 2014; Foster et al., 1996)
	Betamethasone	Solution 0.1-0.5	Anterior Uveitis, inflammation	(Babu and Mahendradas, 2013)
	Loteprednol etabonate	Solution 0.5	Dry Eye disease	(Sheppard et al., 2014)
Nonsteroidal anti-inflammatory drugs	Ketorolac tromethamine	Solution 0.5	Seasonal allergic conjunctivitis, post-operative inflammation	(Perry and Donnenfeld, 2006; Sandoval et al., 2006)

Table 1. Clinical application of ophthalmic products.

Classification	*Active ingredient*	*Formulation and Concentration %*	*Indication*	*Reference*
	Diclofenac sodium	Solution 0.1	Post-operative inflammation, photophobia, pain relief	(Asasutjarit et al., 2015; Colin and Paquette, 2006; Kraff et al., 1994)
	Bromfenac	Solution 0.09-0.075	Post-operative inflammation and pain relief	(Donnenfeld et al., 2007; Hoy, 2015)
Alpha agonist	Brimonidine	Solution 0.2	Open-angle glaucoma, ocular hypertension	(Adkins and Balfour, 1998)
	Apraclonidine	Solution 0.5	Open-angle glaucoma, ocular hypertension	(Toris et al., 1995)
Mast cell stabilizer	Cromolyn sodium	Solution 4	Vernal keratoconjunctivitis	(Avunduk et al., 2000; Foster and Duncan, 1980)
	Olopatadine hydrochloride	Solution 0.1	Allergic rhino conjunctivitis	(Gonzalez-Estrada et al., 2017; Katelaris et al., 2002)
	Ketotifen fumarate	Solution 0.025	Allergic rhino conjunctivitis	(Crampton, 2003)

Table 1. Clinical application of ophthalmic products.

Classification	*Active ingredient*	*Formulation and Concentration %*	*Indication*	*Reference*
Antihistamines	Epinastine hydrochloride	Solution 0.05	Allergic conjunctivitis	(Lanier et al., 2004)
	Azelastine	Solution 0.05	Allergic conjunctivitis	(Spangler et al., 2001; Williams et al., 2010)
Prostaglandin analogues	Latanoprost	Solution 0.005	Ocular hypertension, narrow-angle glaucoma	(Perry and Donnenfeld, 2006)
	Bimatoprost	Solution 0.03	Ocular hypertension, narrow-angle glaucoma	(Easthope and Perry, 2002)
	Travoprost	Solution 0.005	Ocular hypertension, narrow-angle glaucoma	(Denis et al., 2007)
	Unoprostone	Solution 0.12	Ocular hypertension	(Haria and Spencer, 1996)
Carbonic anhydrase inhibitor	Dorzolamide	Solution 2	Ocular hypertension, glaucoma	(Lass et al., 1998)
	Brinzolamide	Suspension 1	Glaucoma, ocular hypertension	(Silver and Group, 1998)

2.1.2 Intracameral administration

Intracameral administration is an invasive method of injecting the drug into the aqueous humour (*Figure 1*). Intracameral injections are used to administer antibiotics after cataract surgery (Barry et al., 2006; Shorstein et al., 2013). In this case, the bioavailability of the drug in the aqueous humour is 100%. Intracameral injection enables the use of very small doses, thus reducing corneal and systemic side effects. However, it is an invasive technique and can cause a sterile postoperative inflammatory reaction called toxic anterior segment syndrome (TASS) (Cetinkaya et al., 2014). It is caused due to infectious substances entering the anterior segment of the eye through the hole formed after the injection is performed (Braga-Mele et al., 2014; Jamil et al., 2014; Yu-Wai-Man et al., 2008).

2.2 TOPICAL OCULAR PHARMACOKINETICS

Topical ocular pharmacokinetics is the assessment of the time-dependent changes in drug concentration after topical instillation on the ocular surface and anterior tissues (Schoenwald, 2003). Following drug installation, some of the drugs will distribute through structural barriers such as the cornea, conjunctiva, blood-aqueous barrier, and ultimately will access the systemic circulation (Agrahari et al., 2016; Schoenwald, 2003). Understanding the nature of these barriers is important for predicting drugs ocular pharmacokinetics.

2.2.1 Barriers

Barriers in the eye are the physical and mechanical barrier i.e., tear fluid is a mechanical barrier and physical barriers including cornea, conjunctiva, and blood-aqueous barrier that will be briefly described in the following sections. They provide protection against exogenous compounds and microbes, but they are also crucial for the stability, dynamics, and homeostasis of the eye. However, these physical and structural barriers also

restrict the permeation of drugs into intraocular tissues after topical administration (Cholkar et al., 2013; Tomi and Hosoya, 2010).

2.2.1.1 Tear fluid

Tear fluid is a mechanical barrier that covers the outer surface of the eye. It provides immune protection, mechanical and environmental support to the cornea and conjunctiva. It gives a smooth and transparent refractive surface for vision (Dartt and Willcox, 2013). The dynamic balance of preocular tear film is maintained by tear production, absorption, evaporation and drainage. Total tear fluid volume is 7.5 µL and tear turnover is 0.47-0.66 µL/min in rabbits (Chrai et al., 1973) whereas in humans it is 6.6 -7.4 µL and 0.18- 0.52 µL/min respectively (Mishima et al., 1966).

Tear fluid is composed of lipid, aqueous and mucous layers. The outer lipid layer provides stability to the outer surface by controlling evaporation and surface tension of the tear fluid. It also acts as a tight seal when the eyelid is closed (Rantamäki et al., 2011). The aqueous layer consists of different proteins involved in corneal protection against pathogens, inflammatory processes and wound healing (De Souza et al., 2006). The innermost layer next to the cornea is a mucin-enriched mucous layer which maintains stability and wetting properties of the entire tear film (Rantamäki et al., 2011).

2.2.1.2 Cornea

The cornea is the main anatomical barrier to drug absorption after topical administration. The cornea is approximately 520 µm thick in humans, avascular, and devoid of lymphatic vessels. The cornea is composed of multiple layers including epithelium, epithelial basement membrane, bowman's layer, stroma, Descemet's membrane and endothelium (Barar et al., 2009; Fatt and Weissman, 2013) (*Table 2; Figure 3*).

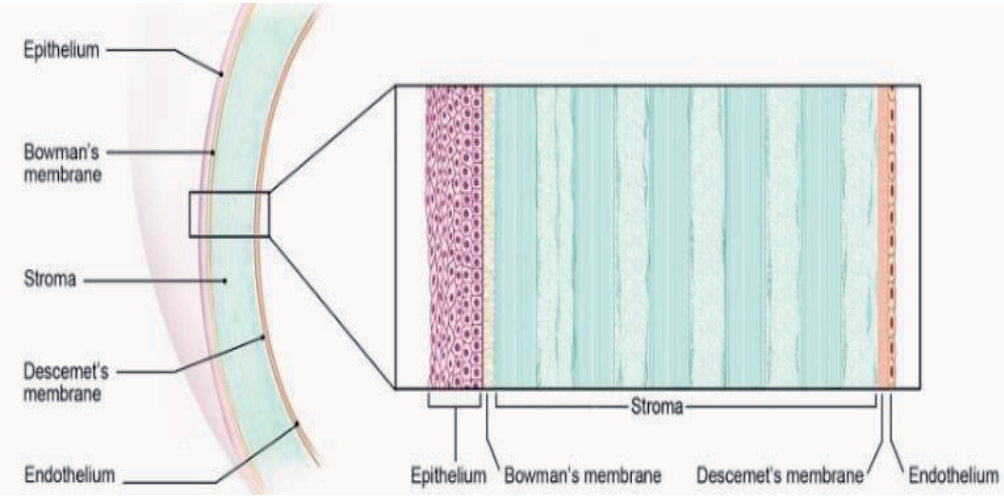


Figure 3. Anatomy of the cornea. The base figure was taken and modified from https://www.flickr.com/photos/nihgov/28173573525.

The human corneal epithelium is approximately 50-60 μm thick and consists of 5-7 cell layers that are unevenly distributed across the cornea (Reinstein et al., 2008). It makes up about 60% of total corneal resistance with trans-epithelial resistance (TER) of 3.3 ± 1.5 kΩ cm^2 (Klyce, 1972) or 7.5 ± 0.2 kΩ cm^2 reported both for rabbits (Marshall and Klyce, 1983). This 5–6 layer structure is composed of three different types of cell layers called superficial, wing and basal. The superficial layer consists of 2-3 layers (2-6 μm thick) with each cell shaped flat and polygonal. This layer acts as a watertight seal for preventing pathogenic organisms access to the cornea. Directly posterior is 2-3 layers of thawing cells with the tight junction between the cells and are named due to their wing like shape and hence called wing layers. At the bottom is the basal epithelial layer composed of a single sheet of columnar cells (Edwards and Prausnitz, 2001; Eghrari et al., 2015).

The basement membrane lays next to the epithelial layer and is primarily composed of collagen and laminins.

The bowman's layer is acellular and comes next to the basement membrane. It is composed of collagen and has more fibronectin compared to the stroma (Fatt and Weissman, 2013; Germundsson et al., 2012; Li et al., 1997; Schmoll et al., 2012; Taylor and Kimsey, 1981).

Stroma is the bulk of corneal tissue and is composed of collagen (type 1; 15 %), water (78%), keratocytes (3-5 %), noncollagenous proteins (5%), glycosaminoglycans (1%), and salts (1%). The main structural component of the stroma is collagen fibers which are present as stacks of 200 bundles running parallel to the surface (about 2 μm in thickness). The diameter is uniform, about 20-30 mm, and has a center spacing of 60 mm (Edwards and Prausnitz, 1998).

The descemet's membrane is about 8-10 μm in thickness and is a fibrous acellular layer adjacent to the endothelial cells (Johnson et al., 1982; Pavelka and Roth, 2010). The endothelium is a monocellular layer of hexagonal cells with 5 μm in thickness and 20 μm wide.

Table 2. Anatomy of the human corneal epithelium.

Layer	Thickness (μm)	Reference
Epithelium	50-60	(Reinstein et al., 2008)
Basement membrane	0.3	(Taylor and Kimsey, 1981)
Bowman's layer	13.7	(Germundsson et al., 2012)
Stroma	478-500	(Reinstein et al., 2009)
Descemet's membrane	8-10	(Johnson et al., 1982)
Endothelium	5	(Eghrari et al., 2015)

2.2.1.3 Conjunctiva

The conjunctiva is a membrane covering the non-corneal surface of the anterior eye globe until the posterior segment of the eyelids (Ramos et al., 2015). It acts as a barrier to the permeation of drugs into the intraocular tissues but is more permeable than the cornea (Ramsay et al., 2018). It has a rich blood supply and lymphatic vessels. The conjunctiva is divided into three parts: 1) the palpebral conjunctiva; 2) the bulbar conjunctiva; 3) the fornix (forniceal conjunctiva) that joins the palpebral and bulbar conjunctiva (*Figure 4)* (Ramsay et al., 2017; Shields and Shields, 2004).

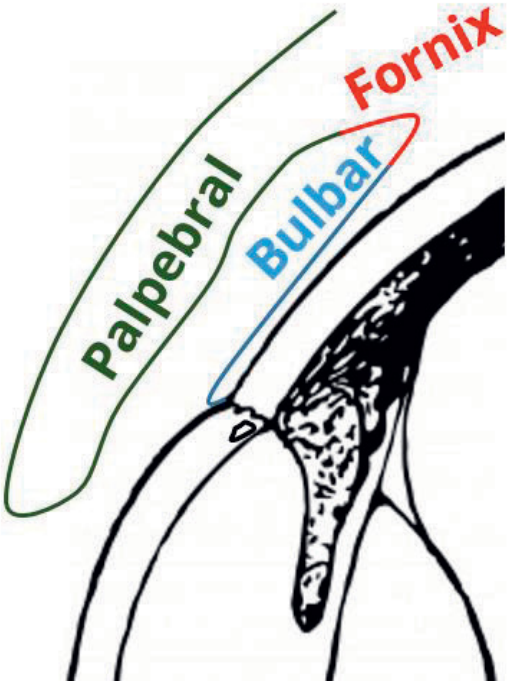


Figure 4. Anatomy of the conjunctiva. The base figure was taken and modified from (https://www.publicdomainpictures.net/en/view-image.php?image=130389&picture= medical-eye).

The palpebral conjunctiva lines the inner surface of the eyelid. The marginal tarsal arteries and descending branches of the peripheral tarsal arteries provide blood supply to the palpebral conjunctiva. The bulbar conjunctiva lines the surface of the globe from the anterior sclera onto the limbus which is a border between the transparent cornea and the opaque sclera (Lee and Holze, 1950; Spencer, 1985; Van Buskirk, 1989). The blood flow to this region in the human eye comes from the anterior ciliary arteries with a velocity of 0.12, 0.026 and 0.056 mm/sec across arterioles, capillaries and venules, respectively (Lee and Holze, 1950; Spencer, 1985).

The fornix conjunctiva (which does not constitute a real barrier) is a continuation of the palpebral conjunctiva and joins the bulbar conjunctiva. The peripheral tarsal arcade provides the blood supply to this part of the conjunctiva (Lee and Holze, 1950; Shields and Shields, 2004; Spencer, 1985).

The conjunctiva is composed of epithelium and stroma layers. The epithelium is composed of stratified squamous and columnar epithelial

cells. The columnar cells are found nearer to the fornix whereas the squamous cells are found nearer to the limbus. The stroma consists of fibrovascular connective tissues and is thin at the limbus and thick in the fornix (Huang et al., 1989; Spinak and Friedman, 1977). The lymphatic system is present in the conjunctiva with drainage taking place from the palpebral aperture into the deep lymphatic system (Shoukath et al., 2017). The TER value of exercised conjunctival epithelium is about 1300 Ω cm^2 for rabbits (Kompella et al., 1993) and 477 ±244 Ω cm^2 for porcine (Ramsay et al., 2017).

2.2.1.4 Blood-aqueous barrier

The blood-aqueous barrier is composed of the capillaries endothelia in the iris and ciliary body muscle, the posterior iris epithelium and the inner non-pigmented ciliary epithelium, which present tight junctions (Coca-Prados, 2014; Del Amo et al., 2017; Raviola, 1977) (*Figure 5*). The tight junctions limit the paracellular space diffusion between the cells of the barrier. However, it is important to notice that only the capillaries in the ciliary muscle (a component of the blood-aqueous barrier) have tight junctions while the ones in the ciliary processes are fenestrated (Hornof et al., 2005). Furthermore, iridial capillaries are continuous and form tight junctions as well (Alm, 1992). Both, iris ciliary muscle vessels and iridial vessels are impermeable to horseradish peroxidase (HRP) (40 kDa ∽ 2.5nm) in the rhesus monkey (Raviola, 1974).

The blood-aqueous barrier epithelial component (non-pigmented ciliary epithelium and posterior iris epithelium) have tight junctions and HRP does not permeate the non-pigmented ciliary epithelium in monkeys (Raviola, 1974; Smith and Rudt, 1975) and the posterior iris epithelium in humans (Tonjum and Pedersen, 1977).

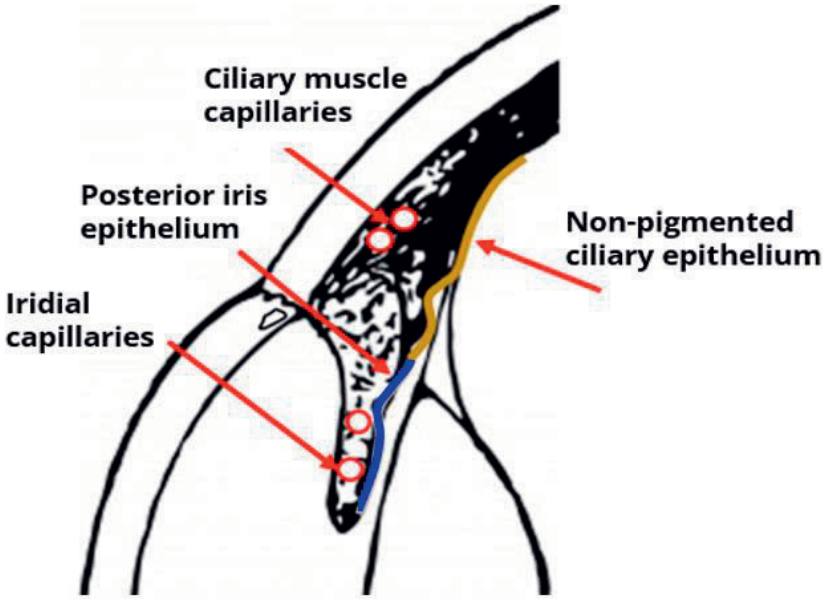


Figure 5. Blood-aqueous barrier components. The thick lines indicate the tight junctions in the epithelium and vascular endothelium. The Basic figure is taken and modified from (https://www.publicdomainpictures.net/en/viewimage.php?image=130389&picture= medical-eye).

The aqueous humour is secreted from the ciliary body epithelium processes into the posterior segment of the eye from where it en-routes to the anterior chamber (Friedenwald and Stiehler, 1938; Stewart and Tuor, 1994). The outflow of aqueous humour via trabecular meshwork to Schlemn's canal is 3-4.7 µL/min in rabbits (Conrad and Robinson, 1977; Schoenwald, 1990; Urtti and Salminen, 1993) and 2-3 µL/min in humans (Hornof et al., 2005; Worakul and Robinson, 1997). The long posterior ciliary artery and anterior ciliary artery are branched off from the major arterial circle of the iris providing blood to the iris and ciliary body (Kiel and Reitsamer, 2010). The blood flow of iridial and ciliary body vessels is between 31-65 µL/min and 62-105 µL/min respectively in rabbits (Bill, 1974; Koskinen and Bill, 1983; Nilsson and Alm, 2012; Thörig and Bill, 1986).

2.2.2 Topical pharmacokinetics

Topically applied drugs have to pass through several barriers to reach the intraocular tissues and undergo pre-corneal loss due to mechanisms such as drainage into tear ducts, conjunctival systemic absorption, or spillage onto the face. Drugs can be absorbed into the eye either through the corneal pathway or the non-corneal pathway (conjunctival-scleral pathway) after topical administration (Ahmed and Patton, 1985; Doane et al., 1978; Huang et al., 1983; Mishima, 1981; Schoenwald, 1987) presented in *Figure 6*.

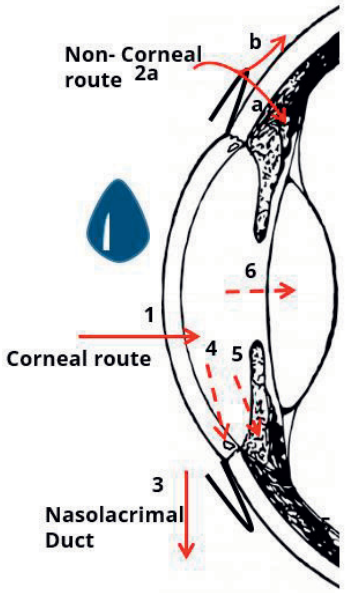


Figure 6. Drug absorption, distribution and elimination after topical administration. 1: corneal absorption; 2a: non-corneal absorption:conjunctival-scleral permeation into the ciliary body and iris, 2b) drug elimination via conjunctival absorption into conjunctival blood vessels reaching the systemic circulation; 3: tear flow and extra volume drainage into the nasolacrimal system; 4: clearance by the aqueous humour flow via the trabecular meshwork into Schlemm's canal; 5: distribution into the ciliary body and iris and the blood circulation therein; 6: distribution to the lens. Basic figure was taken from (https://www.publicdomainpictures.net/en/viewimage.php?image=130389&picture= medical-eye)

2.2.2.1 Precorneal factors

Normal tear flow (basal flow) is 0.53 μL/min (Chrai et al., 1973) whereas most of the applied topical ocular drug dose is eliminated from precorneal tear fluid via rapid drainage flow due to increased volume (volume of the droplet) into the nasolacrimal duct (*Figure 6*) (Agrahari et al., 2016; Djebli et al., 2017). There are different factors affecting the drainage flow of 0.545 1/min from the pre-corneal area: 1) At higher instilled volumes the relative drainage rate will increase (Chrai et al., 1973); 2) Increased viscosity prolongs the drug residence time in the pre-corneal area, but may sometimes lead to a local irritation (Sigurdsson et al., 2005; Zhu and Chauhan, 2008); 3) Eye drops with pH other than 7.4 cause excess tearing and faster drug loss from the precorneal area (Sigurdsson et al., 2005); 4) Deviations from isotonicity in the formulation may also lead to increased elimination rate from the lacrimal fluid (Agrahari et al., 2016). 5) Higher blinking rate causes an increase in drainage and vice versa e.g., rabbits due to a slower blink rate have slower drainage compared to humans (Bentivoglio et al., 1997; Chrai et al., 1973; Maurice, 1995).

2.2.2.2 Corneal absorption pathway

From tear fluid drugs can permeate through the cornea into the aqueous humour (*Figure 6*). Corneal absorption is the main pathway for ocular drug absorption, involving permeation across the corneal epithelium, stroma and endothelium. Each corneal layer causes different resistance to drug permeation, but the corneal epithelium is the dominant barrier, because it has inter-cellular tight junctions with a pore size of about 20 Å and inter-cellular porosity of about 10^{-7} (Chien and Schoenwald, 1990; Hämäläinen et al., 1997; Huang et al., 1983; Lach et al., 1983). The corneal epithelium contains about 90% of the total corneal cells and is highly lipophilic in nature.

Generally, small lipophilic molecules can cross the corneal epithelium relatively easily, but very hydrophobic drugs, hydrophilic drugs, and macromolecules have low permeability in this barrier. Corneal epithelium can act as a depot for very lipophilic drugs releasing them gradually to the stroma. In principle, the movement of drugs across the corneal epithelium

can take place via different pathways: 1) paracellular diffusion; 2) transcellular diffusion; 3) active transport; 4) facilitated diffusion; 5) receptor-mediated transport. However, paracellular and transcellular diffusion are the main modes of drug transport through this layer (Cholkar et al., 2013; Schoenwald, 1990; Zhang et al., 2004). Some examples of transporters present in the human corneal epithelium in the category of solute carrier transporters (SLC) are MATE (Multi-drug And Toxic Compound Extrusion), OATP (Organic Anion Transporter), PEPT (Peptide Transporter), OCT (Organic Cation Transporter), OCTN (Organic cation/carnitine transporter), OAT (Organic anion transporter), and NTCP (Na^+- taurocholate co-transporting polypeptide); and as ATP binding cassette (ABC) transporters (efflux transporters) are MRP (Multi-drug resistance-associated protein), P-gp (P-glycoprotein) and BCRP (Human Breast cancer resistance protein) (Gaudana et al., 2010; Krishna Vadlapatla et al., 2014; Nakano et al., 2014; Vellonen et al., 2018; Vellonen et al., 2010). Of the total gene profiled in the cornea, 68% are SLC (Dahlin et al., 2013). The impact of the transporters on trans-corneal drug absorption seems not relevant in drug absorption being of minimal impact in ocular pharmacokinetics (Vellonen et al., 2018).

The corneal stroma is an acellular hydrophilic matrix (Cholkar et al., 2013; Grass and Robinson, 1988; Prausnitz and Noonan, 1998), and the diffusion through the relatively porous stroma is usually not a rate-limiting step in corneal drug permeation.

The corneal endothelium monolayer is easy to permeate and has shown permeation to 4000 Da fluorescent molecules in ex-vivo human cells (Jumelle et al., 2016). Transcellular and paracellular both are important pathways for drug movement across this monolayer of cells (Prausnitz and Noonan, 1998).

The corneal permeability can be influenced also by formulation related factors (pH, drug ionization) (Brechue and Maren, 1993; Kidron et al., 2010). Weak acid and weak bases are affected by the pH as the un-ionized molecule fraction can permeate through the corneal membrane, for example, the permeability of pilocarpine across the corneal membrane decreased more than three times when pH was decreased from 7.65 to 5.5 (Suhonen et al.,

1998). Moreover, lipophilicity plays an important role in corneal permeability into the aqueous humour availability of drugs (Lach et al., 1983).

Once a drug reaches the aqueous humour it is eliminated from the anterior chamber by two different pathways: 1) clearance via aqueous humour outflow and 2) permeation firstly into the iris-ciliary body and afterwards permeating into the blood vessels entering blood circulation (Bill et al., 1980; Maurice and Mishima, 1984; Nilsson and Alm, 2012). The drug clearance via the aqueous humour outflow corresponds to 3-4 μL/min and is dependent on the physiology of the eye. The clearance via iris-ciliary body blood flow depends on the ability of the drug to permeate across the blood-aqueous barrier, presenting faster clearance values than 3-4 μL/min (equivalent to the aqueous humour outflow), for those that are more permeable (i.e. more lipophilic) (*Table 3*). To determine the drug clearance from the aqueous humour, pharmacokinetic studies after intracameral injection are required. Such studies can provide us with quantitative drug data concentrations in aqueous humour and clarify the role of the different routes of elimination. Even though some *in vitro* studies have suggested the presence of SLC drug transporters in the iris and ciliary body (e.g., OATs and MRPs) (Lee and Pelis, 2016), their overall impact on drug transport between aqueous humour and blood circulation is unclear and probably minimal. From the aqueous humour, drugs can also distribute the lens (Lee and Robinson, 2001).

Several drug metabolizing enzymes (DME) have been identified in the cornea and iris-ciliary body, including cytochrome P450, esterase, peptidases, alcohol and aldehyde reductases (Duvvuri et al., 2004). Even though CYP450 enzymes are the most important DMEs in hepatic metabolism, their expression in the eye and their impact are very low except for esterases (Del Amo et al., 2022; Lee, 1983; Lee et al., 1982a; Lee et al., 1982b; Nakano et al., 2014).

2.2.2.3 Non-corneal absorption pathway

After topical administration, the drug can penetrate the conjunctiva as well from tear fluid. However, the palpebral part of the conjunctiva is highly

vascularized and part of the absorbed drug is lost to the systemic circulation (Ramsay et al., 2017; Urtti, 2006). The fraction of drugs that escape the systemic circulation across the sclera following the route name non-corneal absorption pathway reaches the iris-ciliary body. This is the fraction that may have some therapeutic action in the inner tissues of the eye.

The conjunctiva is more permeable than the cornea (Ramsay et al., 2018). However, lipophilicity still plays a role in the rate of conjunctival permeability as about 100-fold difference in the permeability of hydrophilic sotalol (Log $D_{7.4}$ -0.6) and lipophilic betaxolol (Log $D_{7.4}$ 0.77) was determined (Saha et al., 1996). Even though the conjunctiva has SLC and ABC transporters which may facilitate drug transport across the conjunctiva, due to saturation of tissues with a high concentration of the drug, the impact of these transporters is insignificant (Chemuturi and Yanez, 2013).

The porosity of the conjunctiva is 13-fold larger than that of the cornea and the paracellular pathway through the conjunctiva is relatively less influenced by molecular size compared to the cornea. So, the paracellular pathway in the conjunctiva is a favored ocular absorption route for hydrophilic compounds and macromolecules (Hämäläinen et al., 1997).

The sclera is more permeable than the cornea and is about half as permeable as the conjunctiva. Polyethylene glycols at the molecular weight range of 200-1000 showed a decrease in permeability coefficients ($\times 10^{-6}$ cm^2/s) across rabbit cornea, bulbar conjunctiva, and sclera as follows: 1-0.05, 16-0.4 and 8-0.2. Moreover, sucrose (MW 342) permeates 16 times faster than inulin (MW 5000) through the sclera (Edwards and Prausnitz, 1998; Hämäläinen et al., 1997; Prausnitz and Noonan, 1998). From the sclera, the drug can enter the iris-ciliary body (non-corneal absorption rote). Due to low corneal permeability, hydrophilic compounds tend to have lower bioavailability in the aqueous humour than lipophilic drugs, and limited access to the iris-ciliary body via the aqueous humour (Doane et al., 1978; Urtti, 2006). Moreover, the distribution from the iris-ciliary body to the aqueous humour is not significant because of the limited permeation of drug across the iris-ciliary body to the aqueous humour (Ahmed and Patton, 1985; Doane et al., 1978). Interestingly, *in vivo* data may have suggested

there is an impact of the transporter in limiting the permeation of drug from the iris-ciliary body to aqueous humour.

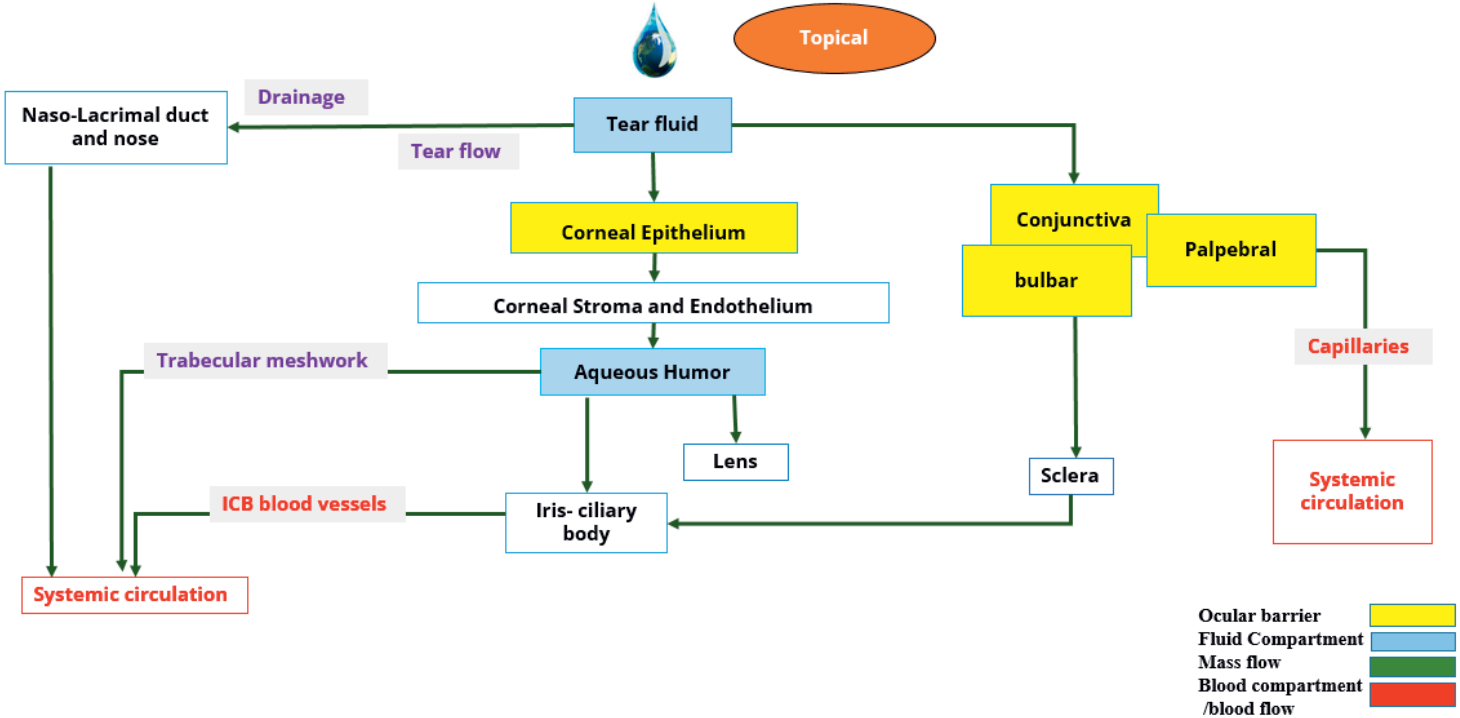


Figure 7. Ocular pharmacokinetics after topical administration of drug onto the ocular surface. ICB: Iris-ciliary body, Conj: Conjunctiva

2.2.3 Ocular bioavailability

To quantitate ocular bioavailability after topical administration it is important to know what percentage of drug gets clear from the aqueous humour. For such purposes, intracameral injection experiments are important to understand the pharmacokinetics of aqueous humour where 100% of the drug dose is available in the aqueous humour. Drug clearance after intracameral injection can be via aqueous humour outflow through trabecular meshwork or by permeating to iris and ciliary body onto elimination via blood flow within these tissues (Maurice and Mishima, 1984; Nilsson and Alm, 2012). Normal aqueous humour outflow is 3 µL/min in rabbits where drugs eliminated from this pathway are dependent on the physiology rather than physicochemical properties of the drug (Conrad and Robinson, 1977; Schoenwald, 1990; Urtti and Salminen, 1993). However, if the drug can easily cross the blood-aqueous barrier (for more detail see section 2.2.1.4) may show faster clearance values (*Table 3*).

Table 3. List of the compounds investigated in pharmacokinetic studies after intracameral injection into rabbit eyes.

Compound	Log P	Log $D_{7.4}$	Rabbit	CL_{IC} (µL/min)	References
Vancomycin	-2.00	-5.14	albino	5.04	(Kodjikian et al., 2010)
FITC-Dextran (MW 4400)	**	**	albino	7.63	(Yamamura et al., 1999)
Pilocarpine	0.39	0.24	albino	33.11	(Conrad and Robinson, 1977)
			albino	34.74	
			pigmented	35.83	
			albino	9.39	(Miller et al., 1980)
Timolol	1.53	-0.35	albino	25.33	(Yamamura et al., 1999)
Tilisolol	1.85	-0.27	albino	16.06	(Yamamura et al., 1999)

Inulin	- 62	-	albino	5.14	(Conrad and Robinson, 1977)
			albino	3.23	
			albino	5.60	
Voriconazole	1.39	1.39	albino	21.07	(Shen et al., 2009)

Hence, such pharmacokinetics experiments provide us with the quantitative value of drug clearance from the aqueous humour. Moreover, the ocular volume of drug distribution after intracameral injection can be determined and compared to the anatomical volumes of aqueous humour and surrounding tissues. This will give information about drug distribution and binding to the ocular tissues (Conrad and Robinson, 1977; Yamamura et al., 1999). Bioavailability can be determined based on pharmacokinetic data from both topical and intracameral drug administrations (*Figure 1*) and is known to be less than 4% after topical administration (Naageshwaran et al., 2020; Yamamura et al., 1999) (equation 1). The absolute ocular bioavailability is the ratio of the dose-normalized areas under the concentration curve (AUC_0 to ∞) in aqueous humour after topical (Top) and intracameral (IC) administration, respectively.

$$\boldsymbol{Aqueous\ humor\ bioavailability} = \frac{\boldsymbol{AUC_{Top} \times Dose_{IC}}}{\boldsymbol{AUC_{IC} \times Dose_{Top}}} \times \mathbf{100} \quad \text{Equation 1}$$

Direct comparison of drug concentrations in the aqueous humour after topical administrations does not inform about bioavailability, because the concentrations depend on both drug absorption into aqueous humour and its elimination from the anterior chamber.

2.3 OCULAR PHARMACOKINETICS IN DRUG DISCOVERY AND DRUG DEVELOPMENT

Drug discovery and development is an expensive and lengthy process usually lasting 12-15 years and costing up to 2.5 billion dollars (Hughes et al., 2011; Morgan et al., 2011). It involves different stages from identification to optimization of lead compounds following preclinical safety to

translational studies and then clinical stages. During clinical studies, the drug is tested in healthy volunteers within the initial phases and in patients during later phases (*Figure 8*) (Hughes et al., 2011; Tamimi and Ellis, 2009).

During the drug discovery phase, physicochemical properties of compounds are profiled and can be used to predict various absorption, distribution, metabolism and excretion (ADME) properties. Often, important parameters such as lipophilicity (partition and distribution coefficients), solubility, metabolizing enzyme, stability and pKa are considered for profiling in drug discovery (Arnott and Planey, 2012; Comer and Box, 2003). These parameters influence ocular tissue absorption and distribution depending on the route of administration and formulation. For passing through the cornea, the drug should have moderate lipophilicity, and also weak hydrogen bond potential, to have better access to corneal epithelium from tear fluid, and form hydrogen bonds with the stromal matrix when reaching the stroma (Kidron et al., 2010; Shirasaki, 2008). For suspensions, molecular weight and particle size can affect the absorption route to ocular tissues and decrease drug diffusion into the aqueous humour (Cunha-Vaz and Maurice, 1969; Thornit et al., 2010). In addition, drug ionization is an important factor affecting the solubility and permeability across the lipid layers of ocular tissues. Formulation having such pH producing a higher concentration of unionized drugs and can increase permeability. Furthermore, the nature of the charge may affect the movement of drugs through membranes. For example, cornea, conjunctiva and sclera are negatively charged above their isoelectric point and the paracellular movement of cationic species may be more favorable (Davies, 2000; Järvinen et al., 1995; Rojanasakul et al., 1992). The stability of the topical formulation depends on the pH, buffer system and packaging. The optimal stability for some drugs is lower than 5 pH. However, to avoid eye discomfort, buffers are added to ensure drug stability and pH change to 7.4 when the drop is instilled (Okafor, 2012). Ocular ADME has been investigated (Duvvuri et al., 2004; Nakano et al., 2014) and some activities have been observed for esterase, peptidases, alcohol dehydrogenase, CYP450, and aldehyde reductase enzymes. However, only esterases seem to play a relevant impact on ocular pharmacokinetics (Del Amo et al., 2022), some of the drugs are

relying on this enzymatic activity to be activated e.g., dipivefrin is a prodrug and is biotransform into epinephrine after topical administration (Anderson et al., 1980).

ADME features for the drug candidates are investigated at various stages during the drug development process using *in silico*, *in vitro* and *in vivo* models. In the case of ocular drug discovery, QSPR (Quantitative Structure Property-Relationship) and mechanistic models are reported in the literature predicting various pharmacokinetic related-properties or parameters such as corneal permeability, conjunctival permeability, vitreous humour clearance and humour volume of distribution among others, (Del Amo et al., 2015; Kidron et al., 2012; Kidron et al., 2010; Ramsay et al., 2017; Zhang et al., 2004). *In vitro* primary and immortalized corneal epithelial and endothelium models are present for studying drug transport, metabolism, permeability, irritation and absorption. *In vitro* conjunctival models provide similar information about the conjunctiva. Moreover, there are primary and immortalized retinal pigment epithelium and endothelium cell models present to assess the tight junctions and functioning of the barriers. In addition, permeability studies, toxicity and polarity studies are performed using cell cultures as well (Shafaie et al., 2016; Steinmetz and Spack, 2009).

It is important to understand the effect of drugs on a living organism and ensure the safety of selected drug candidates. *In vivo* pharmacokinetic studies are utilized in predicting the pharmacokinetics in humans (Brake et al., 2017). However, translation of results from preclinical species to humans is a major challenge and requires a quantitative understanding of both systems i.e. animal and human. In the case of ocular drug development, human ocular pharmacokinetic studies cannot be performed because it is an invasive procedure that requires ocular sampling. Therefore, such studies are done on animal models. The rabbit has been the model of choice for preclinical to clinical translation in ocular drug discovery and development (Del Amo and Urtti, 2015; Y Zernii et al., 2016). A full comparison between rabbit and human eye physiology is listed in *Table 4*.

Table 4. Comparison of anatomy and physiology of rabbit and human.

Pharmacokinetic components	Units	Rabbit	Human	References
Tear volume	μL	7.5	7	(Chrai et al., 1973; Mishima et al., 1966)
Tear turnover	μL/min	0.53	0.1-0.52	(Chrai et al., 1973; Mishima et al., 1966)
Nictitating membrane	**	Present	Absent	(Schoenwald, 2003)
The pH of tear fluid	**	7.3-7.7	7.3-7.7	(Worakul and Robinson, 1997)
Corneal thickness	mm	0.40	0.52	(Barar et al., 2009; Chan et al., 1983)
Corneal surface area	cm^2	1.5-2.0	1.04	(Watsky et al., 1988)
Aqueous humour volume	mL	0.25-0.3	0.1-0.25	(Conrad and Robinson, 1977; Reiss et al., 1984)
Aqueous humour turnover	μL/min	3-4.7	1.5-3.4	(Brubaker, 1982; Schoenwald, 2003)
Aqueous humour pH	***	7.52	7.1-7.3	(Lorget et al., 2016; Veselovský et al., 2001)
The ratio of corneal to conjunctival surface area	***	9	17	(Schoenwald, 2003)
Vitreous volume	mL	1.24-1.41	4	(Silva et al., 2017; Struble et al., 2014)
Choroidal blood flow	mL/hr	62	43	(Nilsson and Alm, 2012; Sebag et al., 1994)
Retinal blood flow	mL/hr	0.66	0.26	(Feke et al., 1989; Nilsson and Alm, 2012)
Iridial blood flow	mL/hr	3.72	1.02	(Alm, 1992; Nilsson and Alm, 2012)
Ciliary body blood flow	mL/hr	4.91	5.34	(Alm, 1992; Nilsson and Alm, 2012)

** no unit

During the drug discovery stage and early drug development, a high throughput screening approach is performed to evaluate the pharmacokinetic properties and choose the compounds more likely to succeed in future phases and speed up the discovery process. For small molecules, this usually involves high throughput screening against a target of interest with a large set of drugs. It is usually intended to eliminate compounds with poor pharmacokinetic properties (Bayliss and Frick, 1999; Smith et al., 2007; Workman et al., 1988).

For such instances, drugs are tested in various animal species where a single drug is given to animals at a time. As an alternative, cassette or cocktail dosing administration of several compounds simultaneously can be used. Cocktail dosing can significantly reduce the number of animals required for the experiments, consequently helping the 3 Rs (reduce, refine, replacement) concept. Moreover, work required for handling animals, sample preparation, and analysis is reduced as well as improving testing efficiency and reducing inter-individual variability (Bayliss and Frick, 1999; Workman et al., 1988). Typically, up to 10 compounds in the form of a mixture can simultaneously be administered to a single animal (Bayliss and Frick, 1999; Frick et al., 1998). However, cocktail dosing in ocular drug research has been only rarely utilized (Gale et al., 2005; Proksch and Ward, 2008), even though its application in the ocular pharmacokinetic field would be ideal because of the invasive nature of these studies.

The physiological-based pharmacokinetic model is a compartmental model where the compartment represents actual organ and tissue spaces with their actual physiological volumes. Moreover, mechanistic physiologically based pharmacokinetic models can integrate the parameters of the compounds and physiological components into an integrated model. Such models can predict pharmacokinetics (and in extended form also pharmacodynamics) of new compounds before experiments. They can be used for the prediction of formulation performance and they are important tools in inter-species translation (Aarons, 2005; Peters, 2012; Uchizono and Lane, 2007). Moreover, appropriate models such as *in silico*, QSPR (Quantitative Structure-Property Relationship) and non-compartmental

models could be utilized for optimal selection of effective compounds and their dosing regimens for the clinical trials (*Figure 8*) (Burman et al., 2005; Derendorf et al., 2000; Gomeni et al., 2001; Rajman, 2008; Thai et al., 2015). Ocular models currently in literature are compartmental, non-compartmental, population pharmacokinetic, infinite and classic pharmacokinetic models (Abduljalil et al., 2008; Causin and Malgaroli, 2016; Deng et al., 2016; Jooybar et al., 2014; Miller et al., 1981; Ranta et al., 2003; Zhang et al., 2000), but more comprehensive mechanistic models are still needed to facilitate drug development and reduce the number of animal experiments.

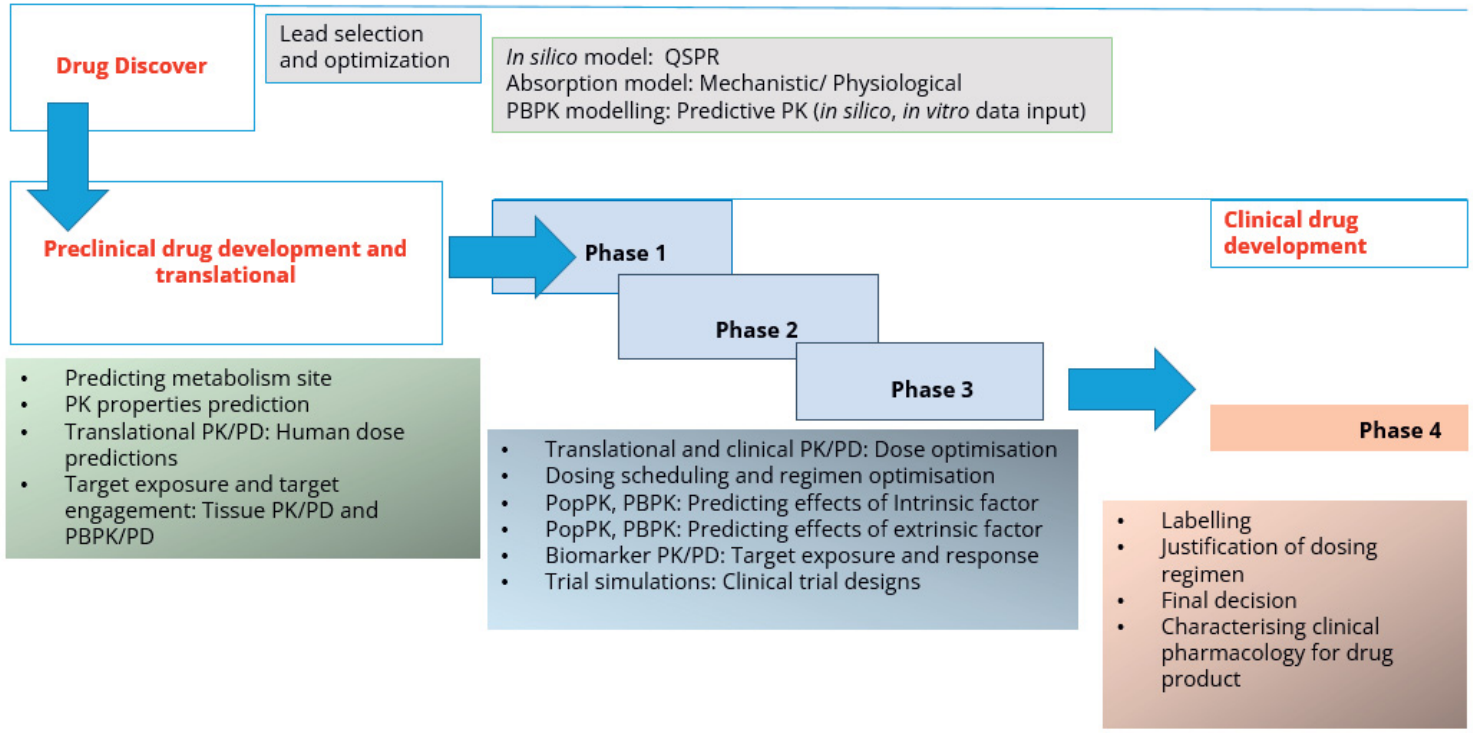


Figure 8. Pharmacokinetic aspects of drug development stages. PopPK: Population pharmacokinetics; PBPK: Physiological based pharmacokinetic models; PK: Pharmacokinetics; PD: Pharmacodynamics.

3 AIMS OF THE STUDY

The overall aim of the current study is to characterize the ocular pharmacokinetics of the three beta-blockers (atenolol, timolol and betaxolol) after topical and intracameral drug administration into rabbit eye, the pharmacokinetic data of these model drugs is of interest from the drug development perspective. The specific aims are as follows:

1) Determine ocular pharmacokinetics after topical and intracameral administration of a cocktail of atenolol, timolol and betaxolol in aqueous humour.

2) Analysis of the pharmacokinetic impact of lipophilicity after topical and intracameral drug administrations.

3) Quantitate intracameral clearance and volume of distribution of the drugs after intracameral administration.

4) Determine topical bioavailability of atenolol, timolol and betaxolol after topical administration.

5) Determining the distribution of the three drugs among the neighboring ocular tissues after topical and intracameral administrations.

4 SUBJECTS AND METHODS

The materials and methods utilized in publications I-III are described briefly in *Table 5* and full detail in the publications.

Table 5. Materials and methods.

In vivo method		
Description	Intracameral study (IC)	Topical study (Top)
Drugs	Atenolol, timolol and betaxolol	
Concentration (nM)	IC: 3mM (1mM beta-blocker each), Top: 40 mM (20mM atenolol, 10mM timolol, 10mM betaxolol)	
Formulation	Aqueous solution	
Buffer (pH)	Phosphate buffer with saline (7.4)	
Animals	Albino rabbits	
Number of animals	14	16
Route of administration	Injection	Instillation
The volume administered (µL/eye)	5	25
Time points of samples (minutes)	10, 20, 30, 60, 120, 240	5, 10, 20, 30, 60, 120, 240
Number of samples per time point	2-4	3-4
Tissues excised	Aqueous humour, iris-ciliary body	Tear fluid, corneal epithelium, corneal stroma-endothelium, bulbar conjunctiva, anterior sclera, iris-ciliary body, aqueous humour, lens, vitreous humour
Tissue analyses	Tissues were dissected and homogenized. The samples were prepared for LC/MS-MS analysis.	
Publication	I, III	II, III
Pharmacokinetic analysis (Phoenix WinNonlin)		

	Analysis method
Pharmacokinetics parameters	CA: Compartmental analysis, NCA: Non-compartmental analysis
	CA: Mean concentration for analysis. 1 and 2 compartment model selection based on AIC (Akaike information criterion), and objective function value. Model evaluation based on weighing schemes uniform, 1/concentration predicted (1/Yhat) and 1/ (concentration predicted)^2 (1/Yhat^2), visualization of residual plots, CV% and SD. NCA: Mean concentration and linear trapezoidal for analysis. Dose normalized concentration of IC and Top studies for BA calculation.
Log $D_{7.4}$	ACD/Percepta
Publication	I, II, III

5 RESULTS

5.1 OCULAR PHARMACOKINETICS AFTER INTRACAMERAL ADMINISTRATION

Intracameral injection of the cocktail of atenolol (Log $D_{7.4}$ = -1.85), timolol (Log $D_{7.4}$= -0.35) and betaxolol (Log $D_{7.4}$= 0.77) was performed in the albino rabbits (5 nmol of each beta-blocker in 5 µL volume). The aqueous humour samples were obtained at different time points. The concentration-time profiles obtained for aqueous humour are presented in *Figure 9* and one compartmental model was the best fit for the three beta-blockers profiles.

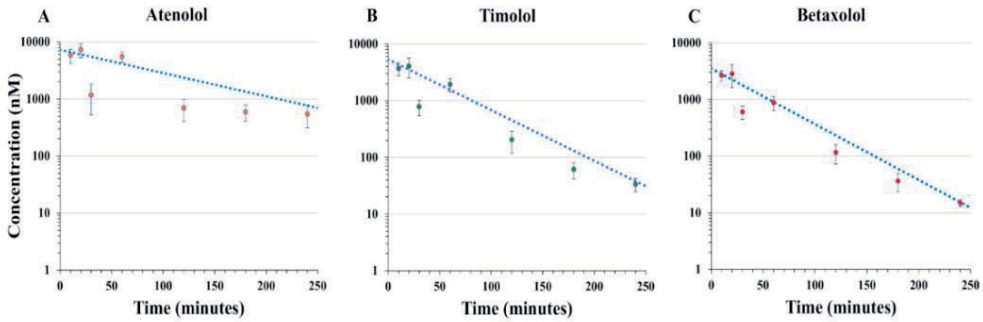


Figure 9. Concentrations of atenolol (A), timolol (B), and betaxolol (C) after intracameral injection. Each point represents the mean concentration (n=2-4) ± standard error of the mean. One compartmental curve fit with $1/Yhat^2$ weighting is shown for each compound (blue lines).

Table 6 shows drug physicochemical properties and the primary pharmacokinetic parameters of three beta-blockers. The results show that the most lipophilic drug, betaxolol, has higher clearance from the anterior chamber than timolol (medium lipophilicity) and atenolol (hydrophilic). A positive trend between drug lipophilicity and drug volume of distribution is also observed, while the half-life in aqueous humour seems shorter with more lipophilic compounds.

Table 6. Estimated pharmacokinetic parameters of the drugs in aqueous humour after intracameral injections in the rabbit eyes.

Drugs	MW	Log $D_{7.4}$	Log P	PSA	HBD	V_{dIC}± SE (µL)	CL_{IC}± SE (µL/min)	$t_{½\ IC}$± SE (min)	AUC± SE (min*nmol/µL)
Atenolol	266.34	-1.85	0.24	84.5	4	687 ± 140	6.44 ± 0.83	73.87 ± 12.16	0.781± 0.100
Timolol	316.42	-0.35	1.53	107.9	2	937 ± 172	19.30 ± 2.66	33.64 ± 2.29	0.266± 0.037
Betaxolol	307.43	0.77	2.94	50.7	2	1421±236	32.20 ± 4.10	30.58 ± 1.71	0.159± 0.020

Log $D_{7.4}$: Octanol water partition (ionized and unionized molecules)
Log P: Octanol water partition (unionized molecules)
PSA: Polar surface area
HBD: Hydrogen bond donor
$V_{d,\ IC}$: Volume of distribution after intracameral injection
CL_{IC}: Clearance after intracameral injection.
$t_{1/2,\ IC}$: Half-life after intracameral injection
AUC: Area under the curve

5.2 OCULAR PHARMACOKINETICS AFTER TOPICAL ADMINISTRATION

Topical administration of a cocktail of atenolol, timolol and betaxolol drop (25 µL) was instilled on rabbit eyes (500 nmol atenolol, 250 nmol timolol and betaxolol), and the aqueous humour samples were obtained at different time points. To the dose-normalized concentration-time profiles one-compartment model was fitted, with a weighting of $1/Yhat^2$ (*Figure 10*). Betaxolol showed to have the highest concentrations in aqueous humour compared to atenolol (lowest dose-normalized concentrations) and timolol (intermediate concentrations).

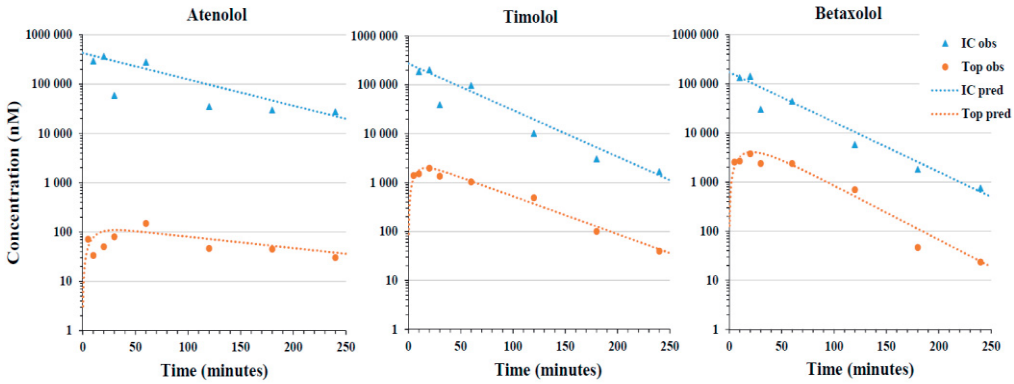


Figure 10. Aqueous humour concentration-time profiles of atenolol, timolol and betaxolol after topical and intracameral administration. The concentrations have been normalized to the dose of 250 nmol.

Pharmacokinetic parameters and bioavailability are presented in *Table 7*. The $AUC_{inf,\ Top}$ (CA) of betaxolol was 2, 6 and 12 times higher compared to timolol and atenolol (dose-normalized value), respectively. The ocular bioavailability of all three drugs was low (below 4%). Betaxolol showed the highest bioavailability with 43-55-fold higher bioavailability than that of atenolol. The bioavailability of timolol was between atenolol and betaxolol.

Table 7. The aqueous humour bioavailability of atenolol, timolol, and betaxolol using non-compartmental (NCA) and compartmental (CA) analyses.

	Topical administration			Bioavailability	
Drugs	Dose	$AUC_{inf,Top}$ ± SE (min*nmol/ mL)		(%)	
	(nmol)	CA	NCA	CA	NCA
Atenolol	500	48.6 ± 15.8	39.2	0.07	0.07
	250[a]	24.3[a]			
Timolol	250	152 ± 14	151	1.22	1.38*
Betaxolol	250	280 ± 47.8	252	3.82	3.87*

(*): values corrected after publication

The bioavailability values estimated for the three beta-blockers are compared with the reliable ones available in the literature (*Figure 11*). The plot shows a positive trend between lipophilicity and ocular bioavailability. Brinzolamide has been added to the following plot but cannot be used for comparison to the other drugs as it is a suspension and not a solution, the drug needs to be dissolved before the absorption process takes place and this reduces its bioavailability value.

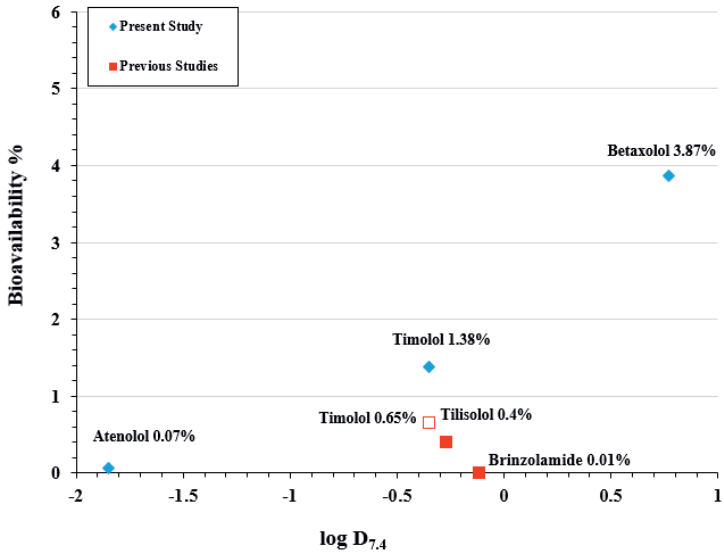


Figure 11. Bioavailability versus log $D_{7.4}$ of ophthalmic topical drugs in rabbit eyes. All values have been determined using NCA. The literature values are from (Yamamura et al., 1999) and (Naageshwaran et al., 2020).

5.3 OCULAR TISSUE EXPOSURE AFTER TOPICAL ADMINISTRATION

After the topical administration described previously (5.2), drug concentrations were also determined from the following tissues: corneal epithelium, corneal stroma-endothelium, bulbar conjunctiva, anterior sclera, iris-ciliary body, vitreous humour, and lens. Concentration profiles which are dose-normalized to 250 nmol of the three beta-blockers in these nine tissues are presented in *Figure 12*. Betaxolol is showing higher concentrations than timolol and atenolol in most tissues. Also, timolol has higher concentrations than atenolol in most tissues. However, in tear fluid, atenolol concentrations are the highest ones.

After the intracameral administration described previously (5.1), drug concentrations were also determined from the iris-ciliary body, and the corresponding NCA pharmacokinetic parameters are presented in *Table 8*.

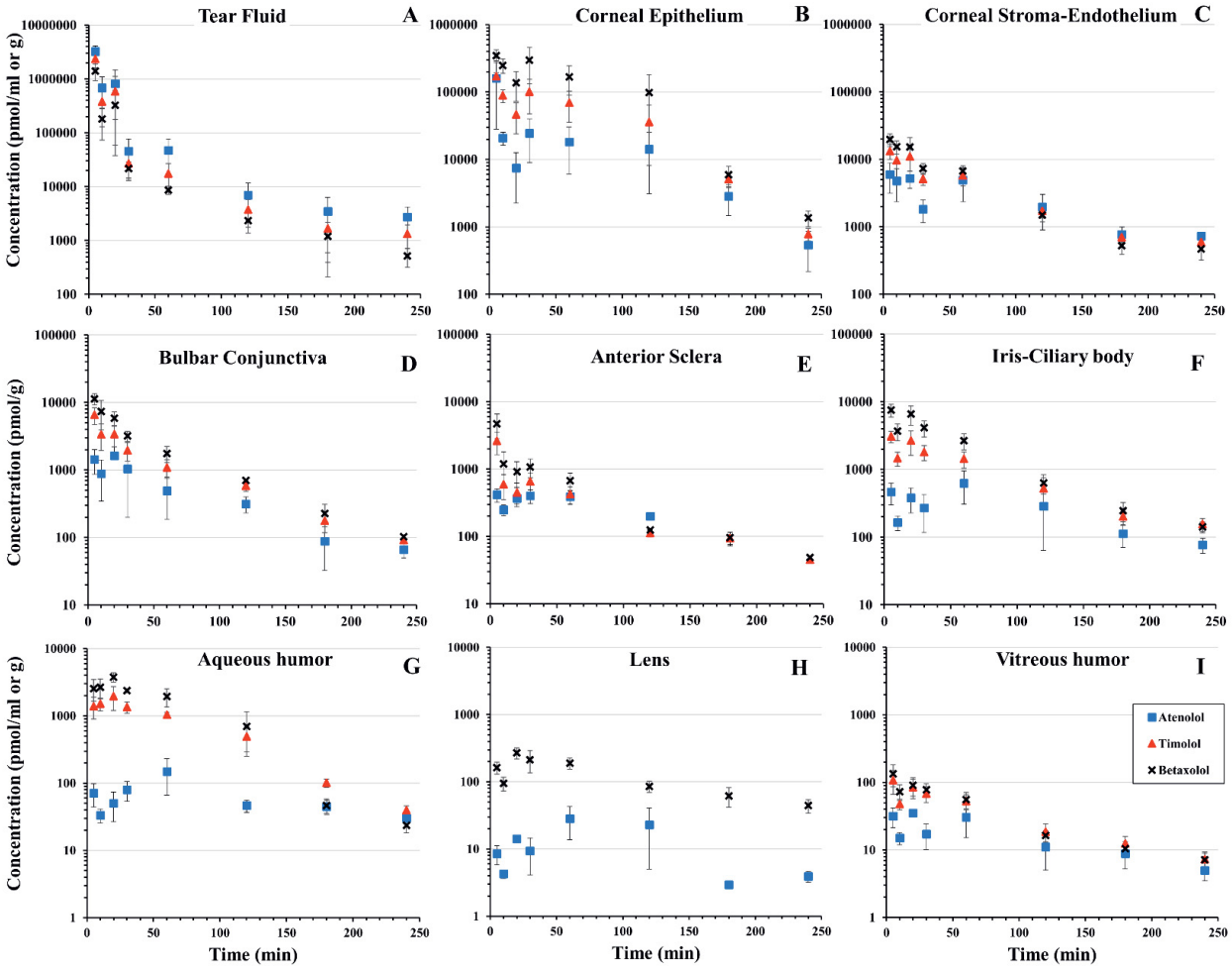

Tear Fluid
A
Corneal Epithelium
B
Corneal Stroma-Endothelium
C
Bulbar Conjunctiva
D
Anterior Sclera
E
Iris-Ciliary body
F
Aqueous humor
G
Lens
H
Vitreous humor
I
Concentration (pmol/ml or g)
Concentration (pmol/g)
Time (min)
Atenolol
Timolol
Betaxolol

Figure 12. Concentration-time profiles of drugs in nine ocular tissues (A-I) of rabbits after topical administration of atenolol (250 nmol dose-normalized), timolol (250 nmol), and betaxolol (250 nmol). Mean drug concentrations ± standard errors of the mean (n=3-4) are presented. Timolol concentration could not be quantified in the lens due to the lack of a reliable control sample.

The pharmacokinetic parameters were calculated for each tissue with NCA (*Table 8*). C_{max} is the highest for all three drugs in corneal epithelium and lowest in lens and vitreous humour. Furthermore, $t_{1/2}$ is the shortest in corneal epithelium for three drugs compared to all the other tissues, but atenolol is showing the longest $t_{1/2}$ in most of the tissues (other than the lens) compared to the other two drugs. The lowest mean residence time (MRT) value for all three beta-blockers is in the tear fluid. Hydrophilic atenolol is showing the highest MRT compared to more lipophilic timolol and betaxolol in most of the tissues (corneal stroma, aqueous humour, vitreous humour, iris-ciliary body).

Table 8. Pharmacokinetic parameters of the beta-blockers in the ocular tissues after topical administration. Pharmacokinetic parameters in the iris-ciliary body and aqueous humour are presented after intracameral administration. All values are dose-normalized to a dose of 250 nmol.

Topical	Atenolol						Timolol						Betaxolol					
	AUC_{inf} (min*nmol/ml or g)	AUC_{last} (min*nmol/ml or g)	C_{max} (nmol/ml or g)	t_{max} (min)	$t_{1/2}$ (min)	MRT	AUC_{inf} (min*nmol/ml or g)	AUC_{last} (min*nmol/ml or g)	C_{max} (nmol/ml or g)	t_{max} (min)	$t_{1/2}$ (min)	MRT	AUC_{inf} (min*nmol/ml or g)	AUC_{last} (min*nmol/ml or g)	C_{max} (nmol/ml or g)	t_{max} (min)	$t_{1/2}$ (min)	MRT
Tear fluid	71720	71542	3240	5	45	9	58398	58311	2345	5	45	6	39799	39769	1398	5	40	5
Corneal epithelium	3387	3361	159	5	34	63	9642	9611	171	5	27	62	24760	24712	348	5	24	60
Corneal stroma - endothelium	627	562	5.98	5	62	104	819	774	13.2	5	53	74	978	949	19.6	5	43	57
Bulbar conjunctiva	208	198 164*	3.24	20	52	74	234	228 197*	6.55	5	47	59	378	372 334*	11.3	5	42	50
Anterior sclera	-	 39.1*	0.41	5	84	-	71.5	67.7 57.3*	2.60	5	59	72	111	108 96.7*	4.70	5	47	55
Iris-ciliary body	72.4	66.2 49.5*	0.63	60	56	105	213	201 169*	3.07	5	52	77	398	390 352*	7.52	5	39	55
Aqueous humor	19.6	15.8	0.15	60	87	148	151	149	1.97	20	37	64	252	251	3.76	20	29	54
Lens	3.65	3.35	0.03	60	53	112	**	**	**	**	**	**	32.8	27.2	0.27	20	88	133
Vitreous humor	4.22	3.69	0.04	20	74	118	8.13	7.52	0.11	5	61	89	8.53	7.97	0.13	5	56	80
Intracameral																		
Iris-ciliary body	9939	7624	85.8	60	85	149	4615	4456	106	10	47	60	5013	4907	145	10	41	51
Aqueous humor	28711	26397	365	20	59	84	10895	10819	204	20	31	45	6520	6486	142	20	31	39
TOP: ICB/AH	3.69						1.41						1.58					
IC: ICB/AH	0.35						0.42						0.77					

AUC_{inf}: Area under the curve from 0 to infinity.
AUC_{last}: Area under the curve from 0 to last sampling time (240 min) unless otherwise indicated.
C_{max}: Maximum concentration.
t_{max}: Time to peak concentration.
$t_{1/2}$: Elimination half-life.
MRT: Mean residence time to infinity.
* AUC_{last} from 0 until 120 min (last sampling time).
** Timolol could not be quantified due to the lack of a reliable control sample.
- Unreliable estimate because the concentration-time profile was shorter than two half-lives.
ICB/AH: AUC_{inf} ratio between iris-ciliary body and aqueous humour after topical (TOP) and intracameral (IC) administrations.

The AUC_{inf} values in various ocular tissues after topical delivery are compared in *Figure 13*. Atenolol had the highest AUC_{inf} in tear fluid, whereas betaxolol showed the highest values in all other tissues. The values of timolol were generally between those of betaxolol and atenolol. The difference between the AUC_{inf} for the three beta-blockers is the highest in corneal epithelium and aqueous humour (*Figure 13*). When looking at the overall exposure of the ocular tissues, we can observe that the values of tear fluid exposure are about four orders of magnitude higher than in the vitreous humour. As expected, the exposure levels are decreasing as the distance from the instilled ocular surface increases.

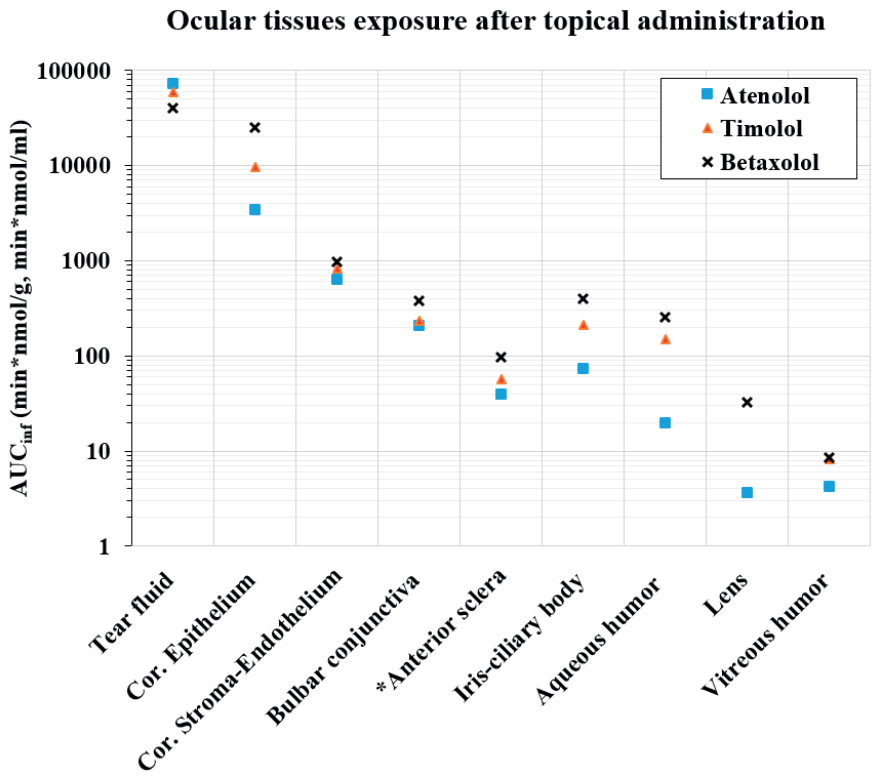


Figure 13. The AUC_{inf} values of betaxolol, timolol and atenolol in the ocular tissues after topical eye drop instillation to the rabbit eyes (normalized to the dose of 250 nmol). For anterior sclera AUC_{last}=120 min values are shown (*). Abbreviation: Cor = cornea.

Figure 14 illustrates concentrations of the drugs in the tissues that are expected to be subjects to corneal (A) and non-corneal absorption (B). For betaxolol, the concentration profile in the tear fluid declines rapidly, but betaxolol absorbs well to the cornea reaching the aqueous humour. On the contrary, the concentration profile of atenolol in tear fluid is higher, but it permeates the cornea poorly. Therefore, the concentration-profile difference of atenolol between tear fluid and aqueous humour is higher than in the case of betaxolol and timolol. The concentration differences between betaxolol and atenolol were higher in the corneal epithelium than in the conjunctiva. Furthermore, the difference between the aqueous humour and iris-ciliary body concentration profiles of atenolol was less pronounced than in the case of timolol and betaxolol, see *Figure 14*. Overall,

the data suggest that the contribution of non-corneal absorption is more pronounced in the case of atenolol than it is for timolol and betaxolol.

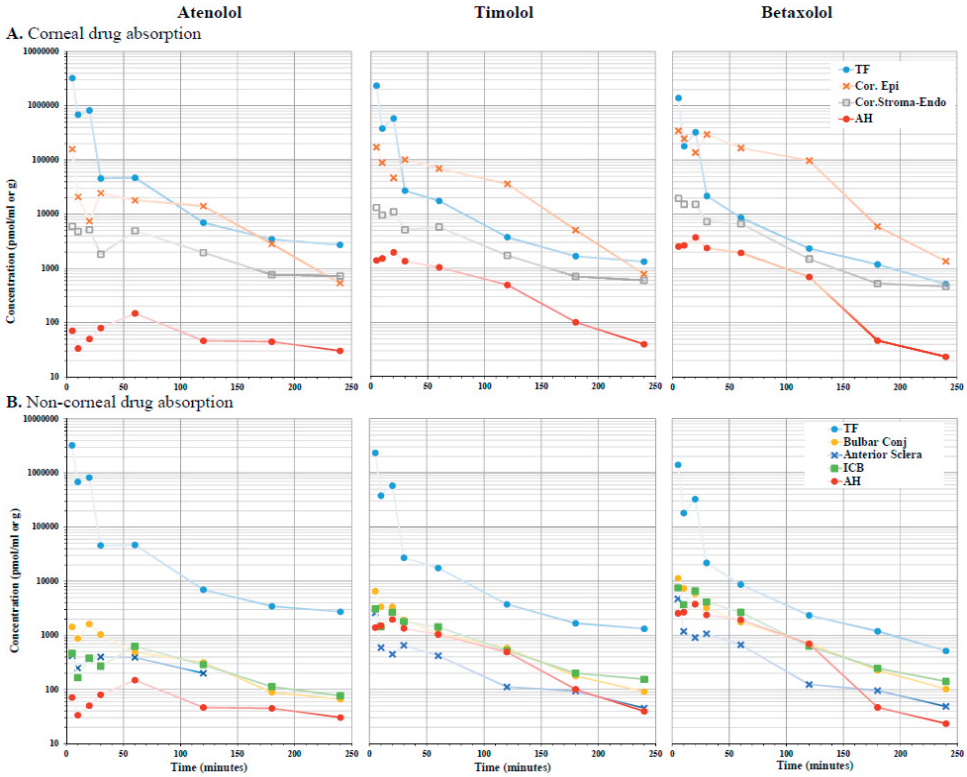


Figure 14. Concentrations of atenolol, timolol, and betaxolol in the ocular tissues of rabbits after topical dosing (normalized to 250 nmol dose). Tissues in the corneal (A) and non-corneal (B) routes of absorption are illustrated.

Figure 15 shows the drug AUC_{inf} ratios between neighboring tissues in the eye after topical drug administration. For most anterior segment interfaces, betaxolol shows the highest partitioning into corneal epithelium (e.g. corneal epithelium/tear fluid), whereas atenolol presents the highest AUC_{inf} ratios between stroma/corneal epithelium. *Figure 15* demonstrates that the corneal absorption pathway is clearly dependent on drug lipophilicity. Interestingly, atenolol shows the highest ratios of iris-ciliary body/aqueous humour.

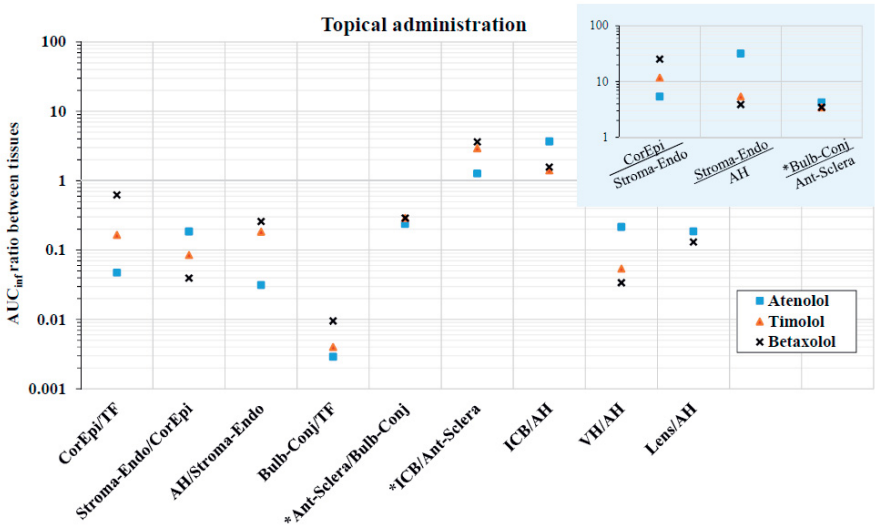


Figure 15. AUC_{inf} ratios between tissues. The AUC_{inf} ratios between neighboring ocular tissues are shown after topical eye drop instillation of betaxolol, timolol and atenolol to the albino rabbit eyes. The inset shows a comparison of drug AUC_{inf} ratio in selected cases (on the x-axis). The AUC_{inf} values were used, except AUC_{last}=120 min was used for anterior sclera (*). Abbreviations: CorEpi = corneal epithelium, TF = tear fluid, Stroma-Endo = corneal stroma-endothelium, AH = aqueous humour, Bulb-Conj = bulbar conjunctiva, Ant-Sclera = anterior sclera, ICB = iris-ciliary body, and VH = vitreous humour.

6 DISCUSSION

6.1 OCULAR PHARMACOKINETICS AFTER INTRACAMERAL ADMINISTRATION

After topical administration, drugs have to pass through barriers to reach their target sites as previously described. The target tissues are dependent on the disease to treat as shown in *Figure 2*. For atenolol, timolol, and betaxolol the targeting tissue is the ciliary body, while aqueous humour is the typical site for sampling and determining the drug bioavailability, for drug concentrations are easier to sample and analyse than from solid tissues. Thus, ocular bioavailability is typically determined based on drug concentrations in the aqueous humour. However, topical pharmacokinetic studies alone are not enough to describe the drug bioavailability in aqueous humour, as drug concentration in aqueous humour reflects both absorption and elimination processes. Therefore, it is not possible to determine the aqueous humour bioavailability from topical administration alone. Moreover, data to determine the importance of the iris-ciliary body in total drug clearance from the aqueous humour is still poorly known. Due to these reasons, determination of pharmacokinetics after intracameral injection is needed to obtain AUC_{inf} and calculate ocular bioavailability after topical drug administration.

$$\boldsymbol{Aqueous\ humor\ bioavailability} = \frac{\boldsymbol{AUC_{Top} \times Dose_{IC}}}{\boldsymbol{AUC_{IC} \times Dose_{Top}}} \times \mathbf{100} \quad \textit{Equation 1.}$$

Moreover, the obtained primary pharmacokinetic parameters such as drug clearance and volume of distribution values allow understanding of both intracameral and topical drug pharmacokinetics and are required parameters for building pharmacokinetic simulations. Such *in silico* tools can be used in the drug design and development of ophthalmic delivery systems (Subrizi et al., 2019).

Intracameral pharmacokinetic studies have been rarely reported in the literature (Kodjikian et al., 2010; Miller et al., 1981; Shen et al., 2009; Yamamura et al., 1999). The reliable literature on intracameral

pharmacokinetic parameters (clearance, volume of distribution) was compiled in *Table 3*, (Kodjikian et al., 2010; Miller et al., 1981; Shen et al., 2009; Yamamura et al., 1999) together with our data in *Figure 16* to show lipophilicity impact on their pharmacokinetics parameters, with a positive trend for both clearance and volume of distribution. This is the first time that these intracameral pharmacokinetic parameters are reported for atenolol and betaxolol. For timolol, our study shows the concentrations for four hours longer than the previous reports (Yamamura et al., 1999), thereby improving the reliability of the kinetic parameters. Encouragingly, timolol results from our cocktail study (CL = 19.30 µL/min, V_d = 937 µL) are similar to the previous studies for timolol administration alone (CL= 25 µL/min, V_d = 860 µL) (Yamamura et al., 1999) supporting the reliability of using cocktail method administration.

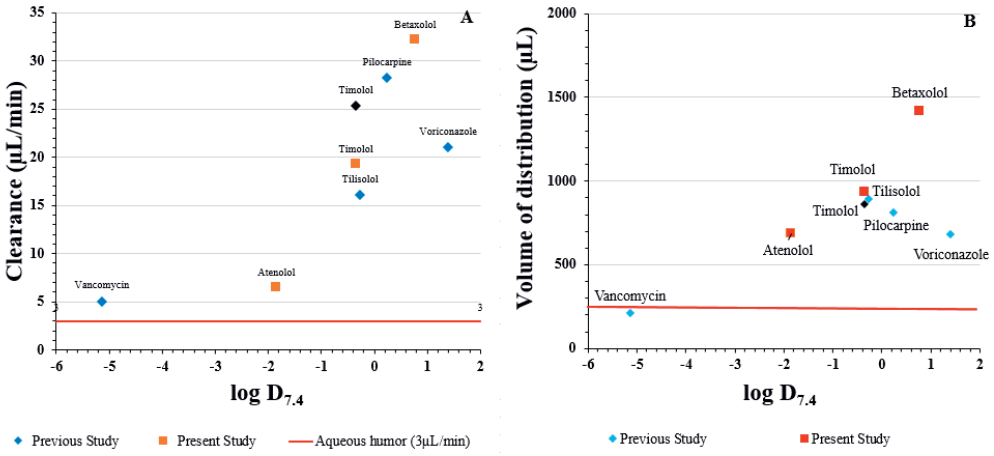


Figure 16. Log $D_{7.4}$ vs pharmacokinetic parameters (A: clearance; B: volume of distribution) of intracamerally administered drugs. Both panels show seven compounds i.e., betaxolol, atenolol, timolol (from the present study using orange squares), and compounds reported in the literature, namely, timolol (Yamamura et al., 1999) (black diamond symbol), vancomycin (Kodjikian et al., 2010), pilocarpine (Miller et al., 1980), voriconazole (Shen et al., 2009), and tilisolol (Yamamura et al., 1999) (blue diamonds). The red lines in the graphs represent the outflow of aqueous humour through the trabecular meshwork (3 µL/min) (A) and the anatomical

volume of aqueous humour (250 µL) (B). More detailed information on the intracameral pharmacokinetic parameters and the reasons for the exclusion of some papers (Ling and Combs, 1987; Tang-Liu et al., 1984) is available in Pub I (Supplementary Table S4 and Supplementary figures 1, 2, 3) and is discussed in 6.2 as well.

The intracameral pharmacokinetic studies provide information about the routes of drug elimination from the anterior chamber. The normal aqueous humour outflow in the rabbit eye is 3-4 µL/min (Heys and Barocas, 2002). Values above 3-4 µL/min indicate that the drug is also cleared by other mechanisms, such as elimination via the iris-ciliary body. Our results show that atenolol clearance (6.44 µL/min) is only slightly higher than aqueous humour outflow, whereas the clearances of more lipophilic betaxolol (32.20 µL/min) and timolol (19.30 µL/min) are indicating the much higher contribution of iris-ciliary body drug clearance. More lipophilic compounds permeate easier than hydrophilic compounds into the iris-ciliary body and through the blood vessel endothelia in the iris and ciliary body (partly belonging to the blood-aqueous barrier), eventually reaching the systemic blood circulation. For comparison, the iris-ciliary body blood flow is 62 µL/min in iris and 82 µL/min in ciliary body in rabbits (Alm, 1992; Nilsson and Alm, 2012) suggesting that the clearance is governed by permeability across the blood vessel endothelial and not by the rate of blood flow.

The anatomical volume of the rabbit aqueous humour is 250-300 µL (Conrad and Robinson, 1977). The volume of distribution higher than the anatomical volume indicates that the compound distributes into and possibly binds to the neighboring tissues. The mean volumes of distribution of atenolol (687 µL), timolol (937 µL) and betaxolol (1421 µL) suggest extensive and lipophilicity dependent drug distribution to the neighboring tissues. The volume of distribution positively correlates with the tissue/aqueous humour partition coefficients. However, *Figure 16* do not show a very good trend between lipophilicity and volume of distribution for pilocarpine and voriconazole compared to other compounds. It can be because both compounds being weak basic will show less interaction with the lipids and surrounding proteins compared to beta-blockers and so lower volume of distribution. However, the impact of lipophilicity can be seen with

atenolol and vancomycin as even though are stronger bases, is showing a very low volume of distribution

6.2 OCULAR BIOAVAILABILITY AFTER TOPICAL ADMINISTRATION

We studied the absorption of atenolol, timolol, and betaxolol after topical administration to the rabbit eye and estimated the pharmacokinetic parameters in the aqueous humour. Absolute bioavailability was determined based on dose normalized AUC_{inf} ratios between topical and intracameral administrations, see *Table 7*. In general, ocular bioavailability data in the literature are very sparse, since it requires analyses of aqueous humour concentrations from both intracameral and topical administration studies. We found two studies like ours in literature (Yamamura et al., 1999) and (Naageshwaran et al., 2020), being the last one a suspension not a solution. Other studies available in the literature but not reliable were Ling et al. and Tang-Liu et al (Ling and Combs, 1987; Tang-Liu et al., 1984) who sampled aqueous humour repeatedly from the same eye i.e. leaking drug from the injection site.

The results showed that betaxolol has higher exposure in the aqueous humour than timolol and atenolol (AUC_{inf}) after topical administration (*Figure 10*). Furthermore, aqueous humour drug concentration profiles after intracameral and topical administration (250 nmol dose-normalized) of timolol and betaxolol are closer to each other compared to atenolol (Supplementary Figure S4 in III), implying that the corneal barrier limits, especially atenolol absorption. The ocular bioavailability of betaxolol was 55 and 3 times higher than that of atenolol and timolol, see *Figure 11*. The more lipophilic drugs, betaxolol and timolol permeate easier through the cornea than atenolol. Therefore, a higher fraction of the betaxolol and timolol dose reaches aqueous humour compared to hydrophilic atenolol. Overall, the low ocular bioavailability of these drugs demonstrates the importance of precorneal drug loss factors in limiting drug absorption.

6.3 OCULAR TISSUE EXPOSURE AND ABSORPTION PATHWAYS AFTER TOPICAL ADMINISTRATION

Pharmacokinetics of atenolol, timolol, and betaxolol in nine ocular tissues after topical administration was studied. In addition, drug concentrations in the iris-ciliary body and aqueous humour were also determined/compiled after intracameral administration (*Table 8*).

In our study, the hydrophilic atenolol compound showed the highest concentrations (and AUC) in tear fluid. Presumably, this is due to the differences in drug clearance from the tear fluid. Tear turnover and drainage of excess fluid are similar factors for all three compounds, but clearance through conjunctiva and cornea is increased at higher lipophilicity (Ramsay et al., 2018; Ramsay et al., 2017), explaining the highest tear fluid AUC for atenolol.

Timolol and betaxolol had higher concentrations than atenolol in the other ocular tissues (*Figure 12*). Betaxolol has the highest and atenolol has the lowest corneal permeability of these three drugs (Wang et al., 1991). This explains the high betaxolol levels in the corneal epithelium and the aqueous humour.

Atenolol levels in the corneal epithelium are the lowest, but its transfer to the hydrophilic stroma is least restricted. However, the distribution of atenolol from stroma to aqueous humour may be restricted more by the endothelium than in the case of timolol and betaxolol (as indicated by the aqueous humour/stroma-endothelium AUC ratios). Thus, the stroma may act as a depot for the hydrophilic atenolol, but not for betaxolol and timolol. From the aqueous humour, the drugs are cleared by aqueous humour outflow and iris-ciliary body blood flow or distributed to the surrounding tissues.

The drug may also permeate from the tear fluid through the conjunctiva into the sclera and further to the iris-ciliary body, thereby passing by the aqueous humour. Even though the conjunctiva is more permeable than the cornea (Hämäläinen et al., 1997), the drug concentrations in the conjunctiva were lower than in the cornea. This explained the effective drug clearance from the conjunctiva into the systemic circulation, since the conjunctiva is

vascularized, but the cornea is not. Also, the differences in the tissue/tear fluid AUC_{inf} ratios between drugs were smaller in the conjunctiva than in the cornea, which is explained by the faster systemic absorption of more lipophilic drugs, partly compensating for their better absorption in the conjunctiva. Drug partitioning from the bulbar conjunctiva to the sclera seems to be similar for all drugs, but lipophilicity seems to favor further permeation to the cellular iris-ciliary body.

For atenolol, 3.69-fold higher AUC_{inf} values were obtained in the iris-ciliary body than in the aqueous humour, whereas the differences were only 1.41-1.58-fold for timolol and betaxolol, see *Table 8*. This is explained by a higher contribution of the non-corneal route (conjunctiva-sclera) to the iris-ciliary body exposure for atenolol compared to betaxolol and timolol. The higher relative role of the non-corneal route in the ocular absorption of hydrophilic compounds is in the line with some previous studies (Abdul Nasir et al., 2016; Acheampong et al., 1995).

Drug distribution from the aqueous humour to the lens is very low. Drug concentrations in this tissue are low after topical delivery. The lens is rather impermeable tissue due to its low lipid content and tight protein structure resulting in lower partitioning and concentrations, even though the lens is exposed to the drug in the aqueous humour (Acheampong et al., 1995; Araie et al., 1982; Heikkinen et al., 2019; Urtti et al., 1990). Moreover, the lens acts as a strong barrier preventing drug distribution from the aqueous humour to the vitreous cavity as well. Furthermore, access to the vitreous is limited by the convective aqueous humour flow from the posterior to the anterior chamber (Heys and Barocas, 2002).

The data generated in this study fill some of those knowledge gaps and can help in developing improved *in silico* models of drug disposition in the eye. Future efforts could also focus on the translation of the disposition from pre-clinical species to the human eye with the overall aim of producing predictive models for the ocular distribution of drugs in both species. For that, the anatomy and physiology of eye-related parameters should be incorporated into the model adding inter-individual variability and better dose scheduling and prediction, hence building a more comprehensive mechanistic model when compared to *in silico* or empirical models.

6.4 IMPACT OF LIPOPHILICITY ON TOPICAL PHARMACOKINETICS

We studied the impact of lipophilicity on ocular pharmacokinetics. Previous work has examined the impact of physicochemical properties on corneal and conjunctival permeability (Hämäläinen et al., 1997; Lach et al., 1983; Prausnitz and Noonan, 1998; Ramsay et al., 2018; Ramsay et al., 2017). Passive permeability has been well characterized and also computational models have been built to predict them based on observed corneal permeability data. The reported physicochemical drug properties that seem relevant in these models are log $D_{7.4}$, molecular weight, hydrogen bond donor groups, molar volume, hydrogen bond acceptors and degree of ionization (Fu and Liang, 2002; Ghorbanzad'e et al., 2011; Kidron et al., 2010; Li et al., 2005; Schoenwald and Huang, 1983; Sharma et al., 2011; Yoshida and Topliss, 1996). However, permeability studies are done using constant flow systems while *in vivo* situation is transient (short contact on the eye surface); so far no one has linked permeability QSPR to in vivo kinetics which is a future challenge.

The drug exposure (AUC_{inf}), $C_{max,}$ and bioavailability of the three beta-blockers in the aqueous humour obtained after topical administration seem to correlate with corneal (and corneal/conjunctiva) permeability values obtained from the literature (Wang et al., 1991) (*Figure 17;* Supplementary data Figure S3 in II). A similar trend has been also observed by other researchers (Huang et al., 1983; Sasaki et al., 1997).

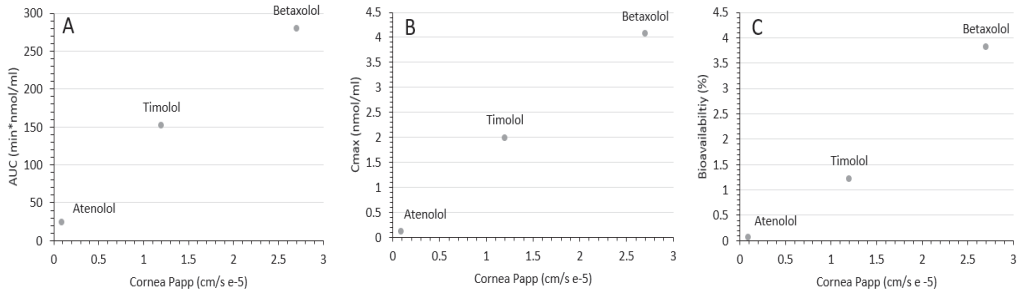


Figure 17. Impact of corneal permeability on the pharmacokinetic parameters topical AUC_{inf}, C_{max}, and BA. The correlation of corneal

permeability (Wang et al., 1991) with the aqueous humour C_{max}, AUC_{inf}, and bioavailability in rabbit eye (present paper).

Corneal permeability is influenced by drug ionization, molecular size, pH, and formulation components (Himmelstein et al., 1978; Lach et al., 1983; Suhonen et al., 1998). Lipophilicity shows a strong correlation with corneal permeability as has been seen in several studies (Chien and Schoenwald, 1990; Lach et al., 1983; Schoenwald, 1987; Wang et al., 1991). Our data show that drugs with higher lipophilicity exhibit higher ocular bioavailability and lipophilicity influences drug distribution between the neighboring tissues. Despite the correlations (*Figure 11; Figure 17*), more data are required to confirm these findings.

Moreover, we have investigated the impact of lipophilicity on intracameral pharmacokinetics, for a detailed description see chapter 6.1. and *Figure 16*. Data on *in vivo* ocular pharmacokinetic parameters after application were collated from both the literature and the current study. The relationship between log $D_{7.4}$ and clearance and the volume of distribution after intracameral administration are shown in *Figure 16: A* where the trend between the clearance and lipophilicity of the drug is seen at Log $D_{7.4}$ of -2 and above. Below this value, the clearance appears to be independent of lipophilicity, being close to the levels of aqueous humour outflow. However, it seems that Log $D_{7.4}$ has a stronger impact on clearance than on the volume of drug distribution, see *Figure 16*. Voriconazole and pilocarpine being weak basic will show less interaction with the lipids and surrounding proteins compared to beta-blockers and so lower volume of distribution. However, the impact of lipophilicity can be seen with atenolol and vancomycin as even though are stronger bases, are showing a very low volume of distribution. We also investigated the impact of other molecular descriptors besides Log $D_{7.4}$ to see if they may correlate with clearance and volume of distribution. However, hydrogen bonding, molecular weight or polar surface area did not show any correlation (Supplementary material Fig 4 in I) implying that the single physicochemical property is not enough to see the trend.

6.5 METHODOLOGICAL ASPECTS

In this thesis, we used a cassette dosing or cocktail approach to study the pharmacokinetics of three beta-blockers simultaneously. This is enabled by LC/MS-MS analytics that is sensitive and selective enabling the analyses of several compounds from the same sample. To the best of our knowledge, this is the first time such an approach has been reported for determining topical ocular bioavailability. In recent years, cocktail dosing has been used to study the activity of several human ADME (Sharma et al., 2004) and to select pharmacokinetically optimal compounds in preclinical drug discovery programs (Zhou et al., 2004). In ocular studies, cassette dosing has been employed to study ocular membrane permeability (Heikkinen et al., 2019; Ramsay et al., 2018; Ramsay et al., 2019; Ramsay et al., 2017), melanin binding (Pelkonen et al., 2017) and *in vivo* pharmacokinetics after Intravitreal and sub-Tenon injections (Gale et al., 2005; Proksch and Ward, 2008). The main advantage of this approach is a significant reduction in the number of needed animals and the decrease of inter-individual and analytical related variability among studies.

Cocktail dosing may be problematic if there are pharmacokinetic interactions between the compounds as this may lead to erroneous parameters. This risk can be minimized by using sensitive analytical methods (such as LC-MS/MS) at low drug doses thereby ensuring that the drug concentrations in the samples are below the K_m or K_i values of relevant metabolizing enzymes and transporters. This should eliminate interactions between the compounds in the cocktail. Success in this sense depends on the drug doses and concentrations, the affinity of the compounds to the enzymes and transporters as well as the importance of drug metabolism and drug transport in the pharmacokinetics of the test compounds (Nagilla et al., 2011). It should be noted such interactions are not relevant if the compounds are not subject to metabolism and active transport, as is the case of the eye. In general, passive kinetics processes are dominant in the eye and no significant roles for drug metabolism and active transport have been demonstrated (Nakano et al., 2014).

In the case of our compounds (betaxolol, timolol, atenolol) no ocular metabolism has been shown and we did not observe any metabolites in our LC/MS-MS assays. Expression of drug transporters in the eye is an emerging field and recently proteomics analyses of transporters in the retina have been published (Pelkonen et al., Mol Pharmaceut 2017; Hellinen et al. IOVS 2019). For example, the studied beta-blocking agents may be substrates of P-glycoprotein which is known to be present in the ciliary body (Vellonen et al., 2018). Nevertheless, the potential of ocular transporter impact on ocular pharmacokinetics is not relevant (Vellonen et al. Adv Drug del Rev 2018). Furthermore, our timolol results are in line with the previous report in which timolol was administered alone (Yamamura et al., 1999).

Saturable binding to protein binding and competition on the binding forms another possible source of error in cocktail studies. Tear fluid (8 mg/mL) (De Souza et al., 2006) and aqueous humour (0.12 mg/mL) have very low protein concentrations (Tripathi et al., 1989) compared to 3% in human plasma (Hegedus et al., 1981). Protein binding has a negligible role in topical ocular pharmacokinetics and, therefore, binding changes have also minimal impact.

Atenolol, timolol and betaxolol are beta-blocking drugs that reduce aqueous humour inflow (Boger, 1979; Brooks and Gillies, 1992). Therefore, these pharmacological effects might influence their elimination from the aqueous humour. However, our results show that the fraction of drug elimination by the aqueous humour outflow is small (3-4 µL/min) compared to the overall clearance from aqueous humour (5.04-32.20 µL/min). Therefore, the changes in the aqueous humour flow should not affect our pharmacokinetic results significantly. Hence, we can conclude that the cocktail approach is adequate and the pharmacokinetic parameters obtain are reliable.

Ocular pharmacokinetic studies with animals are invasive since laboratory animals must be sacrificed at each sampling time point. Therefore, a large number of animals is needed for each drug. Moreover, the variability among individual pharmacokinetics studies investigating the same drug has proven to be significant (see Pub II: Supplementary Table S2, Figure S1). This can be avoided with the cocktail approach which involves the

determination of several compounds in the same animal and the same sample. This reduces variability, yields more reliable kinetic parameters, and enables more reliable comparisons between the study compounds. Importantly, the cocktail approach reduces the required number of animals in ocular pharmacokinetic studies. Completion of this task would speed up the development of new topical ocular treatments and reduce the number of required animal studies supporting the 3 R.

7 CONCLUSION

This thesis provides quantitative data on ocular pharmacokinetics of atenolol, timolol, and betaxolol in albino rabbits. The specific conclusions of this work are outlined below.

1. The intracameral primary pharmacokinetic parameters of the 3 beta-blockers have been reported (being the first time for atenolol and betaxolol). The volume of distribution and clearance from the aqueous humour seems to correlate with the lipophilicity of the drug.
2. Approximately 10% of intracameral betaxolol is eliminated with aqueous humour outflow and 90% by other mechanisms, whereas approximately 50% of atenolol is cleared with aqueous humour outflow.
3. The ocular bioavailability of the investigated drugs has been reported to show a 55-fold range (from 0.07-3.82%) and the rank order of betaxolol > timolol > atenolol. A positive trend between ocular bioavailability and drug lipophilicity was also observed.
4. After topical administration, the cornea is the main absorption route for lipophilic compounds i.e., betaxolol and timolol, while non-corneal absorption (conjunctiva-sclera route) may play a higher role for hydrophilic drugs i.e. atenolol.
5. After topical administration, betaxolol and timolol showed higher AUC_{inf} than atenolol in the investigated eye tissues except for tear fluid.
6. The cassette dosing approach has decreased inter-individual and analytical related variability providing reliable and comparable data among the three compounds.
7. Curated and comprehensive literature data on intracameral and topical pharmacokinetic parameters have been compared with our present data: intracameral studies are sparse, variability among topical studies is high and lipophilicity drug influence on pharmacokinetics is also present.

8. The generated data has brought new insight into the topical pharmacokinetic framework, and drug lipophilicity influence. These data can be further utilized for building ophthalmic mechanistic models to predict drug concentrations in different ocular tissues for physicochemical similar drugs.

Intracameral administration:

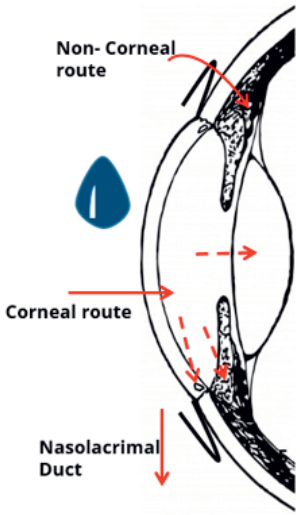
- Aqueous humour clearance and volume of distribution is dependent on lipophilicity.
- Aqueous humour outflow is contributing 50% for atenolol and 10% for betaxolol clearance.

Corneal route of absorption:

- Corneal route is main route of absorption to aqueous humour for lipophilic drugs.
- Aqueous humour bioavailability is linked to corneal route of absorption.

Non-Corneal route of absorption:

- This route is for all drugs but is relatively more prominent for hydrophilic compounds access to iris-ciliary body.



Cassette dosing:

- Decrease inter-individual variability.
- Reduce number of animal.
- Analytical relative variability.

Absolute bioavailability:

55 fold difference between the bioavailability of three beta-blockers.

- Atenolol 0.07%
- Timolol 1.22 %
- Betaxolol 3.82%

AUC_{inf}:

- Betaxolol and timolol showed higher AUC_{inf} in all tissues other than tear fluid.

Data can be utilized for understanding ocular pharmacokinetic framework and in building opthalmic mechanistic model for predicting drug concentration in ocular tissues.

Figure 18. Conclusion of the thesis.

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ORIGINAL PUBLICATIONS

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Ocular Intracameral Pharmacokinetics for a Cocktail of Timolol, Betaxolol, and Atenolol in Rabbits

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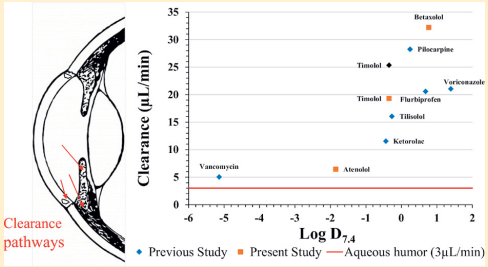
Supporting Information

ABSTRACT: The mechanisms of drug clearance from the aqueous humor are poorly defined. In this study, a cocktail approach was used to simultaneously determine the pharmacokinetics of three β-blocker agents after intracameral (ic) injection into the rabbit eyes. Aqueous humor samples were collected and analyzed using LC−MS/MS to determine drug concentrations. Pharmacokinetic parameters were obtained using a compartmental fitting approach, and the estimated clearance, volume of distribution, and half-life values were the following: atenolol (6.44 μL/min, 687 μL, and 73.87 min), timolol (19.30 μL/min, 937 μL, and 33.64 min), and betaxolol (32.20 μL/min, 1421 μL, and 30.58 min). Increased compound lipophilicity (atenolol < timolol < betaxolol) resulted in higher clearance and volume of distributions in the aqueous humor. Clearance of timolol and betaxolol is about 10 times higher than the aqueous humor outflow, demonstrating the importance of other elimination routes (e.g., uptake to iris and ciliary body and subsequent elimination via blood flow).



KEYWORDS: *intracameral injection, clearance, volume of distribution, timolol, atenolol, betaxolol, ocular pharmacokinetics*

INTRODUCTION

β-Adrenergic antagonists (Figure 1) are used widely for the treatment of glaucoma since they lower the intraocular pressure by reducing the production of aqueous humor (pH 7.5−7.6 [1,2]) in the ciliary body which can ultimately decrease the outflow of aqueous humor in long-term therapy.[3−5] Atenolol and betaxolol selectively inhibit adrenoceptor β_1, whereas timolol nonselectively antagonizes both β_1 and β_2 receptors.[6−8]

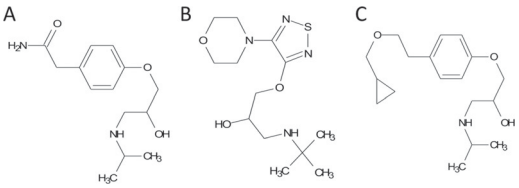


Figure 1. Chemical structure of three β-blockers: (A) atenolol, (B) timolol, (C) betaxolol.

During eye drop treatment, it is important to achieve and maintain adequate drug concentrations in the aqueous humor.[9,10] From the aqueous humor the small molecular weight drugs permeate with relative ease to the iris and ciliary body, and drug concentrations in these tissues typically closely follow the levels in aqueous humor.[11,12] Therefore, drug concentrations in the aqueous humor are widely used to monitor ocular drug absorption and to determine the ocular bioavailability.

Drug clearance from the aqueous humor must be known to accurately determine ocular bioavailability following administration of eye drops. Unfortunately, these values have been

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only rarely determined, mainly because such experiments require intracameral injections and in preclinical species require a large number of rabbits. Thus, aqueous humor clearance of drugs is a poorly understood and missing piece in ocular pharmacokinetics, and lack of intracameral kinetic information makes it challenging to build reliable mathematical models for topical eye medications. Accurate *in silico* models for predicting ocular pharmacokinetics would have the potential to replace and/or refine experiments for new topical agents in the future.

Drug clearance from the aqueous humor can take place via aqueous humor outflow through trabecular meshwork[13] and uveoscleral pathway[14] or permeation to iris and ciliary body and subsequent elimination by the blood flow in these tissues[15−18] (Figure 2). Trabecular meshwork outflow is

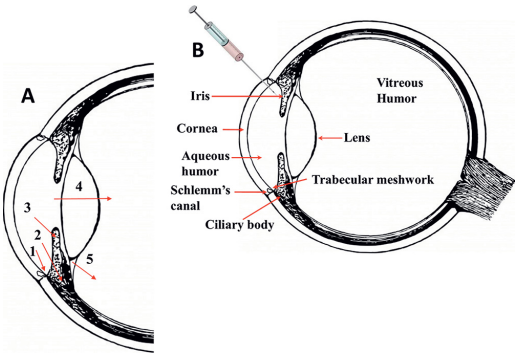

Figure 2. Ocular drug elimination routes after intracameral injection. (A) Elimination of drugs after intracameral injection. (1) Clearance through trabecular meshwork outflow. (2) Clearance through uveoscleral outflow. (3) Elimination to the vasculature of iris and ciliary body. (4) Distribution to lens. (5) Distribution to vitreous humor. (B) Intracameral administration into the aqueous humor. (https://www.publicdomainpictures.net/en/view-image.php?image=130389&picture=medical-eye)

approximately 3 μL/min [19,20] in rabbits, and drug clearance via this pathway is independent of drug's physiochemical properties.[11,21−24] The importance of other pathways depends on the ability of drug to cross the tissues and endothelial vessels. The iridial and muscle ciliary vessels (other ciliary vessels are leaky) present tight junctions, corresponding to the endothelial component of the blood−aqueous barrier.[25,26] Drugs that can easily permeate through these barriers present much faster clearance values.[18,27,28,23,26] Obviously, drugs may also distribute to the neighboring tissues, i.e., iris−ciliary body, vitreous humor and lens, and these may affect the apparent drug volume of distribution.[11,29] (Figure 2).

In this study, ocular pharmacokinetics of three β-blocker agents were determined using a cocktail dosing strategy. The three compounds were given in a single injection solution dosed intracamerally to anesthetized albino rabbits. The cocktail approach reduces the number of animals in the experiments to one-third. To the best of our knowledge, this is the first time a cocktail approach is used for intracameral injection in rabbits.

MATERIALS AND METHODS

Preparation of Drug Solution. solutions of 3 mM atenolol (USP reference standard, Sigma), 3 mM betaxolol hydrochloride (USP reference standard, Sigma), and 3 mM timolol maleate (USP reference standard, Sigma) were prepared in phosphate buffered saline (PBS) at pH 7.4. The solutions were diluted to obtain drug mix solution that contained 1 mM of each β-blocker (atenolol, betaxolol, and timolol). The pH of the β-blocker mix solution was 7.4, and osmolality 267 mOsm/kg was evaluated just prior to use.

Animal Experiments. New Zealand albino rabbits (males, weight 2.7−3.1 kg; Envigo Laboratories UK) were used in the study. The animals were handled in accordance with the statement of the Animals in Research Committee of the ARVO (Association for Research in Vision and Ophthalmology, Rockville, MD, USA), and the experiments were approved by the National Animal Experiment Board in Finland.

Before the experiment, the animals' eyes were examined to confirm the ocular health. The animals were sedated with medetomidine (Domitor vet 1 mg/mL, Orion Pharma, Espoo, Finland; dose 0.5 mg/kg) injection subcutaneously and anesthetized with ketamine (Ketalar/Ketaminol 50 mg/mL, Pfizer Oy Animal Health, Espoo, Finland; dose 25 mg/kg). The pupils were dilated (tropicamide; Oftan Tropicamide 5 mg/mL, Santen Pharmaceutical Co., Ltd., Tampere, Finland), and the surfaces of the eyes were locally anesthetized (Oftan Obucain 4 mg/mL, Santen Pharmaceutical Co., Ltd., Tampere, Finland).

Intracameral injections were performed under direct ophthalmoscopic control through an operating microscope. A beveled, multiplanar self-sealing clear corneal incision[30] was performed in the opposite side of the nictitating membrane. An angled 1.8 mm slit knife was flattened against the eye, and the tip was used to enter the cornea just anterior to the vascular arcade. The blade was advanced tangentially to the corneal surface until the shoulders of the blade were fully buried in the stroma. Then, the point of the blade was redirected posteriorly so that the point and the rest of the blade enter the anterior chamber parallel to the iris. The drug was delivered into the anterior chamber through the clear conceal incision by a 34 G needle. Both eyes of each rabbit were injected with the injection volume of 5 μL/eye.

The animals were sacrificed at designated times (10, 20, 30, 60, 120, 180, and 240 min) by injecting a lethal dose of pentobarbitone (Mebunat vet 60 mg/mL, Orion Pharma, Espoo, Finland; dose 120 mg/kg) into the marginal ear vein. Immediately after death, the aqueous humor was withdrawn from the eyes. The samples were stored at −80 °C until analysis.

Aqueous Humor Samples. Analytical standards (0.1−5000 nM) were prepared from 1 mM β-blocker mix in PBS and diluted with solution containing 20% porcine aqueous humor and 80% of PBS.

Atenolol-d7 (Toronto Research Chemicals, Canada), betaxolol-d5 (Toronto Research Chemicals, Canada), and timolol-d5 maleate (Toronto Research Chemicals, Canada) were used as internal standards (ISTD). Stock solutions (1 mg/mL) were first prepared in DMSO and then diluted to the final ISTD solution containing 100 ng/mL atenolol-d7, 10 ng/mL betaxolol-d5, 10 ng/mL timolol-d5 maleate, and 1% formic acid in acetonitrile.

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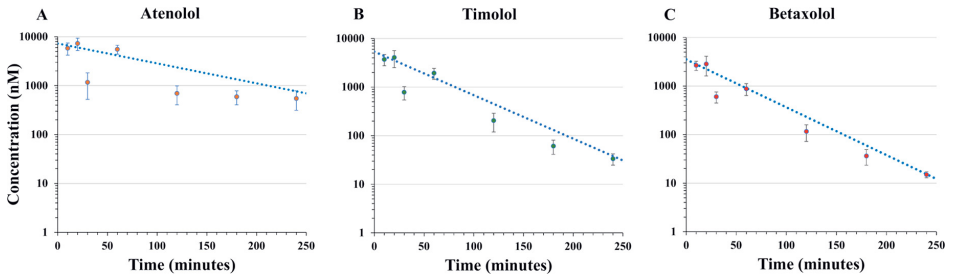

Figure 3. Concentrations of atenolol (A), timolol (B), and betaxolol (C) after intracameral injections. Each point represents the mean concentration (n = 2−4) ± standard error of the mean (concentration−time values are presented in Supporting Information Table S3). One-compartmental curve fit with1/Yhat^2 weighting is shown for each compound (blue lines).

Equal volumes (75 μL) of standard solutions and ISTD solution were mixed by vortexing for 10 s. Then the samples were incubated at room temperature for 15 min to precipitate proteins, centrifuged (5 min, +4 °C, 13 000 rpm), and supernatant was collected for LC−MS analysis. Quality control samples (2.5, 25, 250, and 1500 nM) in triplicates were prepared in the same manner.

Aqueous humor samples were first diluted 1:5 with PBS (containing 14 μL of AH + 56 μL of PBS), and then ISTD solution was added. Thereafter, processing of the samples was as described for the standards. Samples from time points 180 and 240 min were also analyzed without dilution with PBS, and these were chosen for pharmacokinetic analysis since few timolol and betaxolol concentrations in the diluted samples were below LOQ.

The standards, samples, and quality control samples were analyzed using LC−MS/MS (Agilent 1290 liquid chromatograph and Agilent 6495 triple quadruple mass spectrometer, Agilent Technologies Inc., USA). For HPLC separation Agilent Poroshell 120 SB-C18 column (2.7 μm, 2.1 mm × 50 mm) maintained at 50 °C and eluent flow rate of 0.5 mL/min were used. Eluent A was 0.1% formic acid (eluent additive for LC−MS, Fluka) in Milli-Q-water, and eluent B was methanol (Ultra Chromasolv for LC−MS, Honeywell, Riedel-de Haën). The elution gradient was as follows: 2% eluent B for 2 min, then linear rise to 100% B in 5 min, then linear decrease to 2% eluent B in 0.1 min and kept at 2% up to 9 min. Injection volume was 1 μL. Two product ions were monitored for each compound employing MRM mode (multiple reaction monitoring mode). The following MS conditions were used: capillary voltage 3.5 kV, nebulizer 25 psi, gas temperature 200 °C, gas flow 16 L/min, sheath gas heater 350 °C, sheath gas flow 11 L/min, fragmentor voltage 380 V, dwell time 200 ms, and cell accelerator voltage 5 V. The data were analyzed with Agilent MassHunter Quantitative Analysis software (Supporting Information Table S1).

The calibration curve was prepared in duplicate and calculated as a mean of two injections using 8−10 concentration levels. Standard curves had 85−115% mean accuracies compared to nominal concentration. Correlation coefficient of curves were >0.99. QC samples were 85−115% of the nominal concentrations. All measured sample concentrations were >10 nM (Supporting Information Table S2).

Pharmacokinetic Analysis. Compartmental naive pooled data analyses of aqueous humor concentration−time profile (Supporting Information Table S3) after intracameral injection of β-blockers in aqueous humor were performed using Phoenix WinNonlin (build 8.0, Certara L.P.). Values of clearance (CL_{1C}), volume of distribution (V_{dIC}), and half-life ($t_{1/2IC}$) were calculated. One-compartment and two-compartment models were used to fit the data. Different weighting schemes like uniform, 1/concentration predicted (1/Yhat), and 1/(concentration predicted)^2 (1/Yhat^2) were used for curve fitting. Residual plots, CV% (coefficient of variation: an estimate of reliability of the estimated parameter), SD were compared between the three weighting schemes. Objective function values and AIC (Akaike information criterion: a model discrimination indicator) were used to compare compartment models with the same weighting. ACD/Percepta (version 2254, Advanced Chemistry Development, Inc. Toronto, Canada) was utilized to calculate the molecular descriptors, i.e., log $D_{7.4}$, log P, number of hydrogen bond donor (HBD) groups, polar surface area (PSA), and molecular weight (MW), for the three drugs.

RESULTS

From the *in vivo* study, most of the aqueous humor samples could be used except for one eye that received accidentally the injection in the corneal endothelium (20 min) and the other two from the same animal with abnormal eyes (leading to problems in the injection) and markedly different β-blocker concentrations compared with other animals (times 20 and 60 min). A one-compartment model was used to fit the three β-blockers (two-compartment fitting was also conducted but it was unsuccessful). The 1/Yhat^2 weighting scheme was finally used because it obtained low SD, CV% values compared the other weighting schemes (for more detailed information see Supporting Information Figures S1−S3). Figure 3 shows the concentration data of all samples of atenolol, timolol, and betaxolol in the aqueous humor after intracameral administration and one-compartmental fitting. Elimination of atenolol was slower than that of timolol and betaxolol.

Table 1 compiles the physicochemical and pharmacokinetic parameters of the three β-blockers after the intracameral injections. Atenolol with the lowest log $D_{7.4}$ value (−1.85) showed the lowest clearance value of 6.44 μL/min and the longest half-life of 73.87 min. Timolol (log $D_{7.4}$ = −0.35) had faster intracameral clearance (19.30 μL/min) than atenolol but smaller than the value of betaxolol (32.20 μL/min). The results show higher clearance and volume of distribution values and shorter half-life with increasing lipophilicity of the compound.

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Table 1. Estimated Pharmacokinetic Parameters of the Drugs in Aqueous Humor after Intracameral Injections in the Rabbit Eyes[a]

drug	MW	log $D_{7.4}$	log P	PSA	HBD	V_{dIC} ± SE (μL)	CL_{IC} ± SE (μL/min)	$t_{1/2IC}$ ± SE (min)	AUC ± SE (min·nmol/μL)
atenolol	266.34	−1.85	0.24	84.5	4	687 ± 140	6.44 ± 0.83	73.87 ± 12.16	0.781 ± 0.100
timolol	316.42	−0.35	1.53	107.9	2	937 ± 172	19.30 ± 2.66	33.64 ± 2.29	0.266 ± 0.037
betaxolol	307.43	0.77	2.94	50.7	2	1421 ± 236	32.20 ± 4.10	30.58 ± 1.71	0.159 ± 0.020

[a]log $D_{7.4}$: octanol−water partition (ionized and un-ionized molecules). log P: octanol−water partition (un-ionized molecules). PSA: polar surface area. HBD: hydrogen bond donor. $V_{d,IC}$: volume of distribution after intracameral injection. CL_{IC}: clearance after intracameral injection. $t_{1/2,IC}$: half-life after intracameral injection. AUC: area under the curve. SE: standard error.

DISCUSSION

A cocktail approach was developed for the first time for ocular pharmacokinetic rabbit studies. The published rabbit concentrations in rabbit aqueous humor after clinical drug doses in eye drops[31−35] support that the observed drug concentrations of timolol and betaxolol in aqueous humor were in the clinically relevant range. Atenolol is not clinically approved in ophthalmology. Often the main concern in cocktail studies is the potential pharmacokinetic and pharmacodynamic interaction between the given drugs. Drug−drug interaction can be a concern within cocktail studies. Metabolism and protein binding are not affecting elimination from aqueous humor (no metabolites detected; the protein content in aqueous humor is minimal[36]). The three β-blockers are P-glycoprotein transporter substrates which are present in ciliary body but their quantification and cellular location are unknown. Overall we do not expect a pharmacokinetic interaction. Given our experimental setting, dose and duration of the study, we do not expect pharmacodynamics interactions either. The study provided the intracameral clearance of three drugs and their apparent volumes of distribution. A trend was seen between the pharmacokinetic parameters and lipophilicity of the drugs.

Intracameral pharmacokinetics have only been sparsely studied (see Supporting Information Table S4) even though it is an important parameter for the determination of topical ocular drug administration. Our study differs from the previous literature studies in the following ways: (1) Cocktail approach was applied for the first time to evaluate the ocular pharmacokinetics of administered drugs *in vivo*. (2) Ocular pharmacokinetic parameters CL_{IC}, V_{dIC}, and $t_{1/2\ IC}$ were evaluated for the first time for atenolol and betaxolol. (3) Timolol kinetics after intracameral injection was followed for 6 h, previously only 2 h.[27] (4) An indication of relationship was acknowledged between drug lipophilicity and intracameral clearance. Up to 5-fold range difference in clearance was seen between the three β-blockers.

Previously ocular pharmacokinetic studies have been performed using a single drug administration at a time; however this approach requires a large number of animals. We injected atenolol, timolol, and betaxolol simultaneously into the anterior chamber. This approach reduces the experimental variability, and the number of rabbits could be reduced to one-third. This is in support of 3R (reduce, refine, replace) principles in animal experiments. However, performing intracameral injection itself is challenging as the angle of injection, site of injection, and aqueous humor leaking cannot always be the same. Although the injection method was standardized using a well-established cataract surgery technique, aqueous humor leaking could not be completely avoided in every injection. The concentration values do show a drop at 30 min for all three drugs, but there was no experimental justification for excluding these concentrations. Therefore, the data were retained in the pharmacokinetics analysis. This caused some of the variability in our data (Figure 3), but the variability due to injection technique is the same for all drugs in each experiment, unlike in the experiments with a single drug administration. It also facilitates the ease with which comparisons among the compounds can be made.

Atenolol, betaxolol, and timolol are β-blockers with log $D_{7.4}$ values of −1.85, −0.35, and 0.77, respectively. The results showed a clear trend in the CL_{IC} of the three drugs related to their log $D_{7.4}$ values. The higher log $D_{7.4}$ values are associated with the higher clearance values (Figure 4). The 3 μL/min flow line in Figure 4 shows the normal aqueous humor turnover rate in rabbits. Clearance faster than 3 μL/min indicates that drug is cleared after permeating into the iris and ciliary body and vessels by the iris and ciliary body blood flows in addition to the aqueous humor outflow mechanisms.[11,22,28] Lipophilic drugs can more easily permeate into the endothelium of the blood−aqueous barrier than hydrophilic ones.[25] Atenolol showed clearance of 6.44 μL/min that is 4.8 times less than that of betaxolol. This suggests that a large fraction (47%) of atenolol is cleared through the aqueous humor outflow (3 μL/min vs 6.44 μL/min), while only 16% and 9% of timolol and betaxolol respectively are eliminated by aqueous humor outflow (3 μL/min vs 19.30 μL/min and 32.20 μL/min).

Moreover, V_{dIC} values of atenolol, timolol, and betaxolol (687 μL, 937 μL, 1421 μL) suggest that these compounds distribute into the neighboring tissues, i.e., iris−ciliary body, cornea, lens, and vitreous humor, since these values are much greater than the anatomical volume of the aqueous humor (approximately 300 μL).

Timolol has an intermediate lipophilicity; its CL_{IC} was 19.30 μL/min and half-life 33.64 min. These results are similar to the ones obtained by Yamamura and co-workers of 25.33 μL/min clearance, 57.60 min half-life, and 860 μL steady state volume of distribution for intracameral timolol.[27] We used sampling for longer time (6 h versus 2 h in Yamamura et al.'s work[27]) to fully capture the kinetic profile of timolol. Therefore, the V_{dIC} and CL_{IC} values in our study may provide more reliable estimates for timolol kinetics in the anterior chamber. This is in line with the literature showing that timolol distributes from the aqueous humor to surrounding tissues (cornea, iris−ciliary body, lens).[37−40]

The impact of log $D_{7.4}$ on clearance and volume of distribution is illustrated in (Figure 4). The plot includes the compounds investigated in the present study and the ones from intracameral pharmacokinetic studies available in the literature[41,42,11,24,27,43,44] (for more detailed information see Supporting Information Table S4). Figure 4 shows that very hydrophilic drugs such as vancomycin and atenolol are eliminated predominantly via aqueous humor outflow (CL values slightly faster than 3 μL/min), whereas at higher

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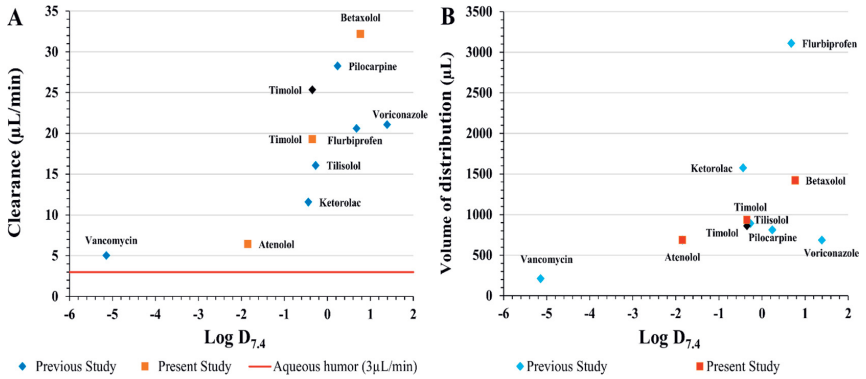

Figure 4. The log $D_{7.4}$ vs pharmacokinetic parameters of IC administered drugs. Both panels show nine compounds, i.e., betaxolol, atenolol, timolol (from the present study presented by orange squares) and compounds reported in the literature such timolol[27] (black diamond symbol) and vancomycin,[42] pilocarpine,[11,24] voriconazole,[43] flurbiprofen,[41] ketorolac,[44] tilisolol[27] (blue diamonds). Panel A shows log $D_{7.4}$ vs clearance of drugs after IC administration. The red line in the graph represents the aqueous humor drug clearance value of 3 μL/min through trabecular meshwork. Panel B shows log $D_{7.4}$ vs volume of distribution of drugs. More detailed information on the IC pharmacokinetic parameters are obtained in the Supporting Information Table S4.

lipophilicity values, the clearance increases several-fold since these drugs are able to potentially permeate more effectively into the iridial and ciliary body endothelial barrier, reaching the blood circulation with blood flows of 62 μL/min in iris and 82 μL/min ciliary body.[15,19] It seems the clearance is permeability-limited since values are below the iris and ciliary body blood flows.

Moreover, the clearance shows a better correlation with log $D_{7.4}$ than the volume of distribution (Figure 4B). The clearance and volume of distribution increase are not fully in line with the log $D_{7.4}$ increase because other factors, such as hydrogen bond donor groups, melanin binding (pigmented rabbits)[12,29,45,26] and partitioning to the lens, may also contribute to clearance and volume of distribution.[29] Correlation to other molecular descriptors was also investigated, proving to be poor (see Supporting Information Figure S4).

In addition to ocular bioavailability after topical drug delivery, clearance and volume of distribution also affect drug concentrations in the anterior chamber. It is important to realize that increasing drug lipophilicity leads to higher corneal permeability and ocular bioavailability after eye drop administration. Nevertheless, lipophilicity also increases the clearance and volume of distribution in the anterior chamber. The 5-fold range in clearance and about 1.5-fold range in volume of distribution are very significant. Therefore, it is important to take intracameral pharmacokinetics into account when designing drugs for ocular administration. This study will help the model building to estimate aqueous humor drug concentrations after topical, subconjunctival, and intravitreal administrations.

CONCLUSION

Intracameral injection of a mix of three β blocking drugs was successfully performed showing that the cocktail approach is feasible in ocular pharmacokinetic studies. Pharmacokinetic parameters of atenolol, timolol, and betaxolol demonstrated a strong dependence of intracameral clearance on the drug lipophilicity (about 5-fold range). The contribution of aqueous humor outflow in ocular elimination ranges from ≈50% (atenolol) to ≈10% (betaxolol) suggesting that most of the drug dose is eliminated by the blood flow of iris and ciliary body. The data can be utilized in building pharmacokinetic models for evaluation and prediction of drug kinetics after ocular administration. The cocktail approach can speed up ocular pharmacokinetic studies and reduce the use of animals.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.9b01024.

Ion exchange monitored with LC−MS; calibration curves of β-blockers for aqueous humor samples; compound list for previous intracameral studies and their pharmacokinetic parameters; pharmacokinetic analysis with one-compartment model for atenolol, timolol, and betaxolol; correlation plots between molecular descriptors versus primary pharmacokinetic parameters (PDF)

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Supporting Information

Ocular Intracameral Pharmacokinetics for a Cocktail of Timolol, Betaxolol, and Atenolol in Rabbits

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Table S1. Ions monitored with LC-MS.

Compound	Qualifier/Quantifier	Precusor Ion (m/z)	Product Ion (m/z)	CE (V)
timolol-d5	Quantifier	322	266	15
timolol-d5	Qualifier	322	79	28
timolol	Quantifier	317	261	20
timolol	Qualifier	317	74.2	20
betaxolol-d5	Quantifier	313	121	25
betaxolol-d5	Qualifier	313	79	30
betaxolol	Quantifier	308.01	116.1	25
betaxolol	Qualifier	308.01	72.1	29
atenolol-d7	Quantifier	274.01	145.1	28
atenolol-d7	Qualifier	274.01	79.2	20
atenolol	Qualifier	267.01	190	21
atenolol	Quantifier	267.01	145	29

Table S2. Calibration curves of beta-blockers for aqueous humor samples.

Compound	Range of calibration curve (nM)	Curve fitting, weighting
Timolol	2.5 - 2000	Quadratic, $1/x^2$
Betaxolol	2.5 - 2000	Quadratic, 1/x
Atenolol	10 - 2000	Quadratic, $1/x^2$

Table S3. Concentration measurement in nM after 5µL intracameral administration of 3 mM cocktail of three beta-blockers.

Rabbit ID	Eye	Dose nmoles	Time point (min)	Timolol Conc (nM)	Betaxolol Conc (nM)	Atenolol Conc (nM)
1	L	5	10	1371	1407	1749
2	L	5	10	5593	3443	9532
8	L	5	10	4918	3833	6922
9	L	5	10	2963	1993	5175
10	L	5	20	5623	4084	9373
11	L	5	20	2518	1612	5245
1	R	5	30	626	473	692
2	R	5	30	750	735	672
8	R	5	30	1359	900	2863
9	R	5	30	391	300	478
3	R	5	60	2560	1171	6208
10	R	5	60	2311	1068	7107
11	R	5	60	955	400	3332
5	R	5	120	446	231	1530
5	L	5	120	197	134	413
12	R	5	120	117	57	629
12	L	5	120	59	41	216
6	R	5	180	46	24	983
6	L	5	180	67	33	680
13	R	5	180	19	15	83
13	L	5	180	113	73	633
7	R	5	240	25	12	280
7	L	5	240	47	20	743
14	R	5	240	13	11	61
14	L	5	240	49	17	1107

Table S4. List of the compounds investigated in pharmacokinetic studies after intracameral injection in rabbit eyes and the corresponding pharmacokinetic parameters, either reported in the original paper and/or calculated with the standard equations (method O) or calculated using Phoenix WinNonlin software and the reported concentrations (method C). GetData Graph Digitizer software (version 2.22. Digital River, Inc., Cologne, Germany) was used in method C when only graphs were available from the original articles. Log P and $logD_{7.4}$ are calculated using ACD/Percepta (version 2254) except for inulin (*) that was obtained from drug bank database. (1) Vd_{IC} corresponds to Vss for 2-compartment model and V_1(central compartment) for one-compartment models.

Compound	Log P	$LogD_{7.4}$	Dose (µg)	Rabbit	Method	Number of compartments	Cl_{IC} (µL/min)	Vd_{IC}^{1} (µL)	$t_{1/2\,IC}$ (min)	References
Vancomycin	-2.00	-5.14	4	albino	C	1	5.04	210	28.86	[32]
Ketorolac	2.58	-0.44	254	albino	C	2	11.56	1574	118.42	[34]
Atenolol	0.24	-1.85	1.33	albino	C	1	6.44	687	73.87	Present study
FITC-Dextran (MW 4400)	**	**	200	albino	O	1	7.63	477	-	[27]
Pilocarpine	0.39	0.24	0.415	albino	O	-	33.11	571	-	[11]
			0.2083	albino	O	-	34.74	579	-	
			0.2083	pigmented	C	1	35.83	1192	23.16	
			0.2083	albino	C	2	9.39	906	89.14	[24]
Timolol	1.53	-0.35	5	albino	O	-	25.33	860	57.60	[27]
			1.58	albino	C	1	19.30	937	33.64	Present study
Tilisolol	1.85	-0.27	4.5	albino	O	-	16.06	891	43.20	6
Inulin	- 62 *	-	0.5	albino	O	-	5.14	286	-	[11]
			1	albino	O	-	3.23	294	-	
			2	albino	O	-	5.60	280	-	

Flurbiprofen	3.82	0.68	30	albino	C	2	13.73	2070	210.47	[31]
			60	albino	C	2	27.47	4150	210.47	
Betaxolol	2.87	0.77	1.53	albino	C	1	32.20	1421	30.58	Present study
Voriconazole	1.39	1.39	25	albino	O	1	21.07	685	22.53	[33]

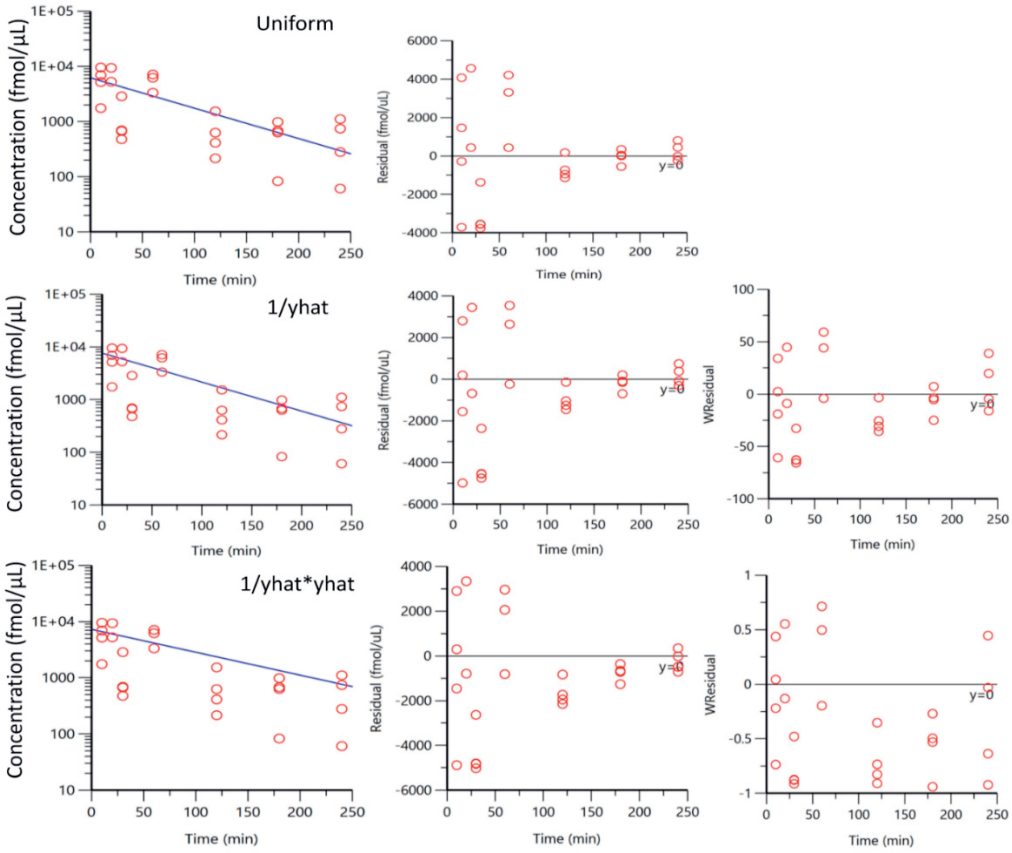

Figure 1: Pharmacokinetic analysis with 1 compartment model: Atenolol semi-log plot and residual plots for uniform, 1/yhat weighting, 1/yhat*yhat weighting.

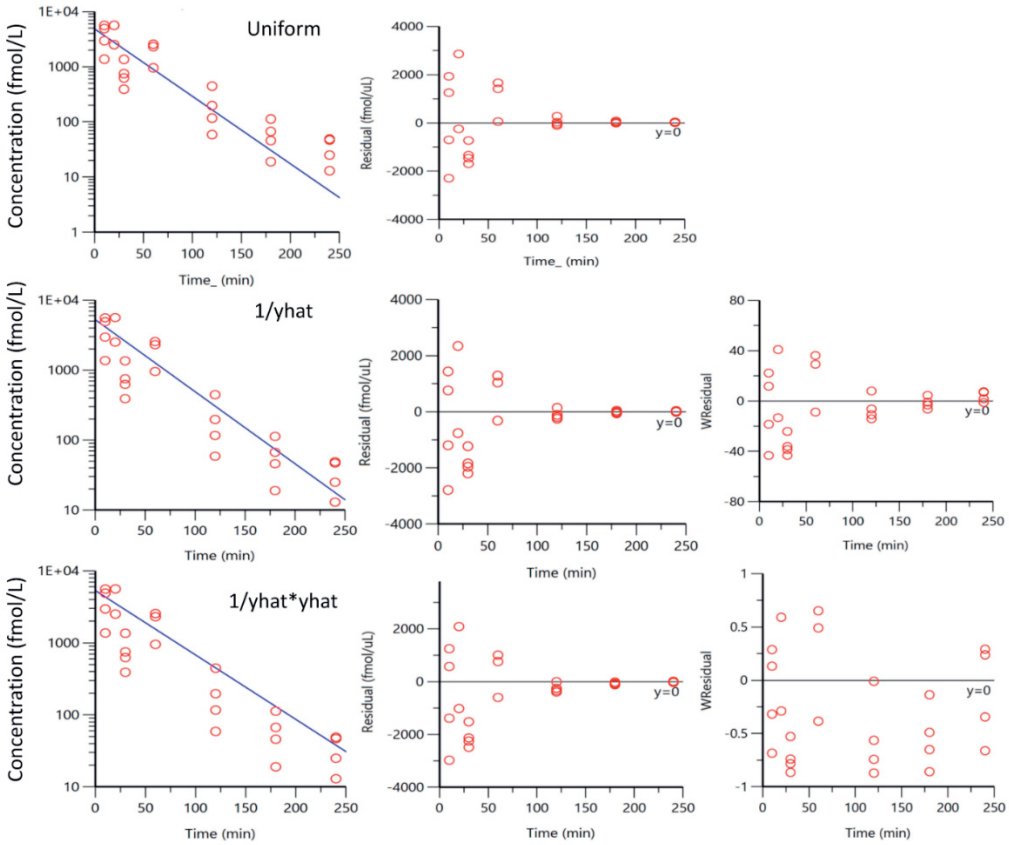

Figure 2: Pharmacokinetic analysis with 1 compartment model: Timolol semi-log plot and residual plots for uniform, 1/yhat weighting, 1/yhat*yhat weighting.

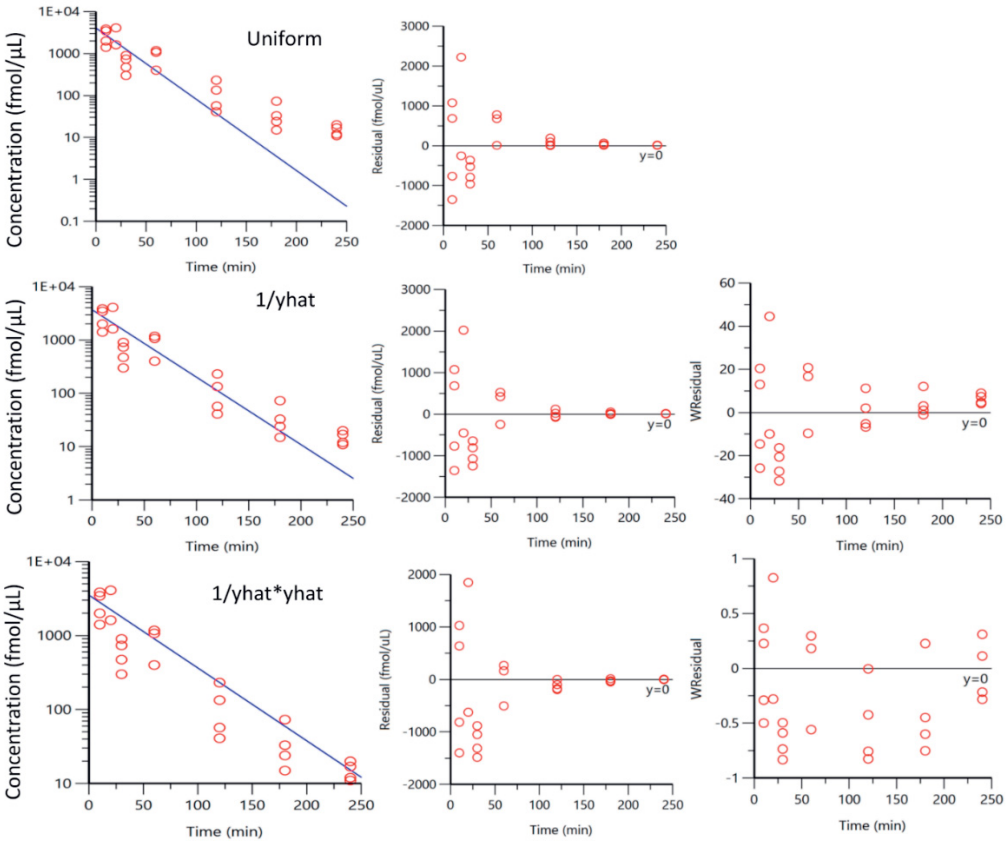

Figure 3: Pharmacokinetic analysis with 1 compartment model: Betaxolol semi-log plot and residual plots for uniform, 1/yhat weighting, 1/yhat*yhat weighting.

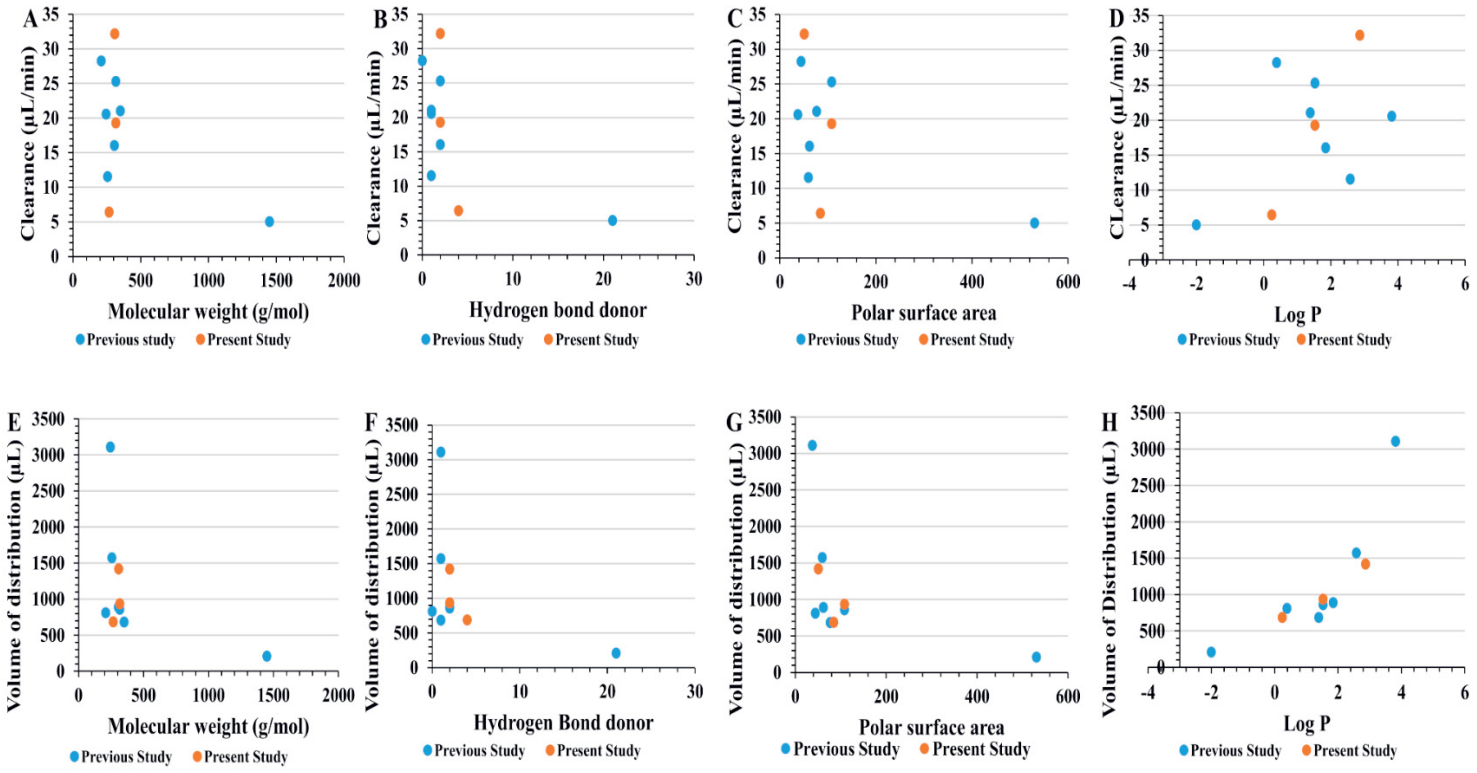

Figure 4: Correlation plots between clearance (A-D) and volume of distribution (E-H) versus molecular weight, hydrogen bond donor, polar surface area and log P.

II

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Topical ocular pharmacokinetics and bioavailability for a cocktail of atenolol, timolol and betaxolol in rabbits

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ABSTRACT

Ocular bioavailability after eye drops administration is an important, but rarely determined, pharmacokinetic parameter. In this study, we measured the pharmacokinetics of a cocktail of three beta blockers after their topical administration into the albino rabbit eye. Samples from aqueous humour were analysed with LC-MS/MS. The pharmacokinetic parameters were estimated using compartmental and non-compartmental analyses. The ocular bioavailability was covering broad range of values: atenolol (0.07 %), timolol (1.22%, 1.51%) and betaxolol (3.82%, 4.31%). Absolute ocular bioavailability presented a positive trend with lipophilicity and the values showed approximately 60-fold range. The generated data enhances our understanding for ocular pharmacokinetics of drugs and may be utilized in pharmacokinetic model building in ophthalmic drug development.

1. Introduction

Topical administration is currently the most common route for the treatment of diseases affecting the anterior part of the eye. Ocular bioavailability after topical administration is stated to be less than 10 %, but bioavailability has been determined only for four compounds in rabbits and not at all in humans. Several pre-corneal factors, such as the drainage of excess fluid, normal tear turnover and systemic absorption through conjunctiva remove the drug from the ocular surface decreasing the drug absorption into intraocular tissues (Himmelstein et al., 1978; Lee and Robinson, 1979). Flow of drug solution into the nasolacrimal duct leads to further systemic absorption from the nasal mucosa and gastrointestinal tract (Himmelstein et al., 1978; Lee and Robinson, 1979; Urtti and Salminen, 1993).

The main ocular absorption routes after topical administration are across the cornea and conjunctiva (Fig. 1). After corneal absorption the drug permeates into the aqueous humour and further into the iris and ciliary body followed by elimination to the systemic circulation. Drug may also be eliminated by aqueous humour turnover into the trabecular meshwork and Schlemm's canal or distribute into the lens. Transfer of drug towards the vitreous humour is hindered by the aqueous humour flow in the posterior-to-anterior direction (Maurice and Mishima, 1984). Drug may also absorb into the eye across the conjunctiva and sclera and then distribute further into the iris and ciliary body (Ahmed et al., 1987; Ahmed and Patton, 1985). Most of conjunctival drug permeation leads to systemic circulation instead of intraocular distribution.

The corneal permeation is the most important ocular absorption route for lipophilic drugs (Doane et al., 1978; Schoenwald, 1987). The corneal epithelium is the main penetration barrier in the cornea (Lach et al., 1983; Schoenwald, 1987; Schoenwald, 1990; Schoenwald and Huang, 1983). Trans-conjunctival drug absorption contributes very little to drug concentrations in the aqueous humour (Ahmed et al., 1987; Ahmed and Patton, 1985) which is the main site in the assessment of topical ocular bioavailability.

The absolute ocular bioavailability is the ratio of the dose-

Abbreviations: AUC_{inf}, area under the curve from time zero to infinity; CA, compartmental analysis; CL, clearance; C_{max}, the maximal concentration; CV%, coefficient of variation; IC, intracameral administration; ISTD, internal standards; LC-MS/MS, liquid chromatography with tandem mass spectrometry; log $D_{7.4}$, logarithm of the octanol-water distribution coefficient at pH 7.4; NCA, non- compartmental analysis; SE, standard error of estimates; $t_{1/2}$, elimination half-life; t_{max}, the time at maximal concentration; Top, topical administration; Vd, volume of distribution

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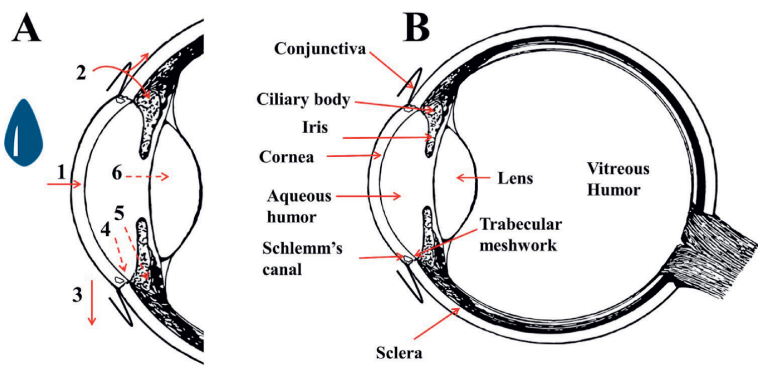

Fig. 1. Ocular pharmacokinetics after topical administration. A: Absorption and elimination pathways after topical drug administration: **1)** corneal absorption; **2)** conjunctival absorption; **3)** clearance through nasolacrimal duct; **4)** elimination to trabecular meshwork; **5)** distribution to iris-ciliary body; **6)** distribution to lens. B: Anatomy of the eye. (https://www.publicdomainpictures.net/en/view-image.php?image=130389&picture= medical-eye).

normalized areas under the concentration curve (AUC) in aqueous humour after topical and intracameral administration, respectively. In the latter case, the drug is directly injected into the anterior chamber and this situation represents 100% bioavailability.

In the present study we determined the topical pharmacokinetics for three anti-glaucoma drugs, atenolol, timolol, and betaxolol that represent a range of lipophilicity values (Fig. 2). These beta-blockers were applied topically as a cocktail on rabbit eyes, aqueous humour drug concentrations were quantified and pharmacokinetic parameters were determined including absolute ocular bioavailability (with intracameral pharmacokinetic data from our previous study (Fayyaz et al., 2019)).

2. Material and Method

2.1. Animal experiments

Animals - Sixteen male albino New Zealand rabbits, age 3–6 months and weight 2.8–3.2 kg, were used in the experiments. The animals were housed in a temperature and humidity-controlled environment with a 12/12 light/dark cycle. The animals were individually housed and fed a normal diet. All rabbits underwent an ocular examination before being accepted into experiments. Animals were handled in accordance with the statement of the Animals in Research Committee of the ARVO (Association for Research in Vision and Ophthalmology, Rockville, Maryland, USA) and all animal experiments were approved by the national Animal Experiment Board of Finland.

Topical application of the beta-blocker cocktail was performed followed by a collection of a single aqueous humour sample from each animal. The sampling times were 5, 10, 20, 30, 60, 120, 180 and 240 min, and the number of eyes at each time point were four (n = 4). The cocktail containing 20 mM atenolol (USP reference standard, Sigma), 10 mM betaxolol hydrochloride (USP reference standard, Sigma) and 10 mM timolol maleate (USP reference standard, Sigma) in phosphate-buffered saline (DPBS, Thermofisher Scientific) (pH adjusted to 7.4; 322 mOsm/kg) was administered onto the upper cornea-scleral limbus of both eyes (25 µL/eye) in each rabbit. The animals were sacrificed by injecting into the marginal ear vein a lethal dose of pentobarbital (Mebunat vet 60 mg/mL; Orion Pharma, Finland) and aqueous humour was aspirated from anterior chamber. All samples were cooled on ice following storage at -80 °C until analysis.

2.2. Analysis of aqueous humour samples

Standards (0.1 – 5000 nM) were prepared from the beta blocker mixture in PBS and diluted with a solution containing 20% porcine aqueous humour and 80% PBS. Atenolol-d7 (Toronto Research Chemicals, Canada), betaxolol-d5 (Toronto Research Chemicals, Canada) and Rac timolol-d5 Maleate (Toronto Research Chemicals, Canada) were used as internal standards (ISTDs). The 1 mg/mL stock solutions were first prepared in DMSO and then diluted to ISTD solution containing 50 ng/mL atenolol-d7, 5 ng/mL betaxolol-d5, 5 ng/mL rac timolol-d5 maleate and 1% formic acid in acetonitrile.

Equal volumes (50 µL) of standard solutions and ISTD solution were mixed by vortexing for 10 sec. After 15 min precipitation step the standards were centrifuged (5 min, +4°C, 13000 rpm) and supernatant was collected for LC-MS analysis. Quality controls (2.5, 25, 250 and 1500 nM) in triplicates were prepared in similar manner. Aqueous humour samples were first diluted 1:5 with PBS and then ISTD solution

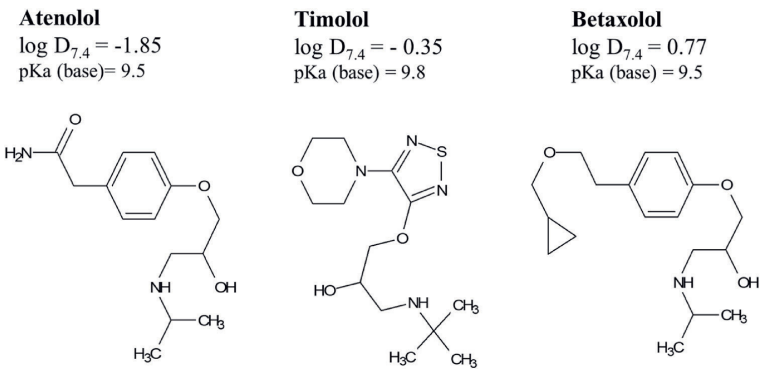

Fig. 2. The chemical structures of atenolol, timolol and betaxolol with the corresponding logarithm of the octanol-water distribution coefficient at pH 7.4 (log $D_{7.4}$) values and pKa values (calculated using ACD/labs, version 2020.1.1, Advanced Chemistry Development, Inc. Toronto, Canada) at pH $_{7.4}$ the relative abundance for ionized fraction versus unionized is > **99/1** for the three drugs.

was added. Thereafter, the procedure was similar to the handling of standards. The standards, samples and quality controls were analysed with LC-MS/MS (Agilent 1290 liquid chromatograph and Agilent 6495 triple quadrupole mass spectrometer, Agilent Technologies Inc., USA) using protocol described earlier (Fayyaz et al., 2019).

The calibration curve was prepared as duplicate and calculated as a mean of two injections using 9–11 concentration levels of total 14 levels. Calibration curves had 85 - 115 % mean accuracies. QC samples were 90 - 110% of the nominal concentrations with imprecision below 10%.

2.3. Pharmacokinetic analysis

Compartmental analysis was performed using Phoenix WinNonlin (build 8.1, Certara L.P.). Mean concentration data was analysed using one- and two-compartment models with first-order absorption kinetics. Akaike's information criterion and visual inspection of the plot of observed and predicted concentrations versus time were used to select the best compartmental model. Curve fitting was performed using three different weighting schemes: uniform, 1/predicted concentration (1/Yhat) and 1/(predicted concentration)^2 (1/Yhat^2). Coefficient of variation (CV%) of estimated parameters and residual plots were utilized for choosing the best weighting scheme within the same compartmental model. AUC from time zero to infinity ($AUC_{inf,Top}$), the maximal concentration ($C_{max,Top}$), time at the maximal concentration ($t_{max,Top}$) and elimination half-life ($t_{1/2,Top}$) were obtained following topical application of the three drugs. Non-compartmental analysis was also performed using mean concentrations and the linear trapezoidal rule (Supplementary data Table S3).

Aqueous humour bioavailability for the three beta-blockers was calculated according to Eq. 1:

$$\textit{Bioavailability} = \frac{AUC_{inf,Top} \times Dose_{IC}}{AUC_{inf,IC} \times Dose_{Top}} \quad (1)$$

where Top and IC refer to topical and intracameral administration, respectively. Data for IC administration were taken from our previous study (Fayyaz et al., 2019).

3. Results

3.1. Topical pharmacokinetic parameters

The mean aqueous humour concentration data was fitted for the three beta blockers. One-compartment model was the best structural model, using the 1/Yhat^2 weighting model for all three drugs. The final model estimated concentrations are presented in Fig. 3 together with the observed data. The more lipophilic compounds betaxolol and timolol achieved higher aqueous humour concentrations than the more hydrophilic compound atenolol. Five measured aqueous humour concentrations across the three drugs were excluded from the pharmacokinetics analysis since they were considered to be outliers (Supplementary data Table S1). The pharmacokinetic parameters estimated from the compartmental analysis are listed in Table 1.

The $AUC_{inf,Top}$ of betaxolol was 12 and 2 times higher than the dose-normalized values of atenolol and timolol, respectively and similar trends are seen for $C_{max,Top}$ (Table 1). The half-life of atenolol in the aqueous humour was 3–5 times longer than the half-lives of timolol and betaxolol.

3.2. Absolute topical bioavailabilities

The dose-normalized comparison of the concentration profiles of the three beta-blockers after topical and intracameral administration in rabbit eye (Fayyaz et al., 2019) is presented in Fig. 4.

Drug bioavailability values in aqueous humour from our topical pharmacokinetic study and the previous intracameral study (Fayyaz et al., 2019) are presented in Table 2. The order of bioavailabilities is betaxolol > timolol > atenolol based on both compartmental and non-compartmental analyses. The results show a substantial 55–62-fold difference between the bioavailability of betaxolol (3.82%, 4.31%) and atenolol (0.07%).

4. Discussion

A cocktail approach was used to determine the ocular exposure of three drugs after topical administration. This approach reduces the number of animals needed and reduces variability arising due to inter-individual differences and analytical factors. Previously, we have used the same approach to investigate the intracameral pharmacokinetics of the same drug set in the rabbit eye (Fayyaz et al., 2019). The combination of both studies allows us to determine the absolute bioavailability in aqueous humour for betaxolol, timolol and atenolol. Both compartmental analysis and non-compartmental analysis were carried out and yielded similar bioavailability values showing robust results. Ocular bioavailability is typically determined for aqueous humour, even though ciliary body is the target tissue for beta blocker anti-glaucoma drugs. The reason is that bioavailability calculation requires a direct injection into the investigated tissue for the determination of the drug clearance from the tissue. This is not feasible for iris-ciliary body, while it is possible for aqueous humour after intracameral injection.

Bioavailability is critically important parameter that provides useful information on the ocular exposure of different drugs and drugs in different formulations. Bioavailability shows the drug fraction absorbed in aqueous humour, while the concentration curves in the aqueous humour after topical administration are not only affected by absorption but also by the clearance from the aqueous humour (CL_{IC} in Table 2, Fig. 4). Unfortunately, there are only a few studies that report pharmacokinetics for both topical and intracameral administration of ophthalmic drugs (Ling and Combs, 1987; Tang-Liu et al., 1984; Yamamura et al., 1999) allowing the determination of absolute drug

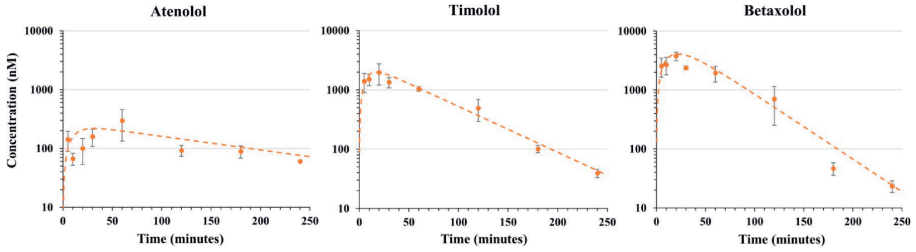

Fig. 3. Aqueous humour concentration-time profiles in rabbits after topical application of atenolol (dose = 500 nmol), timolol (dose = 250 nmol) and betaxolol (dose = 250 nmol) in a cocktail. Each circle represents the mean concentration ± standard error of the mean (n = 3–4). The best fits based on one-compartmental first-order pharmacokinetic model are represented by the dashed line.

Table 1
Compartmental analysis of aqueous humour concentrations after topical administration of atenolol (dose = 500 nmol and dose-normalized values[a]), timolol (dose = 250 nmol) and betaxolol (dose = 250 nmol) in rabbits. SE = standard error of the estimates.

Drug	Dose (nmol)	$AUC_{inf,Top}$ ± SE (min*nmol/ mL)	$C_{max*Top}$ ± SE (nmol/mL)	$t_{max,Top}$ ± SE (min)	$t_{1/2,Top}$ ± SE (min)
Atenolol	500 250[a]	48.6 ± 15.8 24.3[a]	0.22 ± 0.06 0.11[a]	31.6 ± 15.5	130.4 ± 78.4
Timolol	250	152 ± 14	1.99 ± 0.20	17.3 ± 3.7	38.9 ± 2.9
Betaxolol	250	280 ± 47.8	4.07 ± 0.69	21.9 ± 5.1	27.3 ± 3.3

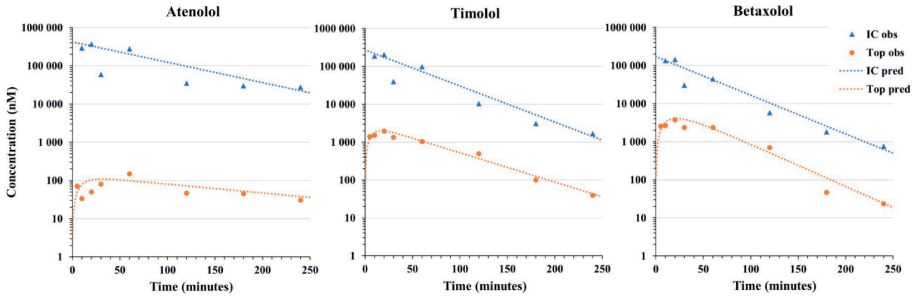

Fig. 4. Aqueous humour concentration-time profiles of atenolol, timolol and betaxolol after topical and intracameral administration. The concentrations have been normalized to the dose of 250 nmol.

Table 2
Aqueous humour bioavailability of atenolol, timolol and betaxolol using compartmental and non-compartmental analyses (CA: compartmental analysis, NCA: non-compartmental analysis).

Drugs	Topical administration			Intracameral administration[a]					Bioavailability (%)	
	Dose (nmol)	$AUC_{inf,Top}$ ± SE (min*nmol/ mL) CA	NCA	Dose (nmol)	CL_{IC} ± SE (µL/min)	Vd_{IC} ± SE (µL)	$AUC_{inf,IC}$ ± SE (min*nmol/ mL) CA	NCA	CA	NCA
Atenolol	500	48.6 ± 15.8	39.2	5	6.44 ± 0.83	687 ± 140	691 ± 141	545	**0.07**	**0.07**
Timolol	250	152 ± 14	151	5	19.3 ± 2.66	937 ± 172	248 ± 52	199	**1.22**	**1.51**
Betaxolol	250	280 ± 47.8	252	5	32.2 ± 4.10	1421 ± 236	146 ± 27	117	**3.82**	**4.31**

Vd_{IC}: Volume of distribution after intracameral injection
CL_{IC}: Clearance after intracameral injection
[a] Fayyaz et al., 2019

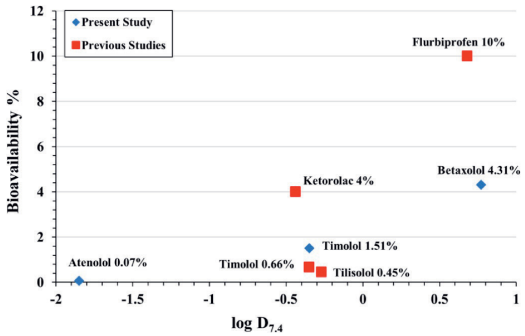

Fig. 5. Bioavailability versus log $D_{7.4}$ of ophthalmic topical drugs in rabbit eyes of six drugs i.e. atenolol, timolol and betaxolol from present study (blue diamonds, bioavailability determined using non-compartmental analysis) and ketorolac (Ling and Combs, 1987), flurbiprofen (Tang-Liu et al., 1984), timolol and tilisolol (Yamamura et al., 1999) (bioavailability determined from non-compartmental analysis) from the literature (red squares).

bioavailability to the aqueous humour. Compiling our data with these literature studies shows a wide range of ocular bioavailabilities ranging from 0.07 to 10 % (Fig. 5). A positive trend of ocular bioavailability can be seen with lipophilicity (log $D_{7.4}$) as lipophilic compounds tend to have a higher ocular bioavailability than more hydrophilic compounds (Fig. 5) presumably due to the higher permeability in the cornea (Kidron et al., 2010). Plotting aqueous humour pharmacokinetic parameters ($C_{max,Top}$, $AUC_{inf,Top}$, bioavailability) against corneal permeability or cornea/conjunctiva permeability ratios in rabbit (Wang et al., 1991) results in excellent correlation (Supplementary data Figure S3 and S4). Several studies have pointed out the importance of drug lipophilicity on corneal permeability (Chien et al., 1990; Lach et al., 1983; Rusinko et al., 2007; Schoenwald, 1987; Schoenwald and Huang, 1983; Wang et al., 1991), but it is important to note that ocular bioavailability of lipophilic drugs is also limited by their fast penetration across the conjunctiva to the systemic circulation (Ramsay et al., 2018; Wang et al., 1991). Corneal penetration may also be influenced by other factors such as drug ionization, molecular size and medium (pH, osmotic pressure, other components) (Brechue and Maren, 1993; Kidron et al., 2010; Pescina et al., 2015).

Since bioavailability data for other beta-blockers are missing, we compared the dose-normalized $AUC_{inf,Top}$ and $C_{max,Top}$ values of drug sets in which timolol was investigated (Araie et al., 1982; Huang et al.,

1983; Ohtori et al., 1998; Ros et al., 1980; Schmitt et al., 1981; Yamamura et al., 1999). We compared the beta-blocker/timolol parameter ratios within each study (Supplementary data Table S2 and Figure S1) and observed higher $AUC_{inf,Top}$ ratio for befunolol (Araie et al., 1982), but not for tilisolol (Yamamura et al., 1999), even though both compounds are more lipophilic than timolol (Supplementary data Figure S1). Based on these pharmacokinetic parameters, we cannot conclude that higher lipophilicity necessarily results in increased drug concentrations in the aqueous humour because clearance from aqueous humour through iris-ciliary body to systemic circulation (Fig. 1) is also faster for more lipophilic compounds (Fayyaz et al., 2019). The faster clearance and shorter half-lives are also shown in Table 2 and Fig. 4. In order to quantitatively understand topical ocular pharmacokinetics, determination of bioavailability is essential.

The data variability amongst topical ocular drug studies is significant and this supports using a cocktail approach that allows generation of reliable and comparable values within the drug set. Moreover, the validity of this approach is proved when showing that one of the cocktail drugs, timolol, presents comparable pharmacokinetics to the ones reported in the literature (Supplementary data Figure S2). Even in our study some drug concentrations at 5 min were unexpectedly high compared with the later time points, especially for atenolol (Fig. 3, Supplementary data Table S1). We cannot exclude the possibility that these aqueous humour samples contained drug traces from tear fluid even though we tried to avoid the cross-contamination of the aqueous humour samples by careful sampling. In any case, these early samples have a minimal contribution to the values of $AUC_{inf,Top}$ and bioavailability, thereby they do not have any influence on our conclusions.

The present data generated for atenolol, timolol and betaxolol can be used to aid further studies with these drugs. Exact values for ocular bioavailability can be useful aid in the development of new drug delivery systems (Subrizi et al., 2019).

Conclusion

Three beta blockers were administered topically and their ocular pharmacokinetics were evaluated. Absolute bioavailability of atenolol, timolol and betaxolol was quantitated in aqueous humour. The data shows broad, about 60-fold, range of bioavailability for topical beta blocking agents. The outcomes of this study is for improved understanding on ocular pharmacokinetics and may inform ophthalmic topical drug dosing and drug development.

CRediT authorship contribution statement

Anam Fayyaz: Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing. **Veli-Pekka Ranta:** Methodology, Writing - review & editing. **Elisa Toropainen:** Methodology, Investigation. **Kati-Sisko Vellonen:** Methodology, Investigation. **Annika Valtari:** Methodology, Investigation. **Jooseppi Puranen:** Methodology, Investigation. **Marika Ruponen:** Project administration, Investigation, Writing - review & editing. **Iain Gardner:** Supervision, Writing - review & editing. **Arto Urtti:** Conceptualization, Supervision, Writing - review & editing, Resources. **Masoud Jamei:** Conceptualization, Supervision, Writing - review & editing, Resources. **Eva M. del Amo:** Conceptualization, Validation, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

Anam Fayyaz, Masoud Jamei, and Iain Gardner are employees of Certara UK Limited, Simcyp Division.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2020.105553.

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Supplementary material

Topical ocular pharmacokinetics and bioavailability for a cocktail of atenolol, timolol and betaxolol in rabbits

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Table S1. Aqueous humour concentrations in rabbits after 25 µL topical administration of 500 nmol of atenolol and 250 nmol of timolol and betaxolol. The concentrations marked with an asterix (*) were considered to be clear outliers, and they were excluded from the pharmacokinetic analysis.

Time point	Atenolol Conc	Timolol Conc	Betaxolol Conc
(Min)	(nM)	(nM)	(nM)
5	15	1355	2571
5	261	1843	3527
5	192	2352	4111
5	102	34	15
10	88	962	1206
10	28	1192	1862
10	95	1447	2434
10	57	2446	5193
20	23	1305	2541
20	140	3419	4518
20	25	127	90*
20	214	3014	4234
30	71	1263	2020
30	219	701	2451
30	276	1979	2772
30	72	1453	2283
60	103	1035	1561
60	23726*	6347*	3693
60	165	873	1337
60	624	1209	1195
120	103	320	323
120	62	198	104
120	144	1084	2036
120	63	369	336

180	58	71	20
180	66	92	75
180	150	102	40
180	83	139	53
240	64	1536*	2435*
240	61	39	20
240	59	29	17
240	58	51	34

Table S2. A literature review (Araie et al., 1982; Huang et al., 1983; Ohtori et al., 1998; Ros et al., 1980; Schmitt et al., 1981; Yamamura et al., 1999) was carried out to analyze the available topical pharmacokinetic studies in the albino rabbit eyes with drug sets which include timolol. Five studies were selected, and $AUC_{inf,Top}$ and $C_{max,Top}$ ratio were estimated using non-compartmental analysis after dose-normalization (0.5 % solution and 25 µL drop) to compare to our study. However, only two studies could be used to compare the trend between lipophilicity of the drug and $AUC_{inf,Top}$ and $C_{max,Top}$ ratio values (Figure S1) (Araie et al., 1982, Yamamura et al., 1999). Huang and coworkers was not a single but multidose study (Huang et al., 1983). Schmitt et al. (Schmitt et al., 1981) used 0.5% of methylcellulose to increase the solubility of the compounds topically administered. These may affect the reliability of the pharmacokinetic parameters of the investigated drugs. Moreover, Ros and co-workers (Ros et al., 1980) investigated only three time points of the concentration profiles of a wider drug set (data not shown), only for metoprolol and timolol the $AUC_{inf,Top}$ and $C_{max,\ Top}$ with non-compartmental analysis could be estimated with the limitation that the last

sampling was already at time sixty minutes. The pharmacokinetic study from Ohtori et al. (Ohtori et al., 1998) which included carteolol and timolol was excluded due to unreliable concentration profiles determined by microdialysis.

(*) $AUC_{last,Top}$

References	Drugs	log $D_{7.4}$	$AUC_{inf,\ Top\ of\ drug}$ / $AUC_{inf,\ Top\ timolol}$	$C_{max,\ Top\ drug}$/ $C_{max,\ Top\ timolol}$
Present study	Betaxolol	0.77	1.60	1.17
	Timolol	-0.35	1	1
	Atenolol	-1.85	0.15	0.11
Yamamura et al., 1999	Tilisolol	-0.27	0.93	1.29
Araie et al., 1982	Befunolol	-0.16	1.67	1.38
Huang, Schoenwald et al. 1983	Bufuralol	1.51	0.37	0.75
	Acebutolol	-0.4	0.09*	0.04
Schmitt et al., 1981	Propranolol	1.51	0.76	1.75
	Alprenolol	0.68	1.38	2.08
	Oxprenolol	0.18	1.75	3.67
	Practolol	-1.27	3.14	1.92
Ros et al., 1980	Metoprolol	-0.29	0.54*	0.50

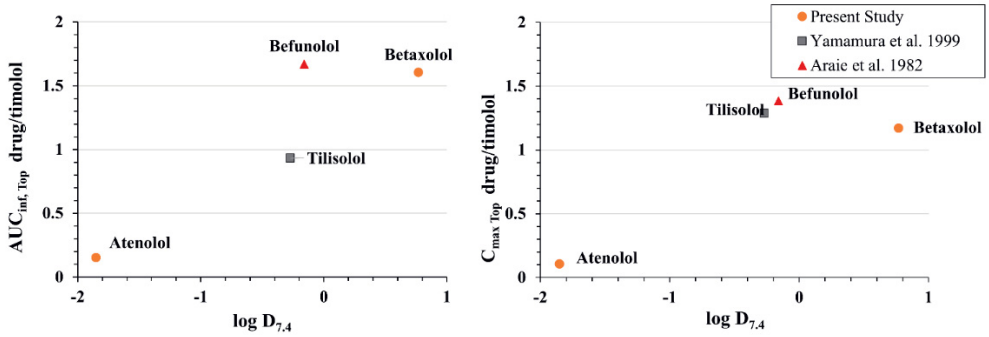

Figure S1. Lipophilicity versus $AUC_{inf,Top}$ and $C_{max,Top}$ ratio values from the drugs of the curated results from table S2 and the present study.

Table S3. Non-compartmental analysis for aqueous humour of three beta-blockers after topical administration into rabbit eye.

Parameters	Atenolol	Timolol	Betaxolol
Dose (nmol)	500	250	250
$AUC_{inf, Top}$ (min*nmol/ml)	39.24	150.67	252.20
$C_{max, Top}$ (nmol/ml)	0.297	1.966	3.764
$t_{max, Top}$ (minute)	60	20	20
$t_{1/2, Top}$ (minutes)	86.98	36.62	28.60

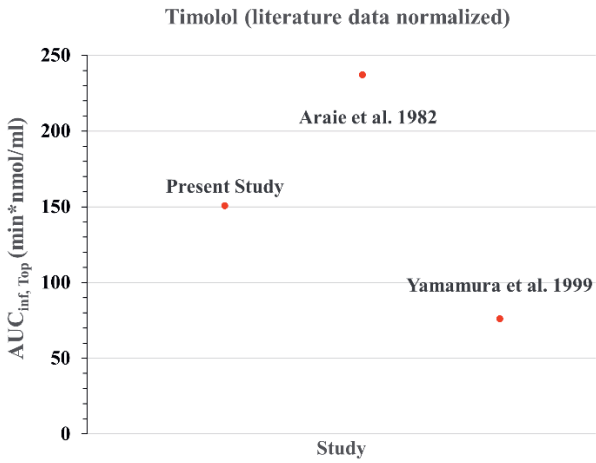

Figure S2. The $AUC_{inf,Top}$ normalized by the timolol dose used in our study (250 nmol) from the literature studies (Araie et al., 1982 and Yamamura et al., 1999)

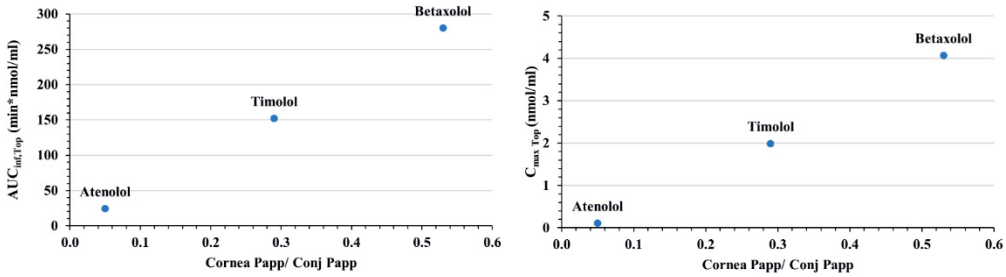

Figure S3. Corneal and conjunctival permeability ratio (Wang et al., 1991) versus dose-normalized $C_{max,Top}$ and $AUC_{inf,Top}$ in rabbit aqueous humour (present study).

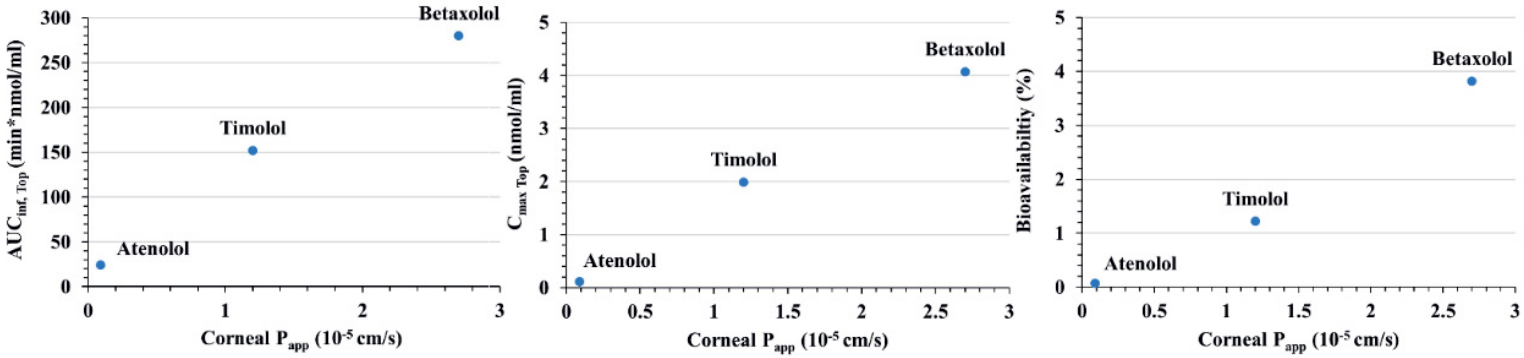

Figure S4. Corneal permeability (Wang et al., 1991) versus pharmacokinetic parameters calculated from aqueous humour in rabbit eye (present paper).

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III

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Ocular pharmacokinetics of atenolol, timolol and betaxolol cocktail: Tissue exposures in the rabbit eye

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ABSTRACT

Quantitative understanding of pharmacokinetics of topically applied ocular drugs requires more research to further understanding and to eventually allow predictive *in silico* models to be developed. To this end, a topical cocktail of betaxolol, timolol and atenolol was instilled on albino rabbit eyes. Tear fluid, corneal epithelium, corneal stroma with endothelium, bulbar conjunctiva, anterior sclera, iris-ciliary body, lens and vitreous samples were collected and analysed using LC-MS/MS. Iris-ciliary body was also analysed after intracameral cocktail injection. Non-compartmental analysis was utilized to estimate the pharmacokinetics parameters. The most lipophilic drug, betaxolol, presented the highest exposure in all tissues except for tear fluid after topical administration, followed by timolol and atenolol. For all drugs, iris-ciliary body concentrations were higher than that of the aqueous humor. After topical instillation the most hydrophilic drug, atenolol, had 3.7 times higher $AUC_{iris\text{-}ciliary\ body}$ than $AUC_{aqueous\ humor}$, whereas the difference was 1.4 and 1.6 times for timolol and betaxolol, respectively. This suggests that the non-corneal route (conjunctival-scleral) was dominating the absorption of atenolol, while the corneal route was more important for timolol and betaxolol. The presented data increase understanding of ocular pharmacokinetics of a cocktail of drugs and provide data that can be used for quantitative modeling and simulation.

1. Introduction

Topical application is the most common route of ocular drug administration. Bioavailability of drugs after topical administration is less than 4% [1–3], since anatomical barriers restrict drug permeation into the inner eye (Fig. 1). Moreover, most of the instilled dose is rapidly removed from the ocular surface by tear turnover, induced lacrimation, and solution drainage into the nasolacrimal duct. During and after the solution drainage, the drug is absorbed into systemic circulation across the conjunctiva, nasal mucosa and gastrointestinal tract [4–6].

Drug absorption into the inner eye takes place through corneal or non-corneal pathways. For the corneal route of absorption, drug penetrates through the corneal epithelium that constitutes the major barrier for drug absorption. Thereafter, the drug reaches the corneal stroma, endothelium, and aqueous humor. The corneal route is considered to be the most common pathway of topical ocular drug absorption [7–9]. On

Abbreviations: AUC_{inf}, area under the curve from time zero to infinity; AUC_{last}, area under the curve from the time of dosing to the time of the last measurable concentration; C_{max}, maximum observed concentration; internal standard, ISTD; t_{max}, time of maximum observed concentration; $t_{1/2}$, elimination half-life; MRT_{inf}, mean residence time; NCA, non-compartmental analysis.

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the other hand, drugs may also be absorbed across the conjunctiva and sclera (non-corneal route) reaching the ciliary body and iris with limited access to the aqueous humor [10–14]. However, the conjunctiva is a highly vascularized tissue and a major part of the drug dose may enter the blood circulation instead of diffusing into the sclera [14–18].

From aqueous humor, the drug may be cleared by aqueous humor outflow via trabecular meshwork into Schlemm's canal [19] or it may enter iris-ciliary body blood vessels and then systemic blood circulation [20–22]. From the aqueous humor drugs also distribute to the lens and vitreous humor, but the concentrations in these tissues are low [13,23].

The corneal epithelium is the main anterior barrier for ocular drug absorption due to the intercellular tight-junctions, particularly limiting permeation of hydrophilic drugs. The acellular corneal stroma allows relatively free diffusion of drugs but partitioning of lipophilic drugs from the lipoidal epithelium to the hydrophilic stroma may be restricted [7–9,24].

In fact, the conjunctiva and sclera are more permeable than the cornea, being sclera less permeable than conjunctiva [14,24–26]. For hydrophilic compounds conjunctiva is ∽ 15–25 times more permeable than cornea, and sclera is half as permeable as conjunctiva [25]. Although drug diffusion across the conjunctiva and sclera is easier than through the cornea [11,14,26], the presence of blood and lymphatic flows in the conjunctiva and episclera results in drug elimination from the eye into the systemic circulation [27–29].

Additionally, iris and ciliary body have a vascular bed with blood flows of 62 μl/min and 82 μl/min in rabbits, respectively [21,30]. Iridial vessels and ciliary muscle vessels with tight junctions are parts of the blood-aqueous barrier. Moreover, the epithelial blood-aqueous barrier (posterior iris epithelium and non-pigmented ciliary epithelium) may limit drug diffusion further into the vitreous [29,31]. Clearance via the iris and ciliary body vessels seems to be faster for lipophilic drugs than hydrophilic compounds [32–34].

The present study was performed to quantitatively understand the ocular pharmacokinetics of the three model drugs: betaxolol, timolol, and atenolol cited in decreasing order of lipophilicity (Table 1). The compounds were administered as eye drops and intracameral injection. Pharmacokinetic parameters were estimated in nine ocular tissues. This study provides a comprehensive description of the drug distribution in the eyes after topical administration, also including the analysis of the corneal vs non-corneal absorption processes.

2. Material and method

2.1. Animal experiments

Animals. Thirty-two albino New Zealand rabbits, age 3–6 months and weight 2.8–3.2 kg were use in the experiment sixteen for the topical study and sixteen for the intracameral one. The animals were housed in a temperature and humidity-controlled environment with a 12/12 light /dark cycle. The animals were individually housed and fed a normal diet. All rabbits underwent an ocular examination before the experiments. The study comply with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and EU Directive 2010/63/EU for animal experiments.

Topical administration. The cocktail containing 20 mM atenolol (USP reference standard, Sigma), 10 mM betaxolol hydrochloride (USP reference standard, Sigma) and 10 mM timolol maleate (USP reference standard, Sigma) in phosphate-buffered saline (PBS, Thermofisher Scientific) (pH adjusted to 7.4; 322 mOsm/kg) was administered onto the upper cornea-scleral limbus of both eyes (25 μl/eye) in each rabbit as previously reported [1]. The tear fluid samples of 1 μl were withdrawn from each eye with disposable microcapillaries (Microcaps, Drummond Scientific). Corneal epithelium, corneal stroma with endothelium, bulbar conjunctiva, anterior sclera, aqueous humor, iris-ciliary body, lens, and vitreous humor were collected and weighted at 5, 10, 20, 30, 60, 120, 180 and 240 min, and the number of eyes at each time point was four (n = 4).

Intracameral administration. A volume of 5 μl of the cocktail solution with atenolol, timolol and betaxolol (each at 1 mM in PBS) was injected into the anterior chamber (aqueous humor) of the rabbit eye [32]. The animals were sacrificed by injecting into the marginal ear vein a lethal dose of pentobarbital (Mebunat vet 60 mg/ml; Orion Pharma, Finland). In these experiments, aqueous humor and iris-ciliary body samples were collected and weighted at time points 10, 20, 30, 60, 120, 180 and 240 min with number of eye per each time point 4 (n = 4) as previously described [32]. All samples were cooled on ice following storage at −80 °C until analyses.

Dissection of ocular tissues. The excised eyeballs were dipped to PBS. Firstly, the corneal epithelium was collected from the corneal surface by gently scraping with a scalpel blade. Then, a piece of approximately 5 mm × 5 mm size of bulbar conjunctiva was collected with small scissors by first cutting a small hole through conjunctiva from the limbus, detaching the conjunctiva from the surface of the eye and

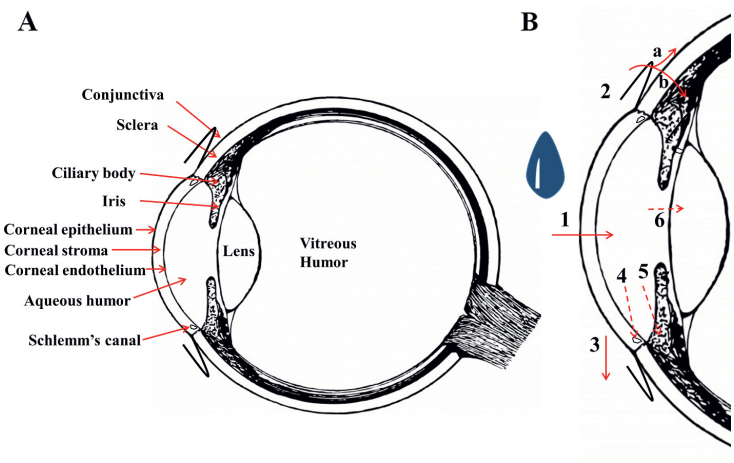
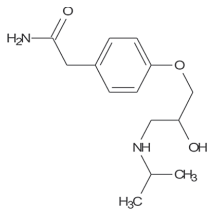
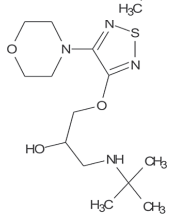
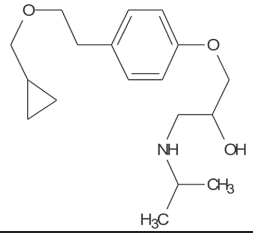


Fig. 1. Ocular absorption, distribution and elimination routes after topical administration. A: Anatomy of the eye. **B:** Absorption, distribution and clearance pathways after topical administration. **1:** corneal absorption; **2: a)** conjunctival absorption into conjunctival blood vessels reaching the systemic circulation and **b)** conjunctiva-scleral absorption into ciliary body and iris; **3:** tear flow and extra volume drainage by the nasolacrimal system; **4:** clearance from trabecular meshwork into Schlemm's canal; **5:** distribution into the ciliary body and iris and the blood circulation therein; **6:** distribution to the lens. (https://www.publicdomainpictures.net/en/view-image.php?image=130389&picture= medical-eye).

Table 1
Physicochemical properties of atenolol, timolol and betaxolol (calculated with ACD/Percepta, version 2254, Advanced Chemistry Development, Inc. Toronto, Canada).

Drugs	Molecular structure	Molecular weight	Log $D_{7.4}$	Polar surface area	Hydrogen bond donor
Atenolol		266.34	−1.85	84.5	4
Timolol		316.42	−0.35	107.9	2
Betaxolol		307.43	0.77	50.7	2

finally removing the conjunctival piece. The sclera was cut open from the same spot as the conjunctiva was collected, and a ~ 5 mm × 5 mm piece of anterior sclera was removed. From the scleral piece, remaining extraocular tissues and retina were removed with scissors. Then, the anterior part (corneal stroma with endothelium, iris-ciliary body and lens) was detached from the posterior tissues (vitreous, retina, choroid, sclera) by cutting with scissors 2 mm posterior from the limbus. Cutting was started from a pre-made hole in the sclera, enabling removal of the iris-ciliary body and lens with the cornea. Then, lens and iris-ciliary body were collected with tweezers, and the sclera was cut off from the corneal stroma and endothelium with scissors to obtain the corneal sample. From the posterior part, vitreous was dissected on a separate dish by gently pulling the vitreous from the eyecup with tweezers. When necessary, the vitreous sample was cleaned from retinal contamination with tweezers and scissors to obtain a clean vitreous sample.

2.2. LC-MS/MS analyses

The corneal epithelial samples were lysed by adding 38-fold volume of 0.1 N NaOH (based on the sample weight) and then pipetted and vortexed until homogenous tissue lysate was obtained. Corneal stroma-endothelium, bulbar conjunctiva and anterior sclera samples were cut into smaller pieces with scalpel before homogenization. The samples were transferred to the 2 ml or 7 ml tubes containing 2.8 mm ceramic beads (Omni International, USA), PBS buffer was added and the tissues were homogenized at 4–6 m/s speed in total for 0.5–12 min using Bead Ruptor Elite (Omni Internationals, USA). For corneal stroma-endothelium, bulbar conjunctiva, anterior sclera, iris ciliary body and lens the homogenization was performed in two steps and PBS buffer was added between steps. Homogenization conditions were optimized for each tissue to obtain smooth tissue homogenate, suitable for pipetting (Supplementary Table S1). Some samples of early time points (corneal epithelium, corneal stroma-endothelium, bulbar conjunctiva, iris-ciliary body) were diluted with similarly prepared blank tissue homogenate.

One part of each sample was mixed with 3 parts of internal standard solution (e.g. 30 μl of sample and 90 μl) by vortexing for 10 sec. Internal standard (ISTD) solution contained 50 ng/ml atenolol-d7 (Toronto Research Chemicals, Canada), 5 ng/ml betaxolol-d5 (Toronto Research Chemicals, Canada), 5 ng/ml rac timolol-d5 maleate (Toronto Research Chemicals, Canada), 1% formic acid in acetonitrile. In the case of the vitreous samples, one part of the vitreous homogenate was mixed with one part of ISTD solution. All the samples were incubated at RT for 10 min and thereafter centrifuged (10 min, +4°C, 13 000 rpm). The supernatant was collected for the LC-MS/MS analysis. Standards (0.5–4000 nM, in duplicates) and quality controls (in triplicates, 4–6 levels) were prepared from the drug cocktail (containing 20 mM atenolol, 10 mM timolol maleate and 10 mM betaxolol hydrochloride in PBS) into blank tissue homogenates in the same way as the samples were treated.

Tear fluid samples were prepared in the same way as aqueous humor samples in our previous article [32] with the exception that ISTD solution was 50 ng/ml atenolol-d7, 5 ng/ml betaxolol-d5, 5 ng/ml rac timolol-d5 maleate, 1% formic acid in 50% acetonitrile. Tear fluid samples were first diluted with ISTD solution (dilution factors were 20–4000) to achieve final concentrations in the range of calibration curve (0.1 – 5000 nM). Standards and quality controls were prepared by diluting the beta-blocker cocktail in PBS by ISTD solution. Additional quality controls (250 nM, n = 2), which were prepared in similar manner as tear fluid samples (dilution of tear fluid 1:20 and 1:2000 with ISTD solution), were included into LC-MS/MS analysis.

All samples, standards and quality controls were analysed by LC-MS/MS (Agilent 1290 liquid chromatograph and Agilent 6495 triple quadrupole mass spectrometer, Agilent Technologies Inc., USA) as described earlier [32]. The calibration curves were generated using 7–13

concentration levels. Mean accuracy of the standards of most standard curve points (≥78%) was 80–120%. The accuracy of QC samples was 80–120% for ≥ 67% of quality controls. For additional tear fluid QC samples the accuracy was 86–110%.

2.3. Pharmacokinetic analysis

Mean concentration–time profiles of drugs in the tissues were analysed using non-compartmental analysis (NCA) with Phoenix WinNonlin (build 8.1, Certara L.P.) using linear–linear trapezoidal interpolation. The following pharmacokinetic parameters were estimated: area under the curve (AUC) from time zero until last sampling point (AUC_{last}), total AUC until infinity (AUC_{inf}), mean residence time (MRT_{inf}), peak concentration (C_{max}), time to peak concentration (t_{max}) and elimination half-life ($t_{1/2}$). The AUC_{inf} and MRT_{inf} were reported for the tissues with the terminal phase of the concentration–time profiles longer than two half-lives.

3. Results

The dose-normalized (to 250 nmol) concentration–time profiles for the topically applied drugs in eight ocular tissues (this study) and the aqueous humor (from our previous study) [1] are shown in Fig. 2. The aqueous humor [32] and iris-ciliary body (present study) concentration–time profiles after intracameral administration of 5 nmol of atenolol, timolol and betaxolol are presented in Supplementary Fig. S1.

Betaxolol concentrations were higher than the levels of timolol and atenolol in most tissues, but in the tear fluid atenolol concentrations were the highest (Fig. 2, Supplementary Fig. S2). Drug concentrations in various tissues show a wide range as the concentrations in the corneal epithelium are at least 1000 times higher than in the vitreous.

The pharmacokinetic parameters for the topical and intracameral beta-blockers are listed in Table 2 (see in Supplementary Table S2 the original data without dose normalization). As expected, the MRT values are shorter in the tear fluid than in the other tissues. In some tissues, the MRT values are relatively constant regardless of the drug (e.g. corneal epithelium), while in other tissues (e.g. aqueous humor, corneal stroma-endothelium, vitreous, iris-ciliary body) hydrophilic atenolol has longer retention times than the more lipophilic timolol and betaxolol (Supplementary Fig. S3).

The AUC_{inf} values of the drugs are shown in Fig. 3. The data shows that AUC_{inf} values span over the range of four orders of magnitude from tear fluid to lens and vitreous. Atenolol has the highest AUC_{inf} in tear fluid whereas betaxolol has the highest AUC values in the corneal epithelium and other tissues. The AUC_{inf} values of three drugs are higher in the iris-ciliary body than in the aqueous humor. AUC_{inf} values of

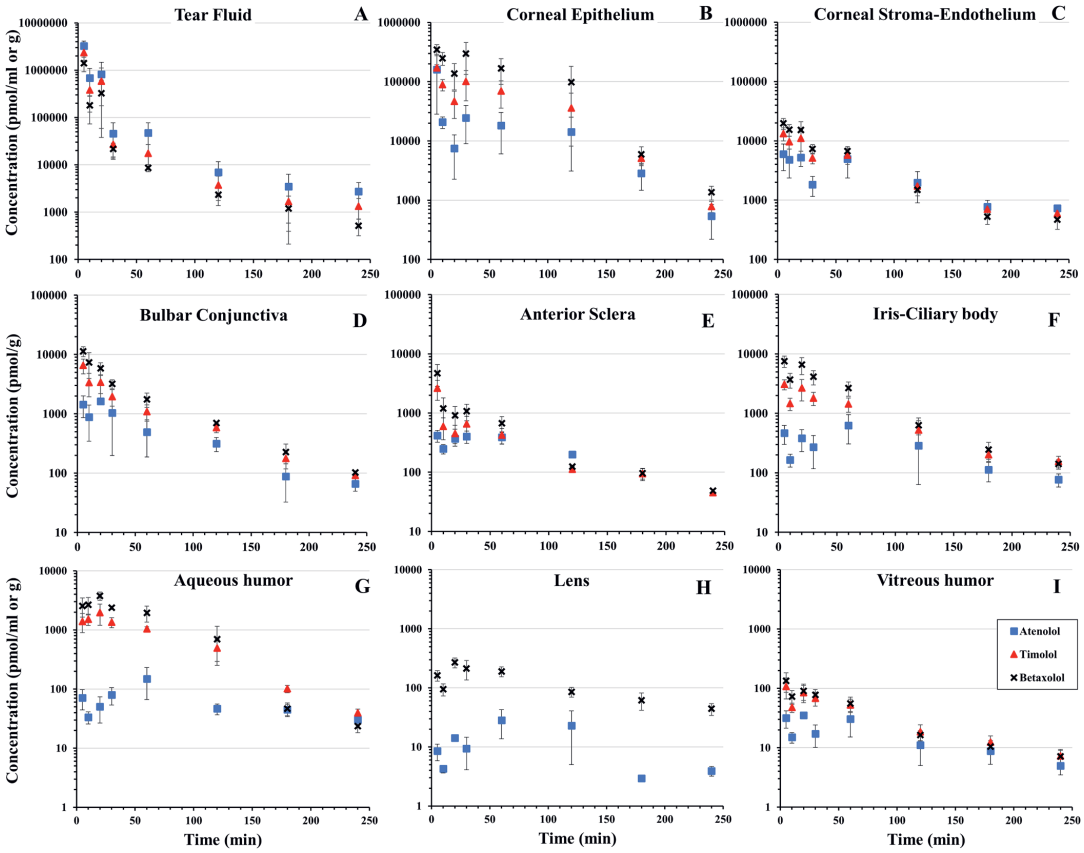


Fig. 2. Concentration-time profiles of drugs in nine ocular matrices (A-I) of rabbits after topical administration of atenolol (250 nmol dose-normalized), timolol (250 nmol dose), and betaxolol (250 nmol dose). Mean drug concentrations ± standard errors of the mean (n = 3–4) are presented. Timolol concentration could not be quantified in the lens due to the lack of a reliable control sample.

Table 2
Pharmacokinetic parameters estimated with NCA analysis of the three beta-blockers concentrations in nine ocular tissues after topical administration including the aqueous humor [1], and iris-ciliary body, and aqueous humor [2] after intracameral administration with a dose-normalization to 250 nmol.

	Atenolol						Timolol						Betaxolol					
Topical	AUC_{inf} (min*nmol/ml or g)	AUC_{last} (min*nmol/ml or g)	C_{max} (nmol/ml or g)	t_{max}	$t_{1/2}$ (min)	MRT	AUC_{inf} (min*nmol/ml or g)	AUC_{last} (min*nmol/ml or g)	C_{max} (nmol/ml or g)	t_{max}	$t_{1/2}$ (min)	MRT	AUC_{inf} (min*nmol/ml or g)	AUC_{last} (min*nmol/ml or g)	C_{max} (nmol/ml or g)	t_{max}	$t_{1/2}$ (min)	MRT
Tear fluid	71720	71542	3240	5	45	9	58398	58311	2345	5	45	6	39799	39769	1398	5	40	5
Corneal epithelium	3387	3361	159	5	34	63	9642	9611	171	5	27	62	24760	24712	348	5	24	60
Corneal stroma -endothelium	627	562	5.98	5	62	104	819	774	13.2	5	53	74	978	949	19.6	5	43	57
Bulbar conjunctiva	208	198	3.24	20	52	74	234	228	6.55	5	47	59	378	372	11.3	5	42	50
		164*						197*						334*				
Anterior sclera	-		0.41	5	84	-	71.5	67.7	2.60	5	59	72	111	108	4.70	5	47	55
		39.1*						57.3*						96.7*				
Iris-ciliary body	72.4	66.2	0.63	60	56	105	213	201	3.07	5	52	77	398	390	7.52	5	39	55
		49.5*						169*						352*				
Aqueous humor	19.6	15.8	0.15	60	87	148	151	149	1.97	20	37	64	252	251	3.76	20	29	54
Lens	3.65	3.35	0.03	60	53	112	**	**	**	**	**	**	32.8	27.2	0.27	20	88	133
Vitreous humor	4.22	3.69	0.04	20	74	118	8.13	7.52	0.11	5	61	89	8.53	7.97	0.13	5	56	80
Intracameral																		
Iris-ciliary body	9939	7624	85.8	60	85	149	4615	4456	106	10	47	60	5013	4907	145	10	41	51
Aqueous humor	28711	26397	365	20	59	84	10895	10819	204	20	31	45	6520	6486	142	20	31	39
TOP: ICB/AH	3.69						1.41						1.58					
IC: ICB/AH	0.35						0.42						0.77					

AUC_{inf}: Area under the curve from 0 to infinity.
AUC_{last}: Area under the curve from 0 to last sampling time (240 min) unless otherwise indicated.
C_{max}: Maximum concentration.
t_{max}: Time to peak concentration.
$t_{1/2}$: Elimination half-life.
MRT: Mean residence time to infinity.
* AUC_{last} until 120 min.
** timolol could not be quantified due to the lack of a reliable control sample.
-: unreliable estimate because the concentration-time profile was shorter than two half-lives.
ICB/AH: AUC_{inf} ratio between iris-ciliary body and aqueous humor after topical (TOP) and intracameral (IC) administrations.

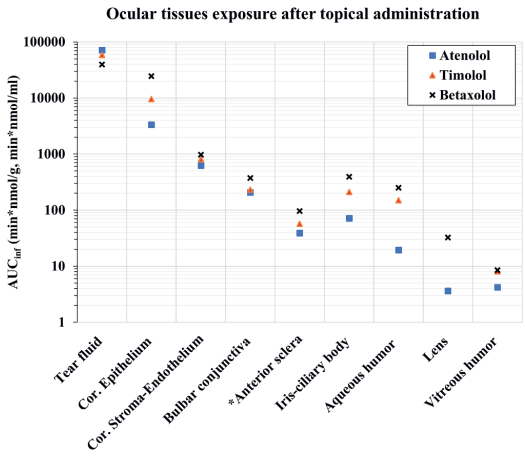


Fig. 3. The AUC_{inf} values of betaxolol, timolol and atenolol in the ocular tissues after topical eye drop instillation to the rabbit eyes with a dose-normalization to 250 nmol. For anterior sclera AUC_{last} = 120 min values are shown (*). Abbreviation: Cor = cornea.

betaxolol in aqueous humor, corneal epithelium, and iris-ciliary body were 12, 7, and 5.5 times higher than the values of atenolol, while betaxolol had 2, 2.5, and 1.9 higher values than timolol.

The AUC_{inf} ratios between the adjacent tissues are presented in Fig. 4. Lipophilic betaxolol shows the highest partitioning from tear fluid to the corneal epithelium and bulbar conjunctiva. Likewise, it shows the highest partitioning from sclera to iris-ciliary body. Interestingly, atenolol shows the highest ratios of iris-ciliary body/aqueous humor, corneal stroma-endothelium/epithelium and vitreous/aqueous humor ratios. Overall, concentration ratios between neighboring tissues show wide range of values from ≈ 0.001 to less than 10.

Iris-ciliary body concentration–time profiles after topical and intracameral administration of the drugs are presented in Supplementary Fig. S4 . Atenolol shows a bigger difference than timolol and betaxolol between the iris-ciliary body concentrations after topical and intracameral administrations. Differences are also seen among AUC_{inf} ratios between iris-ciliary body and aqueous humor (Table 2).

4. Discussion

A cocktail approach was utilized to estimate the pharmacokinetic behavior of betaxolol, timolol and atenolol in the ocular tissues of the rabbit eye. The current study provides pharmacokinetic data and parameters to better understand tissue exposure, partitioning and elimination of the drugs.

4.1. Corneal drug absorption

In the tear fluid, atenolol shows higher levels than the more lipophilic timolol and betaxolol (Table 2, Fig. 2A and 3, Supplementary Fig. S2A). Flow patterns of the instilled solution is identical for all drugs in this study as a cocktail approach was used. Thus, the differences among three drugs can be explained by their different rates of elimination from lacrimal fluid across the membranes (cornea, conjunctiva); with the more lipophilic compounds betaxolol and timolol having higher permeability [14,35]. Since the short initial half-life in the tear fluid cannot be obtained using the NCA analysis, the reported half-lives in Table 2 correspond to later phases in the disposition process that may involve drug equilibration to the tissues, and back-diffusion to the lacrimal fluid [11,24,36].

In the corneal epithelium, betaxolol and timolol showed higher concentrations and corneal partitioning than atenolol, which is expected due to their increased lipophilicity. Corneal stroma is not posing an anatomical barrier for diffusion of small molecules [7]. Interestingly, it seems that hydrophilic atenolol has higher corneal stroma-endothelium / aqueous humor ratio and longer MRT in the corneal stroma-endothelium (inset of Fig. 4, Table 2). This may reflect lower permeability of atenolol in corneal endothelium as compared to timolol and betaxolol.

Betaxolol had the highest AUC_{inf} value in the aqueous humor (Fig. 3, Table 2), probably due to its lipophilicity and higher corneal permeation. Concentration profiles of betaxolol and timolol had similar first-order decline in aqueous humor and in corneal-epithelium (Supplementary Fig. S2A). This supports the notion that corneal absorption is the main route of drug absorption to the aqueous humor and corneal epithelium forms a drug depot after eye drops instillation [1,9,12]. On the contrary, the concentration profile of atenolol is flatter, reflecting

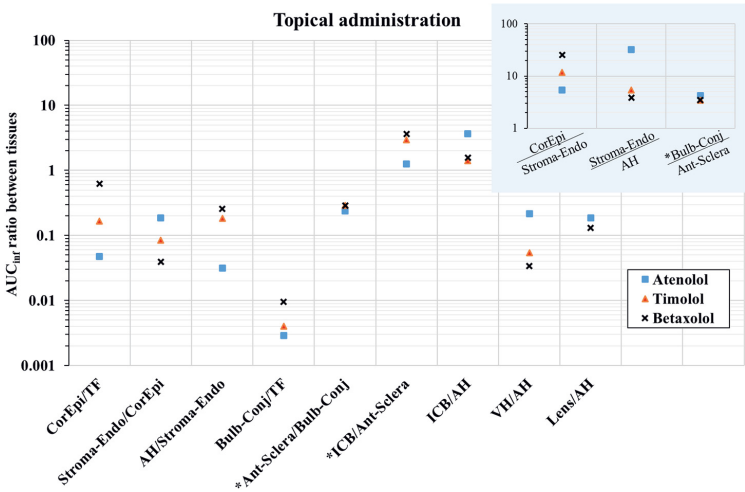


Fig. 4. The AUC_{inf} ratios among neighboring tissues after topical eye drop instillation of betaxolol, timolol and atenolol to the rabbit eyes. The inset shows AUC_{inf} ratios in the direction of drug accumulation into corneal epithelium, corneal stroma-endothelium and bulbar conjunctiva. The AUC_{inf} (Table 2) was used except for anterior sclera-related ratios when $AUC_{last=120min}$ was used (*). Abbreviations: CorEpi = corneal epithelium, TF = tear fluid, Stroma-Endo = corneal stroma-endothelium, AH = aqueous humor, Bulb-Conj = bulbar conjunctiva, Ant-Sclera = anterior sclera, ICB = iris-ciliary body, and VH = vitreous humor.

the first-order decline rate in the corneal stroma-endothelium (Supplementary Fig. S2A). This suggest that corneal stroma, not epithelium, is a depot site for atenolol following topical administration.

As expected [7,9] lipophilicity plays a positive impact on the corneal drug absorption to the aqueous humor. From the aqueous humor, the drugs distribute into the iris-ciliary body, lens, and vitreous. It is known that hydrophilic atenolol has lower clearance from the aqueous humor than timolol and betaxolol [32], this is in line with the longer MRT in the aqueous humor.

4.2. Non-corneal drug absorption

Drugs permeate from tear fluid to the conjunctiva and in this case also betaxolol showed the highest conjunctival concentrations, followed by timolol and atenolol (Fig. 2, Table 2, Supplementary Fig. S2B). Lipophilic drugs permeate faster into cellular conjunctiva, but they are also eliminated faster into the systemic circulation from the conjunctiva [11,14,26]. For this reason, the conjunctival AUC_{inf} values are much lower than the non-vascular corneal ones and the AUC_{inf} differences among the drugs are also smaller in conjunctiva than in cornea (Fig. 2, Fig. 3, Table 2).

Scleral kinetics of betaxolol and timolol showed similar decline as in the bulbar conjunctiva, and no differences among drugs were seen in the $AUC_{last=120\ min}$ ratios between these two tissues (Fig. 4). From sclera the drugs may reach iris-ciliary body. Atenolol concentrations were slightly higher in the iris-ciliary body than the sclera, for the more lipophilic compounds there was a bigger difference in the iris-ciliary body compared to scleral concentrations (Table 2, Fig. 3), possibly due to entry of some drug from the aqueous humor to the iris-ciliary body [20,22,37]. Higher AUC_{inf} ratio of hydrophilic atenolol between iris-ciliary body and aqueous humor may be partly due to its higher non-corneal entry into iris-ciliary body as compared to timolol and betaxolol. The slower clearance of hydrophilic atenolol than timolol and betaxolol via iris blood vessels could also contribute in the cited higher AUC_{inf} ratio of atenolol [32].

After intracameral administration, the concentration time profiles of timolol and betaxolol in aqueous humor and iris-ciliary body are quite similar, but atenolol shows different kinetic behaviour: slower distribution and elimination in the iris-ciliary body (Supplementary Fig. S1). This behavior reflects the slower permeation of atenolol into the blood-aqueous barrier. As intracameral administration results in iridial drug distribution only from the aqueous humor, it is interesting to compare these data with the topical eye drop data that involves also non-corneal drug delivery. Comparison of the AUC_{inf} ICB/AH ratios of hydrophilic atenolol after topical (3.69) and intracameral (0.35) administrations reveals ten times higher ratio for topical administration, suggesting the importance of non-corneal atenolol absorption. The difference was only 2–3-fold for more lipophilic timolol and betaxolol. Therefore, atenolol has the lowest AUC_{inf} in the iris-ciliary body target tissue after topical administration, but the highest contribution from non-corneal route of absorption. Non-corneal absorption was earlier seen also for topical suspension of brinzolamide [2], and timolol insert placed in the conjunctival sac [34]. Comparisons of these ratios inform about the relative importance of the non-corneal route, but these values do not give absolute non-corneal contributions, because *trans*-corneal delivery to the iris-ciliary body may differ from the iris-ciliary body distribution after intracameral injection.

4.3. Lens and vitreous

Atenolol and betaxolol distributed to a lower extent from aqueous humor into the lens (Fig. 3). Poor lenticular distribution is due to its tight structure that resulted in low lens/buffer partition coefficients for various drugs *in vitro* [23]. Thus, the lens acts as a barrier between the anterior chamber and vitreous, but it does not seem to act as a drug reservoir.

Drugs may distribute also from the aqueous humor into the vitreous humor, and the expected route is via permeation across the iris-ciliary body, since lens is quite impermeable and aqueous humor flow from posterior to anterior direction prevents drug transfer to the vitreous. The drug concentrations and AUC_{inf} values in the vitreous humor are 1–2 orders of magnitude lower than in the aqueous humor (Figs. 2-3) as it has been observed earlier [34,38,39]. Ratio of AUC_{inf} values between vitreous and aqueous humor is several times higher for atenolol than for timolol and betaxolol (Fig. 4). Atenolol has much lower clearance to the blood flow in the iris-ciliary body than timolol and betaxolol [32], since atenolol does not permeate across the walls of the uveal blood vessels that represent the blood-aqueous barrier. Therefore, atenolol may reach the vitreous at a higher level than timolol and betaxolol. However, the concentrations of all three drugs in the vitreous humor are four orders of magnitude lower than in the tear fluid at early times and bioavailability of topically applied drugs to the vitreous humor is negligible (Fig. 2).

5. Conclusion

The present study shows the influence of lipophilicity on ocular pharmacokinetic processes after topical administration and gives insights on the relative contributions of the corneal and non-corneal routes of drug absorption. Detailed information on the disposition of three drugs to nine different ocular tissues over time following topical administration can be used to build physiological models of drug distribution in the eye with the ultimate aim of developing predictive *in silico* models that would allow the number of preclinical studies in animals to be reduced or replaced. Ultimately speeding up ocular drug development.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2021.06.003.

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SUPPLEMENTARY MATERIALS

OCULAR PHARMACOKINETICS OF ATENOLOL, TIMOLOL AND BETAXOLOL COCKTAIL: TISSUE EXPOSURES IN THE RABBIT EYE

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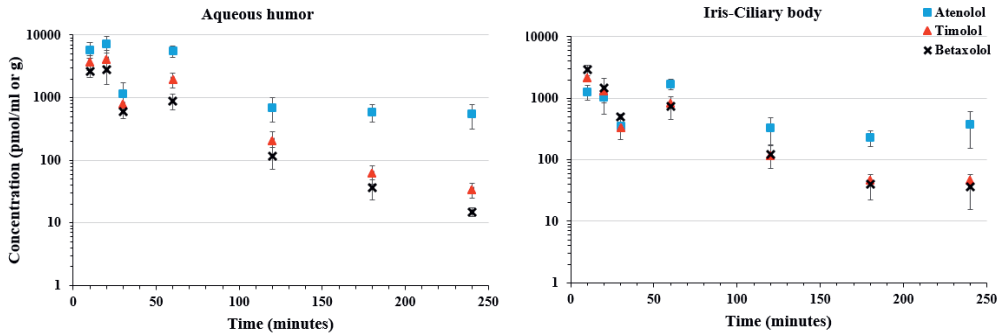
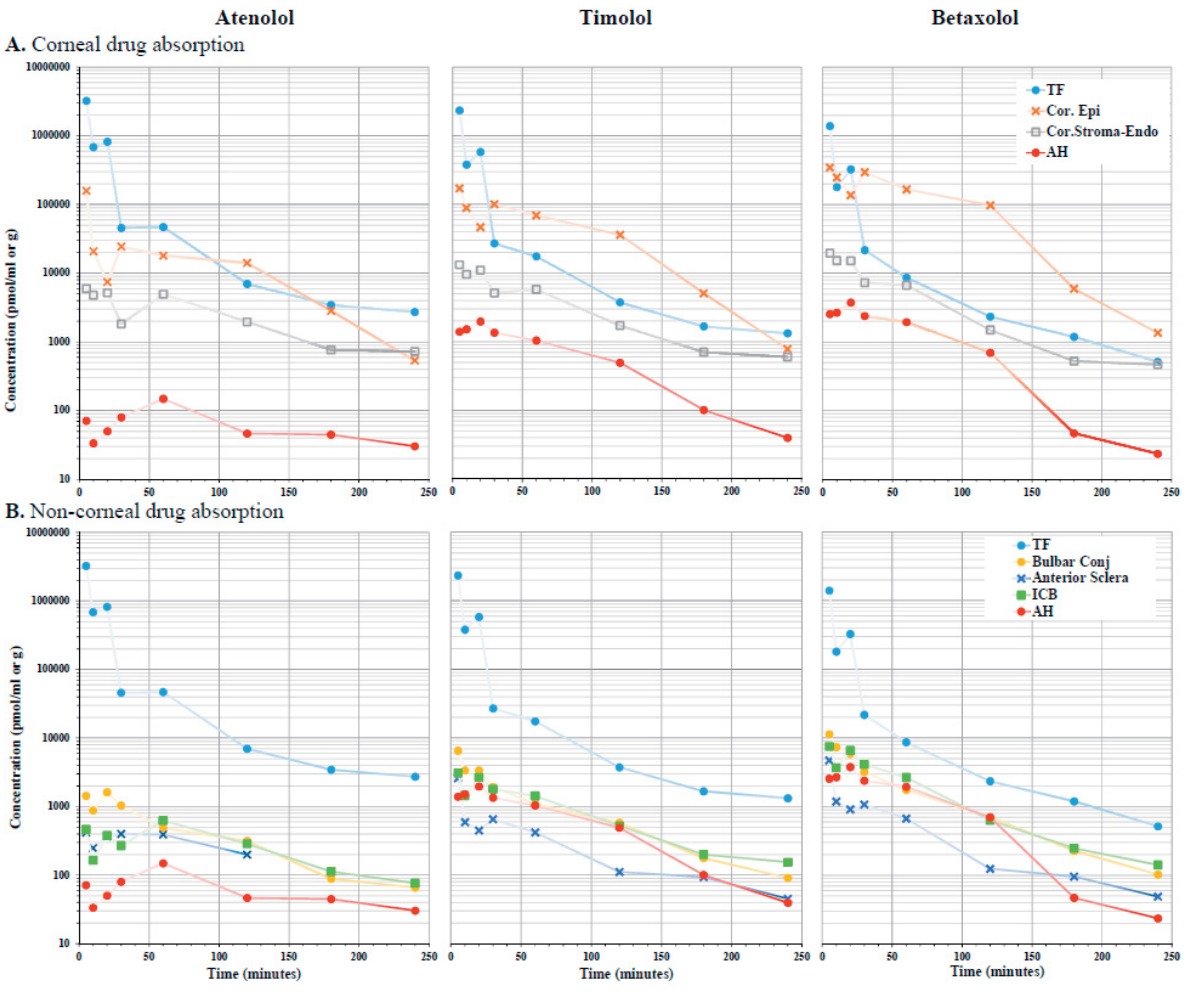
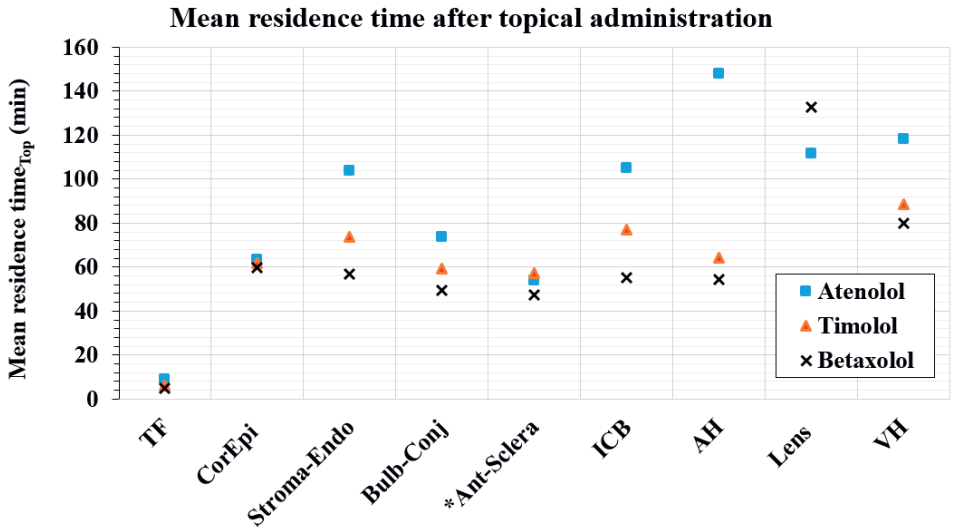


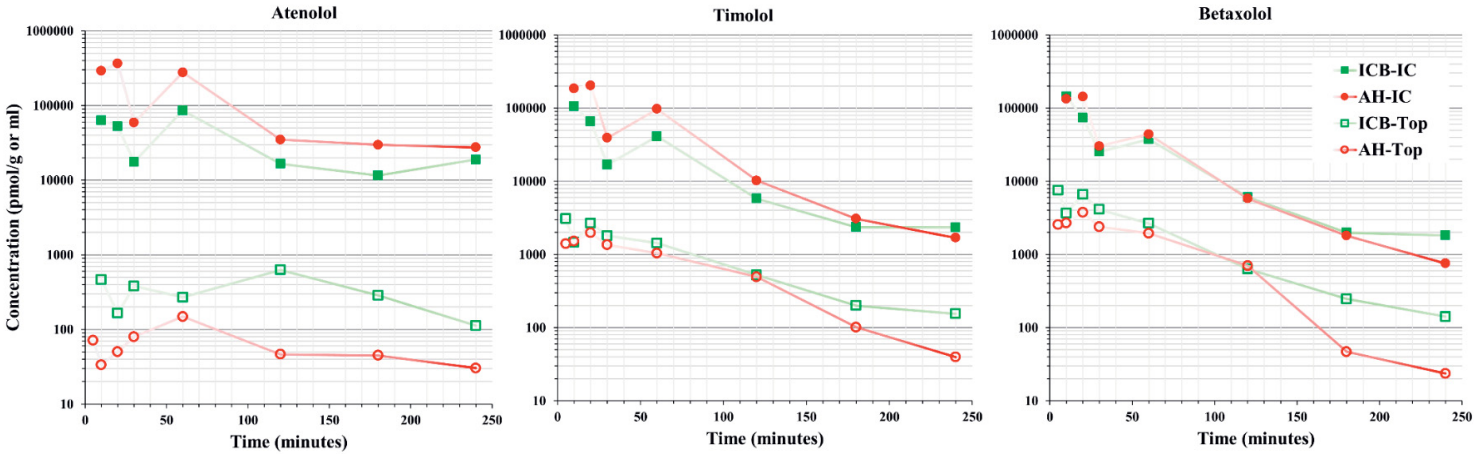
Supplementary Figure S1. The aqueous humor [32] and iris-ciliary body concentration-time profile after intracameral administration of atenolol, betaxolol and timolol (5 nmol) into the rabbit eye. The symbols represent the average concentration for each tissue ± standard error of the mean (n=3-4).


Atenolol
Timolol
Betaxolol
A. Corneal drug absorption
B. Non-corneal drug absorption
Concentration (pmol/ml or g)
Time (minutes)
TF
Cor. Epi
Cor.Stroma-Endo
AH
Bulbar Conj
Anterior Sclera
ICB

Supplementary Figure S2. Concentration-time profile of atenolol (250 nmol dose-normalized), timolol (250 nmol dose), and betaxolol (250 nmol dose) in the ocular tissues of rabbits involved in the corneal route of absorption (A) and the non-corneal route of absorption (B) the after topical administration.



Supplementary Figure S3. Mean residence time (MRT_{inf}) of atenolol, timolol and betaxolol in the ocular tissues after topical administration of the three beta-blockers with dose-normalized to 250 nmol in the rabbit eye. * based on concentration-time profile till 120 minutes (MRT_{last} until 120 min).



Supplementary Figure S4. Aqueous humor (AH) and iris-ciliary body (ICB) concentration-time profiles of atenolol, timolol and betaxolol after intracameral (IC) and topical (Top) administration in rabbit eye. The concentration of all the three beta-blockers were normalized to the dose of 250 nmol.

Supplementary Table S1. Homogenization of ocular tissues.

Tissue	Corneal epithelium	Corneal stroma-endothelium	Bulbar conjunctiva	Anterior sclera	Iris-ciliary body	Lens	Vitreous humor
Pretreatment: Cutting with scalpel	-	X	X	X	-	-	-
Added homogenization buffer/lysis solution before homogenization step 1*	19 x volume of 0.1 N NaOH	2 x volume of PBS	4 x volume of PBS	4 x volume of PBS	4 x volume of PBS	4 x volume of PBS	-
Homogenization step 1	Pipetting and vortexing	Bead Ruptor 4 min, 6 m/s	Bead Ruptor 2 min, 6 m/s	Bead Ruptor 4 min, 6 m/s	Bead Ruptor 1.5 min, 6 m/s	Bead Ruptor 30 s, 4 m/s	Bead Ruptor 30 s, 6 m/s
Added buffer before homogenization step 2*	19 x volume of 0.1 N NaOH	5 x volume of PBS	5 x volume of PBS	5 x volume of PBS	5 x volume of PBS	-	-

Homogenization step 2	Pipetting and vortexing	Bead Ruptor 2x4 min, 6 m/s	Bead Ruptor 2x4 min, 6 m/s	Bead Ruptor 2x4 min, 6 m/s	Bead Ruptor 1 min, 6 m/s	Bead Ruptor 30 s, 5 m/s	-
Further dilution of samples	Samples collected after topical administration were diluted up to 1:50 using tissue lysate	Samples at 5-120 min after topical administration were diluted up to 1:20 using tissue homogenate	Samples at 5-20 min after topical administration were diluted up to 1:5 using tissue homogenate	Dilution 1:2 with PBS	Samples at 5-120 min after topical administration were diluted 1:2 using tissue homogenate	-	-
Ratio of homogenate and ISTD solution	1:4	1:4	1:4	1:4	1:4	1:4	1:2
Centrifugation	10 min, +4°C, 13 000 rpm	10 min, +4°C, 13 000 rpm	10 min, +4°C, 13 000 rpm	10 min, +4°C, 13 000 rpm	10 min, +4°C, 13 000 rpm	10 min, +4°C, 13 000 rpm	10 min, +4°C, 13 000 rpm

* Volumes of PBS buffer and 0.1 N NaOH were based on the weight of the tissue sample.

Supplementary Table S2. Pharmacokinetic parameters were estimated using NCA analysis of the three beta-blockers concentrations in nine ocular tissues including the aqueous humor [1] after topical administration (atenolol dose of 500 nmol, betaxolol dose of 250 nmol, and timolol dose of 250 nmol and the iris-ciliary body and aqueous humor [2] after intracameral administration with a 5 nmol dose for the three beta-blockers.

Topical	Atenolol						Timolol						Betaxolol					
	AUC_{inf}	AUC_{last}	C_{max}	t_{max}	$t_{1/2}$	MRT	AUC_{inf}	AUC_{last}	C_{max}	t_{max}	$t_{1/2}$	MRT	AUC_{inf}	AUC_{last}	C_{max}	t_{max}	$t_{1/2}$	MRT
	(min*nmol/ml or g)		(nmol/ml or g)	(min)			(min*nmol/ml or g)		(nmol/ml or g)	(min)			(min*nmol/ml or g)		(nmol/ml or g)	(min)		
Tear fluid	143440	143084	6480	5	45	9	58398	58311	2345	5	45	6	39799	39769	1398	5	40	5
Corneal epithelium	6775	6722	318	5	34	63	9642	9611	171	5	27	62	24760	24712	348	5	24	60
Corneal stroma - endothelium	1255	1125	11.96	5	62	104	819	774	13.2	5	53	74	978	949	19.6	5	43	57
Bulbar conjunctiva	415	395	6.48	20	52	74	234	228	6.55	5	47	59	378	372	11.3	5	42	50

Anterior sclera	-	78.2*	0.83	5	8 4	-	71.5	67.7	2.60	5	5 9	72	111	108	4.70	5	47	55
Iris-ciliary body	145	132	1.25	6 0	5 6	10 5	213	201	3.07	5	5 2	77	398	390	7.52	5	39	55
Aqueous humor	39.2	31.6	0.30	6 0	8 7	14 8	151	149	1.97	20	3 7	64	252	251	3.76	20	29	54
Lens	7.30	6.69	0.06	6 0	5 3	11 2	**	**	**	**	* *	**	32.8	27.2	0.27	20	88	133
Vitreous humor	8.44	7.38	0.07	2 0	7 4	11 8	8.13	7.52	0.11	5	6 1	89	8.53	7.97	0.13	5	56	80
Intracameral																		
Iris-ciliary body	199	152	1.72	6 0	8 5	14 9	92.3	89.1	2.12	10	4 7	60	100. 3	98.1	2.89	10	41	51
Aqueous humor	574	528	7.31	2 0	5 9	84	218	216	4.07	20	3 1	45	130	130	2.85	20	31	39

AUC_{inf}: Area under the curve from 0 to infinity.

AUC_{last}: Area under the curve from 0 to last sampling time (240 min) unless otherwise indicated.

C_{max}: Maximum concentration.

t_{max}: Time to peak concentration.

$t_{1/2}$: Elimination half-life.

MRT: Mean residence time to infinity.

* AUC_{last} until 120 min.

** timolol could not be quantified due to the lack of a reliable control sample.

- unreliable estimate because the concentration-time profile was shorter than two half-lives.

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Ocular drug development imposes a challenge due to the intricate structure of the eye for which literature is lacking data. Further research is required to improve understanding of ocular pharmacokinetic layout benefiting novel drug development and delivery systems. This thesis addresses ocular pharmacokinetics in terms of topical ocular bioavailability, drug partitioning, exposure and clearance with physicochemical characteristics of the drug using the cocktail approach of drug administration.

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