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PREFORMULATION CHARACTERIZATION AND FORMULATION DEVELOPMENT OF Δ^9-TETRAHYDROCANNABINOL PRODRUGS FOR POTENTIAL TREATMENT OF GLAUCOMA

A Dissertation Submitted
In The Partial Fulfillment of Requirements For
The Doctoral of Philosophy Degree in Pharmaceutical Sciences
with an emphasis in Pharmaceutics

by

TUSHAR HINGORANI

August 2012

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ABSTRACT

Relationship between smoking marihuana and a drop in intraocular pressure (IOP) was first reported in 1971. Research efforts since then have identified a number of constituents that could be linked to this observation. Δ^9-Tetrahydrocannabinol (THC) was one of the ingredients identified. However, four decades and numerous research efforts since the first observation, it has still not been concluded whether THC is effective in the treatment of glaucoma or not. Current therapy for IOP control in glaucoma, though effective, cannot prevent vision loss completely. Thus, identification of agents that can lower IOP as well as protect the retinal ganglion cells from apoptosis could yield a new class of anti-glaucoma molecules. Protection of retinal ganglion cells against apoptosis could yield a new class of antiglaucoma agents. Numerous investigations have demonstrated that THC also acts as a neuroprotective agent making it a very promising lead compound. However noninvasive delivery of THC to the targeted intraocular tissues is challenging due to its poor physiochemical properties.

The aim of the current project was to effectively deliver THC to the intraocular tissues. THC prodrugs were synthesized to improve physicochemical properties. Preformulation characterization and formulation development for the THC prodrugs was undertaken. A high throughput *in vitro* model to screen the various formulations for transcorneal permeability was also developed. Promising formulations were instilled *in vivo* in anesthetized rabbit model and tissue concentrations were determined. Earlier formulations reported in the literature, used for determining efficacy were not observed to deliver THC to the targeted intraocular tissues. The formulations developed in this study were able to deliver significantly higher concentrations to

the intraocular tissues. Future studies need to investigate if these improved formulations of THC demonstrate pharmacological activity against glaucoma.

DEDICATION

I would like dedicate the thesis to my parents Dr. Lal Hingorani and Kanchan Hingorani for their encouragement, unyielding support and love throughout the course of my life.

ACKNOWLEDGEMENTS

Foremost, I would like to thank my adviser Dr. Soumyajit Majumdar for his support, patience and guidance throughout my graduate studies. He has been a source of great innovation and inspiration. I am grateful to my dissertation committee members Dr. Michael A. Repka, Dr. Seongbong Jo and Dr. John O`Haiver for their guidance and time. Finally, I would like to thank my parents, sister and close friends for supporting me throughout my thesis.

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CHAPTER 1

INTRODUCTION

1.1 Anatomy of the eye

The eye, responsible for vision, is one of the most important organs in the human body. It is roughly spherical in shape and averages about 22.4 mm across. The anterior chamber of the eye consists of the cornea, aqueous humor, iris-ciliary body and the lens while the posterior segment consists of the vitreous humor, retina and the sclera (Fig. 1-1).

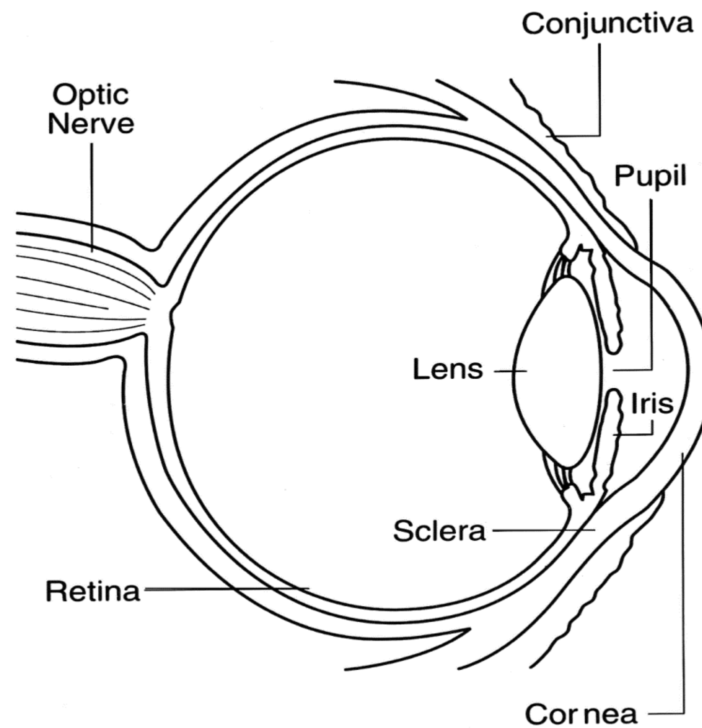

Figure 1-1: Schematic representation of the eye.

Adapted from National Eye Institute, National Institutes of Health

1.1.1. Cornea

The cornea is a multilayered structure composed of lipophilic epithelium and endothelium with a hydrophilic stromal layer sandwiched between them. The corneal epithelium represents the major barrier for transcorneal diffusion (Fig. 1-2). The epithelium in turn is composed of 4-5 cell layers. The uppermost layer consists of squamous cells (polygonal cells) followed by the wing cell and basal cell layers. The squamous layer is tightly linked together by continuous intercellular junctions(1). Transmembrane proteins, claudins and occludins form the tight junctions between the cells. These tight junctions act as a barrier to the passage of hydrophilic molecules through the intercellular spaces. The squamous layer also contains microvilli which is responsible for tear film stability. The basal cells layer is made up of columnar cells that replace the epithelial cell layer by undergoing mitotic division. Underlying the epithelium is a layer of collagen fibers, known as the Bowman's membrane, which also restricts permeability of drug molecules(2). The stroma is hydrophilic in nature and is quite porus allowing passage of large hydrophilic molecules. The stroma consists of 90% water. Because of its hydrophilic nature, the stroma acts as a barrier for lipophilic molecules(3). The endothelium does not possess significant barrier properties, allowing even large molecules to pass through it.

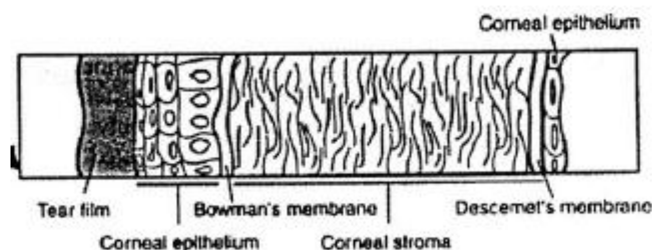

Figure 1-2: Schematic representation of the different lavers of the cornea.
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1.1.2. Aqueous Humor

The aqueous humor is a clear transparent liquid present between the lens and the cornea. It forms an important part of the eyes optical system. The chamber containing the aqueous humor is divided into two portions namely the anterior chamber and the posterior chamber by the iris. The aqueous humor is produced in the posterior chamber by the iris ciliary body and flows over the lens through the opening in the iris to the anterior chamber. It is then drained by the uveoscleral outflow or through the trabecular meshwork in the Schlemm`s canal(5). Aqueous humor production and drainage in the eye has been represented in Fig. 1-3. The total volume of aqueous humor in the anterior chamber is 200-300 μL and the turnover of aqueous humor in the eye is 2.4 ± 0.6 μL/min(6). The main functions of the aqueous humor are nourishment and waste removal of the tissues it comes in contact and maintenance of intraocular pressure.

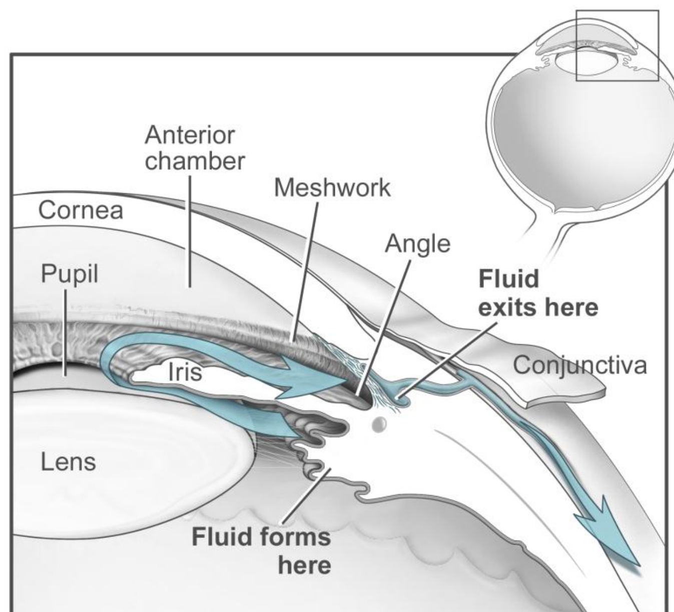

Figure 1-3: Schematic representation of the aqueous humor formation and drainage in the eye. Adapted from the National Eye Institute, National Institutes of Health.

1.1.3. Iris Ciliary Body

The iris divides the chamber containing the aqueous humor of the eye into anterior chamber and the posterior chamber. The amount of melanin present in the iris defines the color. There is no epithelium present on the iris. The sphincter and the dilator muscles in the iris let it close or open depending on the light conditions.

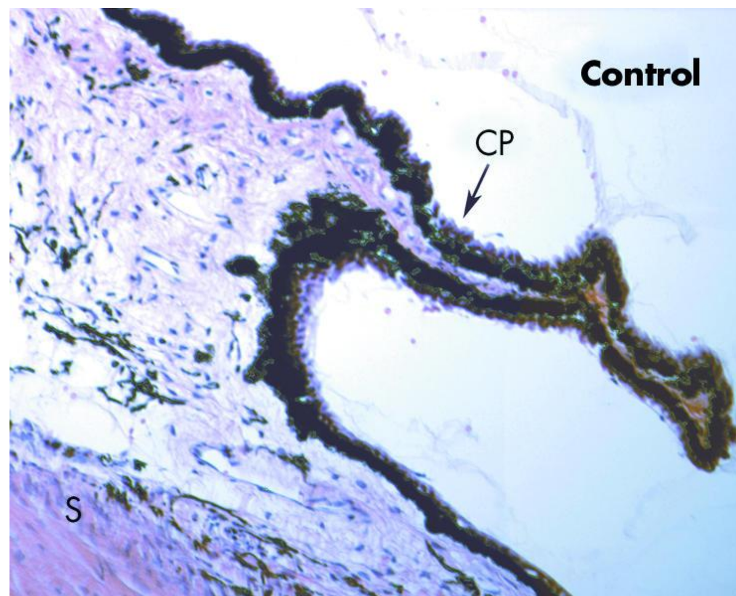

Figure 1-4: Ciliary processes present on the ciliary body.
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The ciliary body plays an important role in aqueous humor production and drainage. The ciliary body consists of ciliary muscle and ciliary processes with basal and later indigitation are present on the surface of the ciliary body (Fig. 1-4)(5). The epithelium of the ciliary process consists of an outer pigmented layer and an inner non-pigmented layer. The aqueous humor is produced by the inner non pigmented layer. There are two major pathways for the drainage of

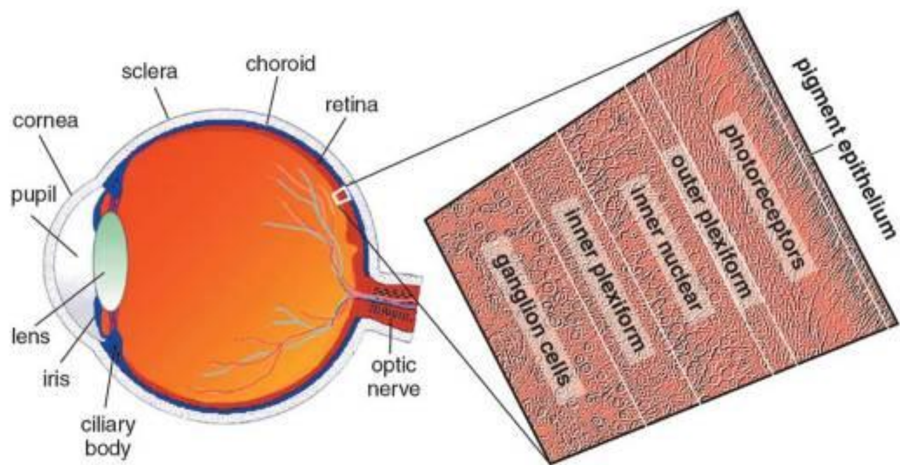
aqueous humor from the anterior ocular segment: drainage through the Schlemm's canal or the uveoscleral route. The major outflow pathway for the aqueous humor is drainage through the schlemm's canal. The trabecular meshwork (TM), a mesh like structure present in front of the schlemm's canal, presents the major resistance to the outflow of aqueous humor(8). Ligaments from the ciliary muscle extend into the TM and contraction/relaxation of the ciliary muscle leads to modulation of the intercellular spaces in the TM, and thus aqueous humor outflow(8). The non-pigmented ciliary epithelium forms the blood aqueous barrier with the vascular endothelium of retinal blood vessels.

1.1.4. Vitreous Humor

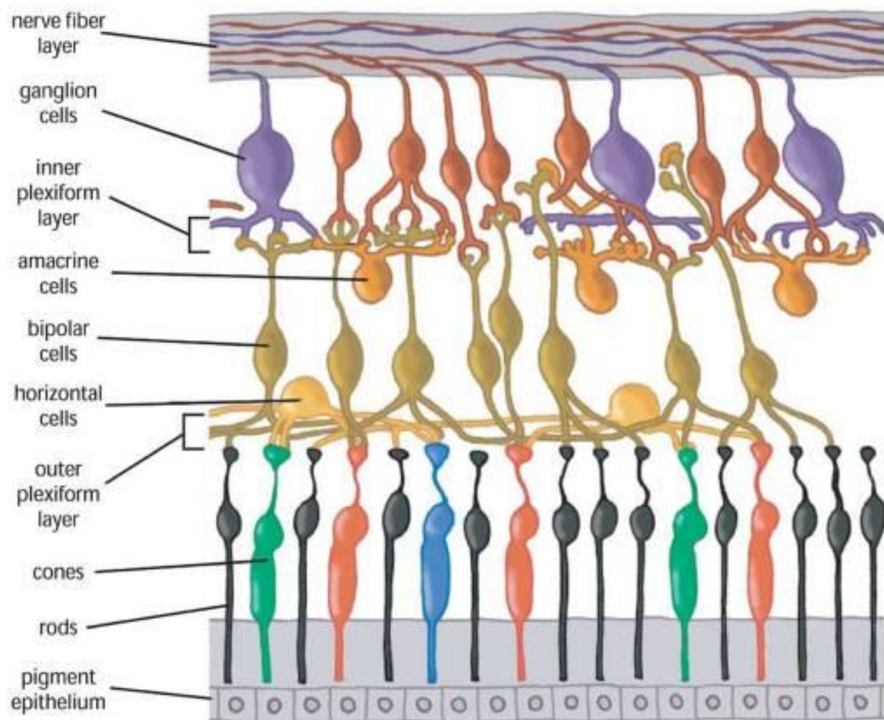
The vitreous humor is a clear extracellular matrix with gel like consistency present in the posterior chamber of the eye. It is present between the lens and the inner limiting lamina of the retina. The pH of vitreous humor is about 7.5 and is largely devoid of any cellular material. It occupies about 80% of the total volume of the eye. A meshwork of collagen fibrils (12-15 nm in diameter) lead to the gel like consistency of the vitreous humor. Hyaluronic acid, a mojor component of vitreous humor, occupies the space between the collagen fibers(9).

1.1.5. Retina

Light is focused by the cornea and the lens on the retina which converts it into a neural signal that is processed as an image by the brain. A schematic representation of the retina is depicted in Fig. 1-5. The retina is divided into the retinal pigmented epithelium and the neural retina. The retinal pigmented epithelium consists of cuboidal epithelium cells (Fig. 1-6). Choriocapalliries are leaky and usually allow drugs to equilibrate in the choroidal extracellular space. However, tight junctional proteins are present between the cuboidal cells forming a tight barrier known as the outer blood retinal barrier(10, 11).



(a)



(b)

Figure 1-5: Schematic representation of the retinal layers

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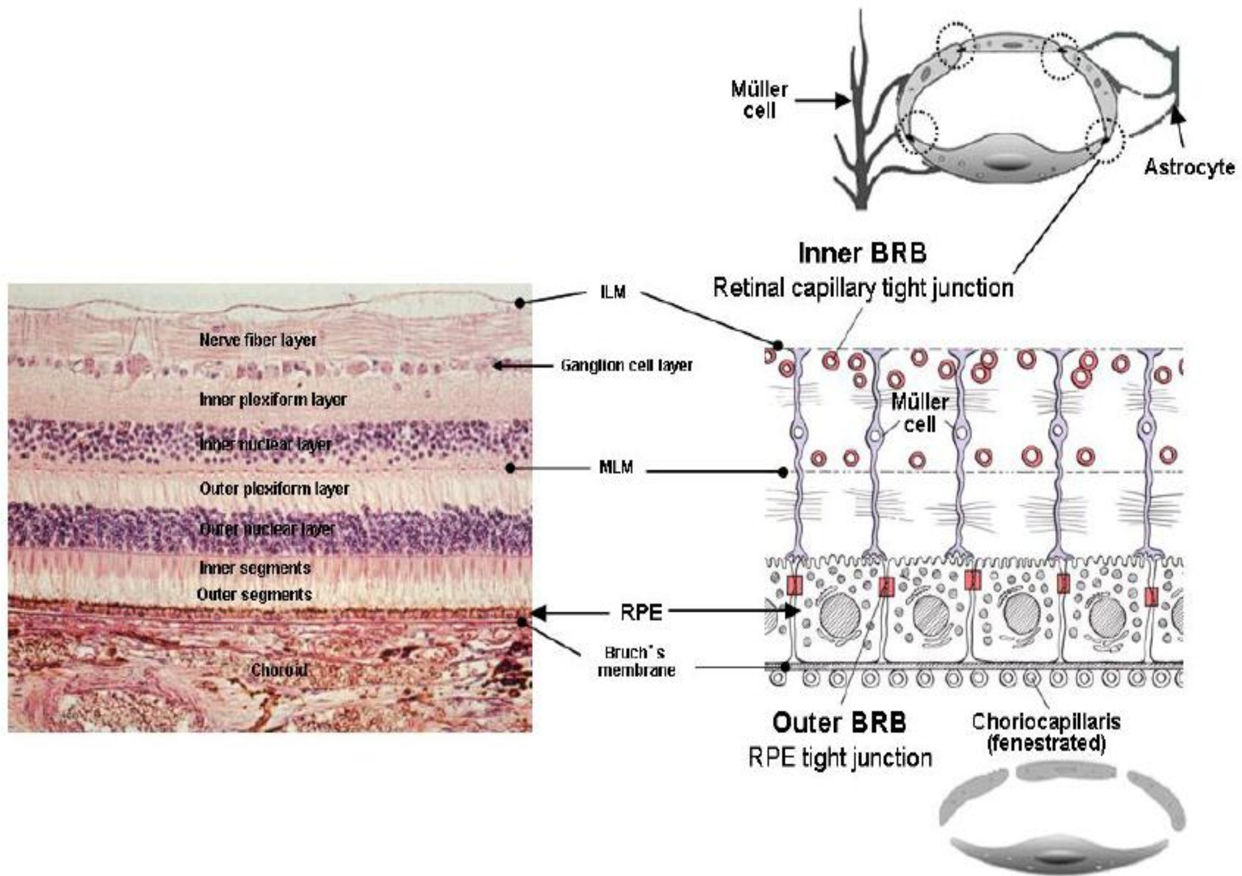

Figure 1-6: Schematic representation of the blood retinal barrier.

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The neural retina consists of the photoreceptor layer, the outer plexiform layer, the inner nuclear layer, inner plexiform layer and the ganglion layer. The ganglion cell layer consists of the rods and the cones. Retinal capillaries are present in the inner nuclear layer and the outer plexiform layer. The endothelial cells junctions of retinal capillaries is lined by tight junctions. These tight junctions do not allow xenobotics to enter into the retina forming the inner blood retinal barrier. Astrocytes are closely associated with tight junctional proteins present on the retinal endothelial cells and support their function(11).

1.1.6. Sclera

The cornea and the sclera together form the outer covering of the eyeball. Together they help maintain the shape of the eyeball. The sclera is composed of collagen fibers and proteoglycans. The scleral thickness varies from the limbus to the equator. It is relatively thick near the limbus (0.53 ± 0.14 mm) and thin near the equator (0.39 ± 0.17 mm). Although both cornea and the sclera are made up of collagen fibers, sclera is opaque in nature(13).

1.2. Glaucoma

Glaucoma is one of the world's primary causes of vision loss and is ranked third in the list of people's major fears (after cancer and heart disease)(14). It affects nearly 70 million people worldwide, including an estimated 2.2 million Americans. An additional 2 million Americans remain undiagnosed. African-Americans over the age of 40 and all adults over the age of 60, especially Hispanics, are at a higher risk for the condition(3). In terms of economic impact, Glaucoma accounts for over 7 million visits to physicians each year and is estimated to cost the U.S. government over $1.5 billion annually (15).

The term glaucoma encompasses a group of disorders that cause damage to the optic nerve involving loss of retinal ganglion cells, leading to vision loss or blindness if left untreated. An increase in the intraocular pressure (IOP) is considered to be a significant risk factor in the progression of the disease (16, 17). The most common form of glaucoma is primary open-angle glaucoma (POAG), in which fluid builds up in the front chamber of the eye(18). The resulting increase of pressure within the eye damages the optic nerve leading to a disruption in the transmission of visual information to the brain(19).

A number of therapeutic candidates are currently available that reduce the IOP (20). The classes of medicinal agents and their mechanism of action have been depicted in Table 1-1.

Table 1-1: Drugs used in the treatment of glaucoma and their mechanism of action.

Drug class	Mechanism of action of decreasing IOP
Prostaglandin analogs (e.g. latanoprost and bimatoprost)	Increases uveoscleral outflow and/or trabecular outflow of aqueous humor;
Beta-adrenergic receptor antagonists (timolol, betaxolol	Decreases aqueous humor production
Carbonic anyhydrase inhibitors (drozolamide)	Lowers secretion of aqueous humor by inhibiting carbonic anhydrase in the ciliary body.
Alpha2-adrenergic agonists (brimonidine)	Decreases aqueous humor production and increases uveo-scleral outflow
Sympathomimetics (epinephrine)	Increase outflow of aqueous humor through trabecular meshwork and possibly through uveoscleral outflow pathway
Parasympathomimetics (pilocarpine)	Contraction of the ciliary muscle, tightening the trabecular meshwork and allowing increased outflow of the aqueous humour

However, a reduction in IOP is often not enough to prevent or arrest the development or progression of glaucoma related optic neuropathy (21, 22). It has been suggested that neuroglial cell cytotoxicity in the optic nerve and retina leads to visual field loss in glaucoma (23). Neuroglial cell toxicity impairs macroglial glutamate metabolism and causes microglia and macroglia to release inflammatory cytokines following ischemia due to compression or vascular occlusion. The released glutamate acts on receptors including the NMDA subtype on the retinal ganglion cells to induce calcium influx and the release of toxic ROS leading to apoptosis (23).

The drawback of currently available therapeutic options in the treatment of glaucoma is that they do not exert any neuroprotective activity. Moreover, there is a patient population that does not respond to the above listed classes of therapeutic agents. Thus, a new class of compounds that would not only lower IOP but would also possess neuroprotective action is direly needed in the continued fight against glaucoma. One candidate that exhibits a lot of promise is Δ^9-Tetrahydrocannabinol (23, 24).

1.3. Δ^9-Tetrahydrocannabinol

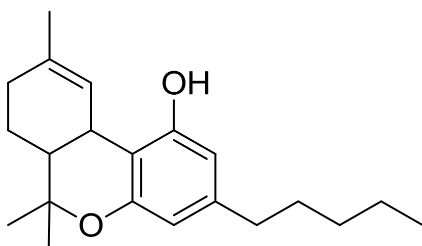

Figure 1-7: Chemical structure of Δ^9-Tetrahydrocannabinol

Δ^9-Tetrahydrocannabinol (THC, Fig. 1-7) is the primary active ingredient of the plant *Cannabis sativa* (marijuana) and is responsible for the majority of the pharmacological effects. People have utilized the plant (that includes numerous cannabinoids) since ancient times for medicinal purposes as well as for its intoxicating properties. While marijuana is primarily known as an abused drug, there are important pharmacological properties of THC that could be directed to specific therapeutic effects, given an appropriate delivery mechanism. To date, the clinical applications approved by the Food and Drug Administration (FDA) are for the control of nausea and vomiting associated with chemotherapy and for appetite stimulation of AIDS patients

suffering from anorexia and wasting syndrome (25, 26). However, THC's therapeutic potential in a host of other medical areas, including glaucoma, has been a subject of intense research.

In 2005 an *ad hoc* expert committee report to the National Eye Institute (NEI) and a publication from the American Association of Ophthalmology concluded that there was insufficient evidence supporting any added advantages of THC over other glaucoma medications (27, 28). At the time when these reports had been prepared a systematic investigation into topical delivery of THC had not been carried out in the scientific community. In fact, the literature reports available (29, 30), suggested that THC did not demonstrate any therapeutic activity when topically administered (31, 32). However, we did demonstrate that the more polar derivatives, THC hemisuccinate ester prodrugs N-methylglucamine salt, did produce a significant reduction in IOP. Unfortunately, this preliminary compound was highly unstable in aqueous solutions and caused ocular discomfort, possibly because of the alkaline pH necessary for solubilization (30). Because of the inconclusive activity reports following topical application and lack of a clear understanding of the mechanism of action (i.e. whether THC acted through the CNS or local cannabinoid receptors, CB1 and/or CB2) (33-37), most of the conclusions drawn by the committee, with respect to the utility of THC in glaucoma, were based on the administration of THC either through inhalation (smoking) or through systemic administration; routes of delivery that is associated with the systemic side-effects of THC.

Over the last few years a significant amount of research, supported by the NEI and other funding agencies, has been focused on the pharmacology of cannabinoids and cannabinoid receptors expressed on ocular tissues and glaucoma. It has now been established that CB1 and CB2 receptors are expressed on the trabecular meshwork (TM) of the eye and on the retinal ganglion cells (38-42). Moreover, studies have demonstrated that the endocannabinoids, THC

and a synthetic analog WIN 55-212-2, can bind to the CB1 and CB2 receptors expressed on the TM and lead to increased aqueous humor outflow (38-45). The mechanism of action appears to be changes induced in the actin cytoskeleton of the TM as a result of activation of the MAPK pathway with the binding of cannabinoids to the CB1 and CB2 receptors expressed on the TM. This leads to increased drainage of aqueous humor through the TM and a resultant drop in the IOP (42-44, 46-48).

What makes THC particularly attractive is its neuroprotective action. El-Remessy et al. demonstrated the effectiveness of THC in NMDA induced retinal neurotoxicity (23). In these experiments THC was injected intravitreally. Other reports also demonstrate the effectiveness of THC in the preservation of the retinal ganglionic cells (24, 49).

Thus, there is now little doubt about the potential of THC in the treatment of glaucoma and preservation of vision. The challenge lies in the effective and efficient delivery of THC to the anterior and posterior chamber ocular tissues through topical THC instillation. So far, this has not been examined in a methodical manner. The renewed interest in THC for the treatment of glaucoma has produced exciting new evidence and motivated us to reinvestigate topical delivery of THC by developing more stable and water soluble THC derivatives.

1.4. Challenges in topical delivery of THC

Delivery of THC to the deep seated ocular tissues (TM and retinal ganglion cells) through topical administration is a challenging task. THC is an extremely lipophilic compound with a high logP value. Although THC is reported to be absorbed across the intestinal membranes, the cornea is very different since it is comprised of a lipophilic epithelium, a hydrophilic stroma (90% water) and a lipophilic endothelium. For efficient transcorneal permeation, the therapeutic agents need to possess optimum physicochemical characteristics suited for this complex

structure. Moreover, there are a host of physiological barriers that minimize penetration of topically applied agents to the back-of-the eye.

Early trials in 1977 examined the use of various oils for topical THC application. Amongst these, light mineral oil (LMO) was found to be the most effective. A 0.1% (50 µg in 50 µL) THC solution in LMO produced an aqueous humor concentration of 0.05 µg/mL which correlated to a 6-20% drop in IOP in rabbits (50). Green et al. conducted a study examining the effectiveness of a single drop of 0.1% THC (in LMO) in humans. In this study a decrease in IOP was not observed in the normal human volunteers (31). Studies conducted (29, 30), with THC dissolved in various oily vehicles and topically applied, also did not demonstrate any reduction in IOP in the rabbit model. Subsequently, in the year 2000, Kearse and Green evaluated transcorneal permeability of THC, *in vitro,* from various vehicles including LMO (51). With LMO as the vehicle, corneal permeability of THC was only 0.18×10^{-9} cm/s, which is extremely poor by any standard and could be a reason for the observed lack of any IOP lowering effect *in vivo*. Amongst the multiple vehicles tested by Green, maximum permeability (2.4×10^{-6} cm/s) was observed when hyaluronic acid (0.5%) was present in the formulation. Incidentally, when the authors used 30% HPβCD in the vehicle, transcorneal THC permeability was observed to be only 0.33×10^{-9} cm/s. In 1992, Muchtar et al. demonstrated that a 0.4% THC in a submicron emulsion formulation produced intense and long lasting reduction in IOP in a rabbit model of ocular hypertension (chymotrypsin treated) (52). Since *in vitro* transcorneal permeability of THC from the submicron emulsions or THC concentrations generated in the aqueous humor *in vivo* from this vehicle was not determined in the studies conducted by Muchtar et al., it is not possible to conclude whether the improved IOP lowering effect was as a result of increased dose (from 0.1% to 0.4%) or increased transcorneal / trans-scleral permeability. These initial literature

reports demonstrates that there is sporadic evidence of the effectiveness of topically applied THC in glaucoma and highlights the need for a concentrated effort in developing a proper transcorneal and transretinal delivery strategy for this very attractive therapeutic candidate.

Significant effort has not been made to improve the physicochemical characteristics of THC, through prodrug modification, to enhance transcorneal permeability characteristics. Thus, we will first design and identify a significantly more polar and stable THC prodrug with optimized transcorneal permeability characteristics leading to significant IOP lowering capability *in vivo* in rabbits.

Ophthalmic formulations are usually formulated at high concentration (1-4 % w/v) to overcome the short contact time on the ocular surface. THC, due to its water insoluble nature, is particularly difficult to deliver topically to the intraocular tissues. Its high lipophilicity also provides a challenge to transcorneal permeability due to hydrophilic stroma sandwiched between a lipophilic bilayer. THC tends to accumulate in the lipophilic epithelium of the cornea and not pass through the hydrophilic stroma. This necessitates structural modification of THC to improve physicochemical characteristics.

1.5. Prodrugs

Chemical modification of a therapeutic agent by way of prodrug design has become an important drug delivery tool (53-55). This is one of the most widely accepted and successful strategies for modifying physicochemical characteristics, aqueous solubility, chemical and enzymatic stability of drug candidates through linkage of appropriate promoieties. A significant positive aspect of the prodrug approach is that it does not induce any change in the membrane

structure, fluidity or characteristics. The prodrugs are cleaved *in vivo* to generate the active drug and the harmless promoiety, which is eliminated from the body (Fig. 1-8).

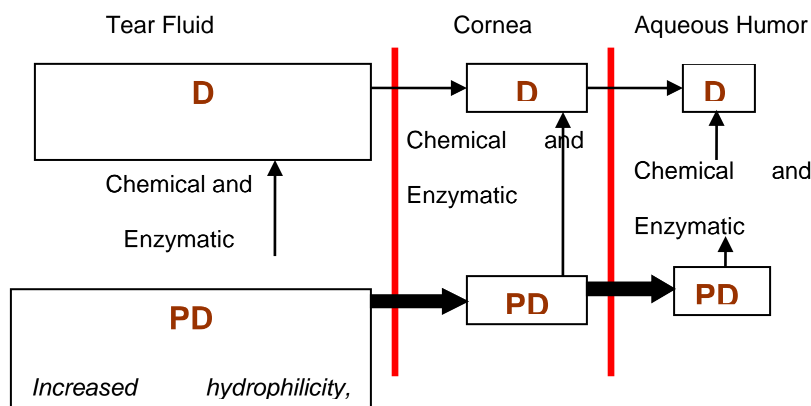

Figure 1-8: Schematic representation of the utility of the prodrug concept. Prodrug (PD) derivatization of the parent drug THC (D), in combination with the formulation excipients improves overall permeability. Transcorneal permeability and bioreversion are illustrated by arrows. Line thickness represents the extent and higher or lower rates of permeability.

Earlier studies, evaluating topical THC administration, it was observed that the polar derivatives partitioned better across the cornea and produced a significant drop in the IOP(30). These initial results support lend direct support to the hypothesis of this application and synthesis of more stable and hydrophilic THC prodrugs.

Cytochrome P 450 (CYP) mediated (primarily CYP 2C9 and CYP 3A4) metabolism of THC is well established (56, 57). The cornea is also known to express CYP enzymes, although at much lower concentrations than that in the liver (58). A possible benefit that could be derived from THC prodrug approach could be diminished CYP mediated metabolism (59, 60) and thus greater THC penetration into the aqueous humor.

THC has an acidic phenolic hydroxyl group (pka 10.6) that does not ionize at physiological pH but is amenable to chemical modification. Attachment of an ionizable group in the low pH range would improve aqueous solubility and permeability across the hydrophilic stroma.

1.5.1. Dicarboxylic acid (DCA) ester prodrugs

DCA's are commonly used promoieties in prodrug derivatization approaches. In earlier studies evaluating the effect of topically administered THC, the water soluble salt (N-methylglucamine) of THC-HS produced a significant drop in the IOP(30). However, the prodrug was observed to degrade rapidly and further studies had not been pursued. The improved transmembrane permeability characteristics of THC-HS was also observed with transrectal delivery: THC-HS demonstrated significantly higher blood levels compared to the other prodrugs tested(61). Two esters, Tetrahydrocannabinol hemisuccinate (THC-HS) and Tetrahydrocannabinol Hemiglutarate were synthesized. These prodrugs improved aqueous solubility due to presence of ionizing group with a low pka. At low pH values when the prodrugs were unionized, they demonstrated an inherent improvement in permeability. At physiological pH values the permeability was significantly decreased to values even lower than THC since they were completely charged.

1.5.2. Amino acid prodrugs:

In the past decade amino acids have taken center stage as promoieties for transporter targeted prodrug derivatization of hydrophilic drug molecules (62-68). Some studies exploiting this mechanism for circumvention of efflux proteins have also been published (69-72). A few studies exploring the use of single amino acid based prodrug derivatization to enhance hydrophilicity of lipophilic molecules and improve oral absorption have also been reported (73-

80). This application proposes to systematically design, synthesize, screen and test a matrix of mono- and di- amino acid based THC prodrugs. Literature reports indicate that amino acids with an electron donating side chain form ester linkages that are more stable. Valine conjugates have thus been observed to form extremely stable prodrugs. On the other hand, polar amino acid based THC prodrugs may demonstrate greater solubility. Thus, a combination of THC-$AA_{nonpolar}$-AA_{polar} may yield significantly more stable and soluble THC prodrugs. Besides modulation of lipophilicity, addition of a peptide bond has been observed to enhance the stability of the ester linkage (65). Thus, decreased lipophilicity, increased solubility and enhanced stability may be achieved via this approach. Amino acid-dicarboxylic acid esters demonstrated significant improvement in physiochemical parameters. *In vivo* bioavailability of THC from prodrug formulations was compared to formulations of THC that were previously studied to determine *in vivo* bioavailability.

CHAPTER 2 AIMS OF THE STUDY

The goal of the project is to achieve optimal concentrations of Δ^9-Tetrahydrocannabinol in the iris-ciliary body and in the retina through topical delivery. Δ^9-Tetrahydrocannabinol being a highly lipophilic drug, a combination of chemical modification and formulation approaches will be explored. An *in vitro* model for the high throughput screening of Δ^9-Tetrahydrocannabinol prodrugs and formulations also needs to be developed.

SPECIFIC AIMS:

1. To develop a high throughput *in vitro* screening tool for Δ^9-Tetrahydrocannabinol prodrugs and formulations (Chapter 3).
2. To screen Δ^9-Tetrahydrocannabinol dicarboxylic acid prodrugs for solubility, stability, physicochemical properties and *in vitro* permeability across the cornea (Chapter 4).
3. To formulate Δ^9-Tetrahydrocannabinol dicarboxylic acid prodrug exhibiting good solubility and permeability characteristics in micellar formulations and determine *in vivo* bioavailability (Chapter 5).
4. To screen and formulate amino acid, amino acid- amino acid and amino acid dicarbocylic prodrugs of THC and determine *in vivo* bioavailability (Chapter 6).

CHAPTER 3

EVALUATION OF ACTIVE AND PASSIVE TRANSPORT PROCESSES IN CORNEAS EXTRACTED FROM PRESERVED RABBIT EYES.

3.1. ABSTRACT

In vitro transcorneal permeability studies are an important screening tool in drug development. The objective of this research is to examine the feasibility of using corneas isolated from preserved rabbit eyes as a model for permeability evaluation. Eyes from male New Zealand White rabbits were used immediately or were stored overnight in PBS or HBSS over wet ice. Integrity of isolated corneas was evaluated by measuring the TEER and by determining the permeability of paracellular and transcellular markers. Active transport was assessed by measuring transcorneal permeability of selected amino acids. Esterase activity was estimated using p-nitrophenyl assay. In all cases, corneas from freshly enucleated eyes were compared to those isolated from the day-old preserved eyes. Transcellular and paracellular passive diffusion was not affected by the storage medium and observed to be similar in the fresh and preserved eye models. However, amino acid transporters demonstrated lower functional activity in corneas excised from eyes preserved in PBS. Moreover, preserved eyes displayed almost 1.5-fold lower esterase activity in the corneal tissue. Thus, corneas isolated from day-old eyes, preserved in HBSS, closely mimics freshly excised rabbit corneas in terms of both active and passive transport characteristics but possesses slightly reduced enzymatic activity.

3.2. INTRODUCTION

Transcorneal absorptive pathway is the most important route of absorption for drugs applied topically to the eye (81). A major challenge for pharmaceutical scientists attempting drug delivery to the anterior segment of the eye, however, is overcoming the epithelial tight junction barriers (82) and also tailoring the pharmaceutical properties of the compound to meet the diffusional restrictions imposed by the unique corneal structure comprising of both hydrophilic and lipophilic layers. To exert therapeutic activity, a topically administered agent must demonstrate sufficient corneal permeability so as to generate adequate drug concentrations at the site of action in the anterior ocular chamber. Thus, *in vitro* transcorneal permeability studies play a critical role in the screening and selection of drug molecules and formulation components during the design phase.

Compared to *in vivo* experiments, *in vitro* experiments avoid and isolate confounding physiological mechanisms from transmembrane diffusion; are less time consuming; amenable to high throughput screening; and are thus preferred. Currently, the most favored model for *in vitro* corneal permeability determinations are freshly excised animal corneas (83).

Rabbit eyes are physiologically very similar to the human eyes and have been extensively used to study corneal permeation of therapeutic moieties and to optimize topical formulations before preclinical and clinical testing. Unfortunately, these experiments require sacrificing of animals procured specifically for the purpose of *in vitro* testing and also incur high costs associated with the caring for laboratory animals (84). In some cases investigators use corneas isolated from rabbits used in other protocols. This practice does reduce the number of animals required but leaves the investigators unsure about the number and time of availability of the tissues and suffers from a lack of control on parameters such as age and sex. Moreover, the

experiments carried out in the original protocol may affect ocular permeability characteristics. It would be a significant step towards reducing the number of animals required for research purpose if the pressing need to specifically sacrifice rabbits for the purpose of carrying out *in vitro* transcorneal experiments could be eliminated.

Cell cultures, as *in vitro* models, have not met with much success with respect to projection of corneal permeability rates. This is primarily because of the lack of tight junction expression, variability in the expression and polarization of transporter proteins (85) and difficulty in accurately mimicking the multi-layered and multi-component corneal structure in the currently available cell culture models. The cell lines also exhibit time dependent TEER values, have a high cost associated with producing and maintaining the cultures and are susceptible to microbial contaminations (86). Becker *et al*. have compared different epithelial cell culture models available as well as corneal constructs for *in-vitro* drug permeation(87). The authors compared Statens Serum Institute Rabbit corneal cells (SIRC), transformed human corneal epithelial cells (HCE-T) cell lines as well as commercially available SkinEthic reconstituted human corneal epithelium (HCE-S) and Clonetrics cultured human corneal epithelium (HCE-C). The results indicated that SIRC, HCE-S and HCE-T could not differentiate between the permeabilities of molecules with different physicochemical properties. Only HCE-C remained a viable option but has to be used within 24 hours of receipt from the manufacturer. Since ^{14}[C] mannitol permeability across HCE-C was not reported a direct comparison cannot be made between the corneal tissue and the HCE-C cell line in terms of the paracellular diffusional barrier properties. Moreover, the authors did not evaluate the cell culture models with respect to molecular expression and functional activity of carrier-mediated systems, which plays a critical role in transcorneal diffusion.

Alternatively, eyes obtained from local abattoirs have been evaluated for studying transcorneal permeability (88-90). Various means to transport the excised eyes from the slaughterhouse to the laboratory have been reported. Eyes have been transported directly on ice or have been placed in solutions and transported over ice(88-90). In all studies, the eyes were used within a couple of hours post isolation and knowledge about the effect of prolonged storage is currently lacking. Considering that the abattoirs would sacrifice the animals only once or twice a week, an option for preserving the procured eyes overnight in specified solutions, would significantly help in the research process as well as bring down the need for sacrificing laboratory animals. Storage may, however, lead to alterations in the corneal epithelial structure and protein expression which would impact permeability of drug molecules. To date, the effect of the storage conditions and storage solutions on corneal integrity and permeability characteristics has not been fully investigated.

Literature indicates that eyes stored in phosphate buffered saline (PBS) and hanks balanced salt solution (HBSS) have been successfully used for the preparation of primary cell cultures (27, 28, 91), indicating that corneal cells remain viable in these medium for the duration tested. However, for these studies, maintenance of the integrity of the corneal epithelial barriers was not important and was thus not tested. Corneas stored in unfavorable media would swell, display decreased integrity due to the loss of tight junction proteins and may also demonstrate diminished functional activity of transporters expressed on the cornea. As a result studies with these tissues may predict erroneous permeability values for the test compounds.

Incidentally, a lot of research has been focused on developing media for the storage and transportation of human corneas. Preserved corneas have been evaluated with respect to biological and histological characteristics (92-95). Although corneas are shipped for

transplantation in human subjects, the storage / transport solutions available are very expensive and the sheer volume required for shipping whole animal globes makes these media even less attractive for *in vitro* corneal permeability studies in the drug development process. Moreover, surprisingly, the storage solutions currently used have never been evaluated with respect to the preservation of the corneal epithelial barrier and transporter characteristics.

In this study, we have evaluated PBS and HBSS, two commonly used cell culture media, as storage solutions for whole rabbit eye globes. Mannitol and diazepam have been routinely used as markers for passive paracellular and transcellular pathways and were thus selected for this study. Additionally, corneal permeability of the hydrophilic nucleoside antiviral agent, acyclovir, known to diffuse across the cornea by passive diffusion mechanisms(96) , was also studied. Quinidine, a lipophilic compound traversing biological membranes by the transcellular route using a combination of passive and active diffusional processes(97), was also included as a marker compound. Moreover, the functional activity of the three amino acid transporters previously reported on the corneal epithelium: the $B^{0,+}$, ASCT1 and L-type amino acid transporters were also tested (98-100). In addition to passive and active transport processes, activity of esterase enzymes was also compared since these enzymes regulate bioreversion of ester prodrugs in the corneal tissue and, thus, can impact net corneal permeability. It was assumed that since the eyes would generally be preserved over wet ice, the metabolic rate would be minimal. Thus, addition of nutrients, e.g. essential amino acids, would not be necessary. Also, since whole animal globes, rather than isolated corneas, would generally be preserved, no osmotic agent such as dextran was added to the medium.

3.3. EXPERIMENTAL SECTION

3.3.1. Materials

Acyclovir was obtained from Hawkins Inc. (Minneapolis, USA) and quinidine from Sigma Chemical Co. (St. Louis, USA). Radiolabelled amino acids were obtained from Moravek Biochemicals, Inc. (Califonia, USA) and [^{14}C]mannitol and [^{3}H]diazepam were from Perkin Elmer Life and Analytical Sciences (Boston, USA) respectively. All other solvents and chemicals were obtained from Fisher Scientific (Fair Lawn, NJ), and used as such.

3.3.2. Animals

Male, albino, New Zealand rabbits weighing between 2 to 2.5 kg were procured from Myrtles' Rabbitry (Thompson Station, TN). Animal experiments conformed to the tenets of Association for Research in Vision and Ophthalmology (ARVO) statement on the Use of Animals in Ophthalmic and Vision Research.

3.4. METHODS

3.4.1. Corneal Permeation Studies

Animals were anesthetized with ketazime/xylazine given intramuscularly and euthanized by an excess of pentobarbital injected through the marginal ear vein. Some of the globes so obtained were either stored in PBS or HBSS, over wet ice, or were taken for immediate isolation of the corneas and further experimentation. Corneas from the preserved globes were isolated 24 hours after storage initiation. Corneas were excised, following previously published protocols (101), with approximately 1 mm scleral portions remaining for ease of mounting. The corneas were mounted between standard, 9 mm, side-by-side diffusion cells (PermeGear Inc., Bethlehem,

PA) with the epithelial layer facing the donor side. Temperature was maintained at 34°C during the transport studies with the help of a circulating water bath. Dulbeccos phosphate buffered saline (DPBS) was used as the transport medium. Volume of the receiver solution (3.2 mL DPBS) was slightly higher than that of the donor solution (3 mL drug solution) to maintain the natural curvature of the cornea. Contents of both chambers were stirred continuously. Aliquots, 200 µL, were withdrawn at appropriate time intervals and immediately replaced with an equal volume of DPBS and stored at -80° C until further analysis. Unlabeled samples were analyzed using an HPLC system.

To the radioactive aliquots five milliliters of scintillation cocktail (Scintisafe Econo 2, Fisher Scientific, USA) was added and the radioactivity was measured using a Liquid Scintillation Analyzer (Perkin Elmer Life and Analytical Sciences, Model TriCarb 2900TR, CT, USA).

3.4.2. Light Microscopy

Corneas were stained with hematoxylin-eosin using previously published procedures(87). Briefly the corneas were dehydrated in increasing ethanol concentration, embedded in paraffin wax and then cut into 4µM sections. These sections were stained with hematoxylin and eosin solutions and used for light microscopy.

3.4.3. Trans-epithelial Electrical Resistance (TEER)

Ag/AgCl electrodes 2 mm in diameter were shaped in the form of circular rings and placed approximately 2 mm from the cornea in both the donor and the receiver compartments and the chambers were filled with DPBS solution. The electrical resistance across the corneas was measured, every hour for a period of three hours, using an experimental setup consisting of a waveform generator and digital multimeter (Agilent Technologies, Santa Clara, CA).

3.4.4. Paracellular and Transcellular Permeability

Tight-junction characteristics was compared using acyclovir (1mM) and [^{14}C]mannitol (0.5 μCi/ml, specific radioactivity 55 mCi/mM) in DPBS, as paracellular diffusion markers. The transcellular permeability markers quinidine (0.5μM), and [^{3}H]diazepam (0.5 μCi/ml, specific radioactivity 70 mCi/mM) in DPBS, were used to compare the properties of the lipoidal cell membrane. The studies compared fresh versus preserved corneas.

3.4.5. Transport Activity of Amino Acid Transporters

To compare functional activity of corneal amino acid transporters, transcorneal permeability of [^{14}C]L-Arginine (specific activity 57 mCi/mM), [^{14}C]L-Phenylalanine (specific activity 391 mCi/mM) and [^{14}C]L-Alanine (specific activity 162 mCi/mM) was determined in both freshly excised and preserved rabbit corneas. L-Arginine and L-phenylalanine are known to be transported solely by the $ATB^{0,+}$ and LAT1(98, 99) transporters, respectively. Although multiple systems are involved in the transport of L-alanine, it permeates across the rabbit cornea primarily through the ASCT1(100) transporter. Procedures as described under corneal permeation studies were followed. The donor solutions contained 0.5 μCi/mL of the radioactive agents.

Choline chloride and K_2HPO_4 were used in equimolar quantities to replace NaCl and Na_2HPO_4, respectively, in the transport medium to study sodium dependency of the transport process. 2-Amino-2-norbornanecarboxylic acid (BCH) was used as a specific L-amino acid transporter inhibitor.

3.4.6. Enzymatic (esterase) Activity

A method described by Armstrong *et al.* was modified to determine the esterase activity in the ocular tissues obtained from fresh and preserved rabbit eyes (102). Eyes were carefully

dissected and the isolated tissues were stored at -80°C. Aqueous humor and vitreous humor were used after centrifugation at 13,000 rpm at 4°C for 5 minutes. Cornea, iris-cilliary body and retina-choroid were homogenized over an ice bath by using methods described elsewhere (103). The protein content of the tissue homogenates was standardized to 1 mg/mL using the method of Bradford (104). Total esterase activity was determined spectrophotometrically using p-nitrophenyl acetate (3 mM) as a substrate. In 3 mL acetone, 54.3 mg p-nitrophenylacetate was dissolved and the volume was made up to 100 mL using 10 mM phosphate buffer (pH 7.2) to obtain a final concentration of 1mM p-nitrophenylacetate. To 1 mL of this solution, 1.9 mL of buffer and 100 µL of the tissue homogenate were added in a quartz cell. The contents were mixed by briefly inverting the cell and the kinetics of hydrolysis was evaluated at 348 mµ, using a Thermo Scientific *GENESYS 6*™ UV-Vis spectrophotometer, for 5 mins. For these studies, p-nitrophenyl acetate in the buffer solution, in the absence of the enzyme, was used as a blank.

3.4.7. HPLC Analytical Method

Samples were analyzed using an HPLC system consisting of Waters 717 plus autosampler, Waters 2475 Multi λ Fluorescence detector (ex λ 270, em λ 380) and Agilent 2295 integrator. Waters C18 Symmetry column, 4.6 × 250 mm, was used. Mobile phase consisted of 15 mM phosphate buffer (pH 2.5) containing 1% acetonitrile. Retention times were 10.5 ± 0.2 min for ACV, 7.4 ± 0.1min for L-Asp-ACV and 7.5 ± 0.1 min for D-Asp-ACV.

3.4.8. Data Analysis

Flux and permeability calculations were performed as previously reported (101, 105).Briefly, steady state flux values were calculated from the plot of cumulative amount of drug in the receiver phase (C_{cum}) with respect to time (Eq. 1). Steady flux values were normalized to donor concentration (C_d) to calculate drug permeability (Eq. 2).

$$\text{Flux (J)} = dC_{Cum}/dt \quad (1)$$

$$\text{Permeability } (P_{app}) = \text{Flux} / C_d \quad (2)$$

All experiments were carried out at least in triplicate. Student’s t test for unpaired sample was used for statistical analysis. Data obtained for multiple groups was subjected to statistical analysis using One Way Analysis of Variance (ANOVA). Variation between the groups was checked using Levenes’ test and significant difference between group means was determined using Tukeys HSD test. Results were considered statistically significant if p-value was < 0.05.

3.5. RESULTS

3.4.3. Light Microscopy

Hematoxylin-eosin stained cross sections for corneas extracted from ocular globes stored in PBS/HBSS did not show any significant difference from the fresh corneas (Fig. 3-1).

3.4.4. Trans-epithelial Electrical Resistance

TEER values represented in Fig. 3-2 for corneas from eyes preserved in PBS (4.7 ± 0.3 $K\Omega.cm^2$) and HBSS (4.8 ± 0.25 $K\Omega.cm^2$) were slightly lower but not significantly different from corneas from freshly isolated eyes (5.2 ± 0.3 $K\Omega.cm^2$). The TEER values remained constant throughout the three hour duration of the experiment.

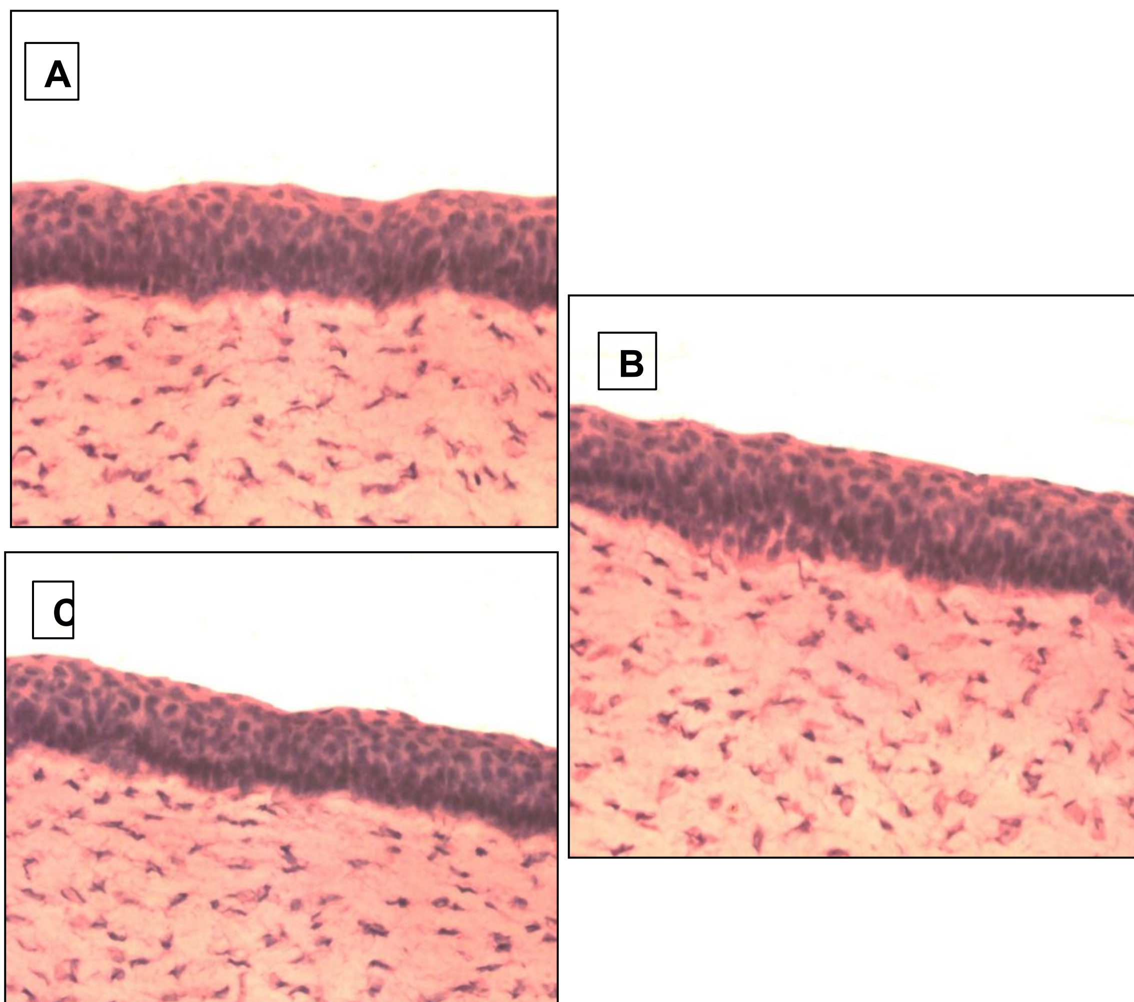

Figure 3-1: Hematoxylin-eosin stained cross section of rabbit cornea. (A) Freshly excised rabbit cornea. (B) Cornea extracted from eyes preserved in PBS over wet ice for 24h. (C)Cornea extracted from eyes preserved in HBSS over wet ice for 24 hours.

3.4.5. Paracellular and Transcellular transport:

Permeability of the paracellular marker [^{14}C]mannitol was found to be approximately three times higher across corneas preserved in PBS or HBSS compared to freshly excised corneas (3.96 ± 1.44 x 10^{-6} cm/sec). Ca^{+2} plays an important role in maintaining the integrity of tight junctions (106). Since Ca^{+2} is present in HBSS it was thought that their presence would help maintain the integrity of the tight junctions. However, significant difference was not

observed (Fig. 3-3) in the permeability of [^{14}C]mannitol between eyes preserved in PBS (14.88 ± 1.69 * 10^{-6} cm/sec) and HBSS (13.6 ± 0.26 x 10^{-6} cm/sec).

Corneal diffusion of acyclovir (Fig. 3-4), however, was not affected by the storage medium. Corneal permeability of acyclovir across corneas from fresh eyes (3.25 ± 0.11 x 10^{-6} cm/sec) was not significantly different from those stored in PBS (4.01 ± 0.38 x 10^{-6} cm/sec).

Permeability of the transcellular marker, [^{3}H]diazepam, in fresh corneas (2.19 ± 0.4 x 10^{-5} cm/sec) was not significantly different from corneas extracted from eyes preserved in PBS (2.35 ± 0.59 x 10^{-5} cm/sec) or HBSS (2.59 ± 0.34 x 10^{-5} cm/sec)(Fig. 3-3). Also permeability of quinidine (2.0 ± 0.1 x 10^{-5} cm/sec) was not affected when eyes were preserved in PBS (2.5 ± 0.4 x 10^{-5} cm/sec) as depicted in Fig. 3-4.

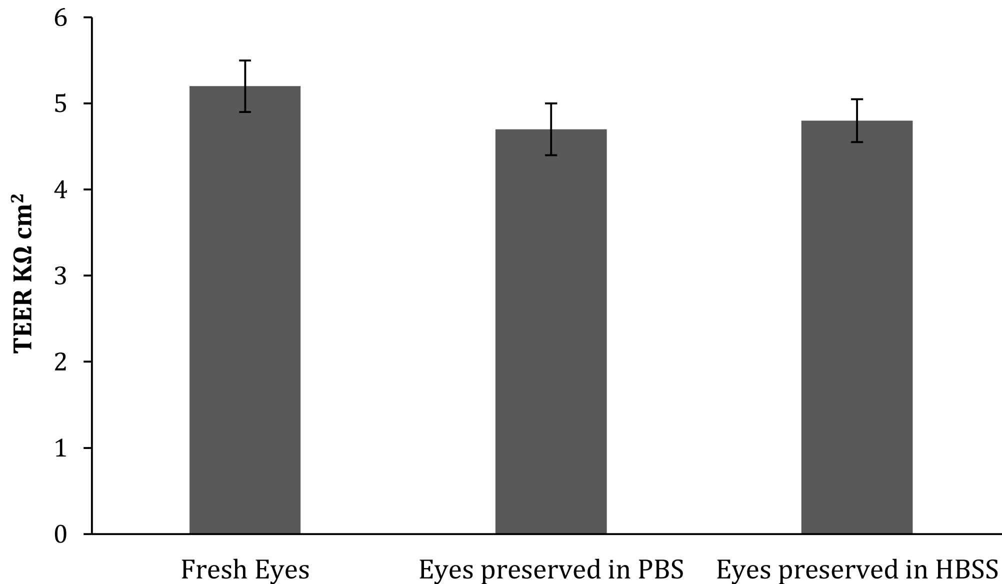

Figure 3-2: Trans-epithelial electrical resistance (TEER) values across freshly excised corneas and corneas obtained from eyes preserved in PBS/HBSS over wet ice for 24h. Results are depicted as mean ± S.D (n=4).

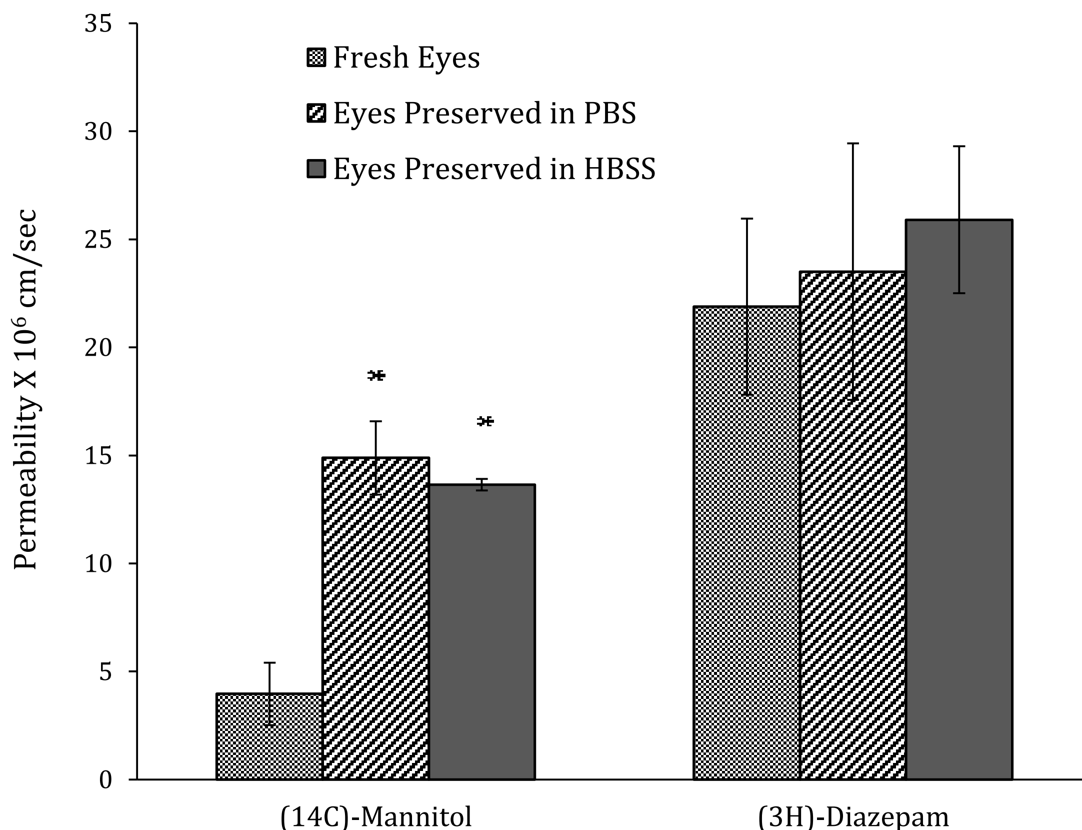

Figure 3-3: Transcorneal permeability of [14C]Mannitol (0.5 μCi/ml) and [3H]Diazepam (0.5 μCi/ml), across corneas from fresh or preserved (in PBS or HBSS over wet ice for 24h) rabbit eyes. Results are depicted as mean ± SD (n=3). * $p<0.05$

3.4.6. Amino Acid Transporter Activity

Eyes preserved in PBS and HBSS exhibited functional activity of the amino acid transporters. In the case of eyes preserved in PBS, transport of $[^{14}C]$L-Arginine (Fig. 3-5) across fresh eyes ($6.46 \pm 2 \times 10^{-6}$ cm/sec) was not significantly different from those obtained from eyes preserved in PBS ($6.59 \pm 2.2 \times 10^{-6}$ cm/sec) or HBSS ($7.06 \pm 1.68 \times 10^{-6}$ cm/sec). $[^{14}C]$L-Arginine transport was inhibited significantly by the specific L-amino acid transporter inhibitor, BCH, in eyes preserved in both PBS and HBSS (Fig.3-6A & B).

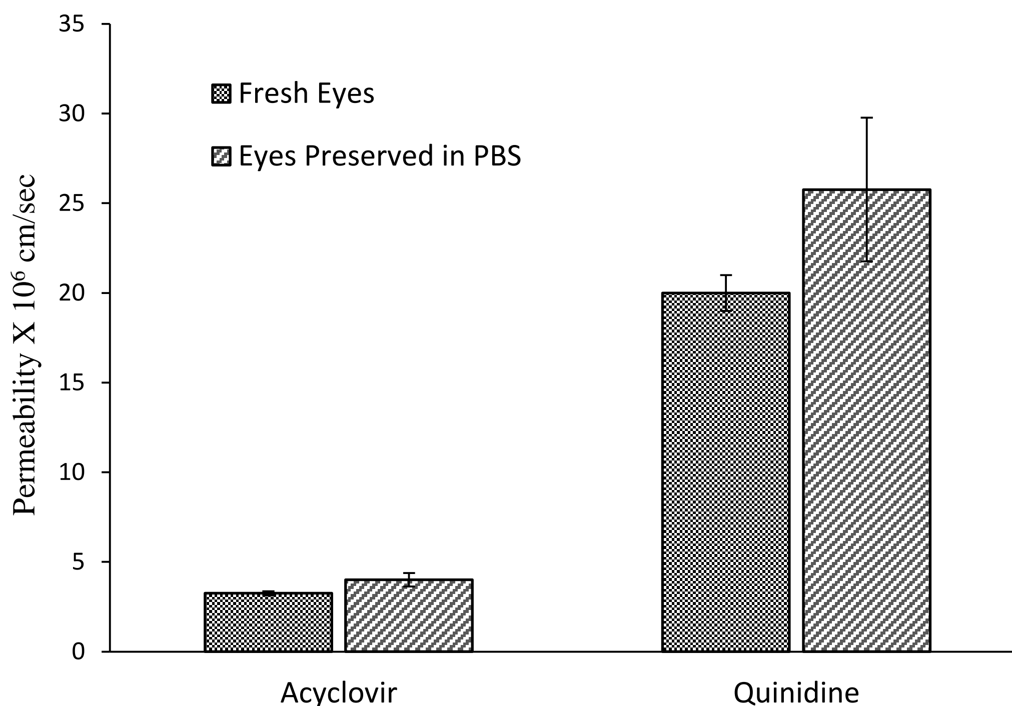

Figure 3-4: Transcorneal permeability of acyclovir and quinidine across corneas from eyes preserved in phosphate buffered saline (PBS) over wet ice for 24h compared with freshly excised rabbit corneas. The studies were conducted at 34°C. Results are depicted a mean ± SD (n=3). * $p<0.05$

Corneal permeability of [^{14}C]phenylalanine (9.89 ± 0.49 x 10^{-6} cm/sec, freshly excised corneas) was found to be significantly lower when the eyes were preserved in PBS (6.9 ± 0.54 x 10^{-6} cm/sec). However, permeability values across the corneas from eyes that were preserved in HBSS (9.45 ± 0.67 x 10^{-6} cm/sec) (Fig. 3-5) were not significantly different from the fresh corneas. In all cases transport of [^{14}C]phenylalanine was significantly inhibited in the presence of the inhibitor, BCH (Fig. 3-6A & B).

Active transport of [^{14}C]L-Alanine across corneas that were freshly excised (1.87 ± 0.35 x 10^{-5} cm/sec) was found to be similar to those from eyes preserved in PBS (1.57 ± 0.90 x 10^{-5}

cm/sec) or HBSS ($1.99 \pm 0.15 \times 10^{-5}$ cm/sec)(Fig. 3-5). When the studies were carried out in sodium free medium permeability was significantly reduced (Fig. 3-6A & B) in all cases.

The observed corneal permeability values (from fresh eyes) of the amino acids are about 2 to 2.5 fold lower than literature reports (98-100). This difference may be attributed to use of a different radiolabel (^{14}C instead of ^{3}H) being used in this study.

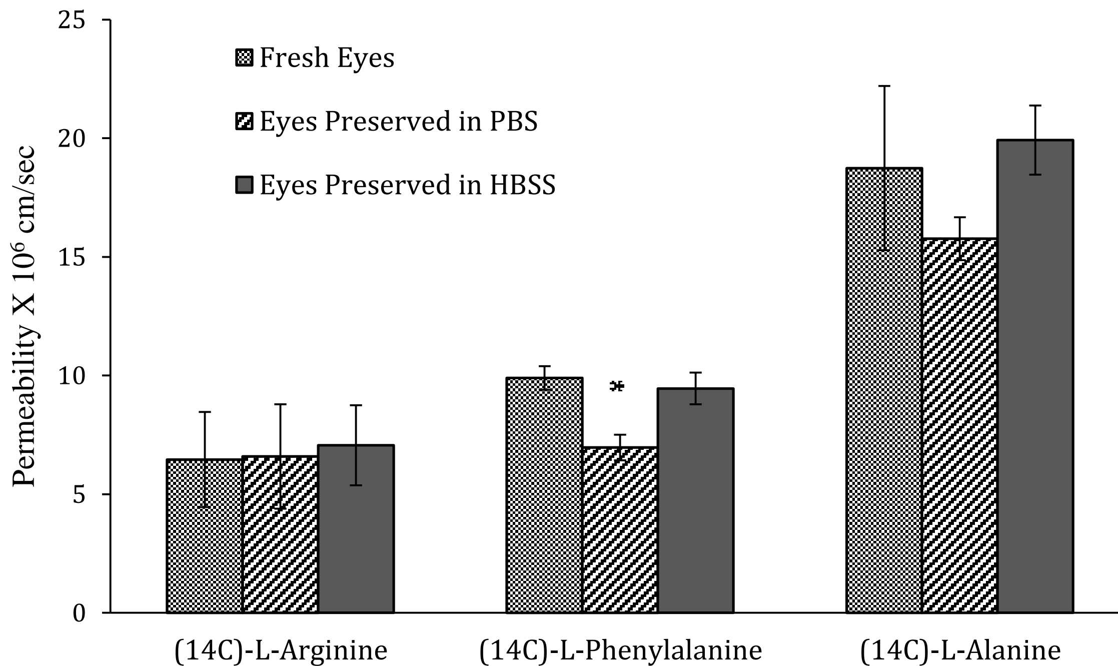

Figure 3-5: Transcorneal permeability of [14C]L-Arginine (0.5 μCi/ml), [3H]L-Phenylalanine (0.5 μCi/ml) and [14C]L-Alanine (0.5 μCi/ml) across corneas from fresh or preserved (in PBS or HBSS over wet ice for 24h) rabbit eyes. Results are depicted as mean ± SD (n=3). * $p<0.05$

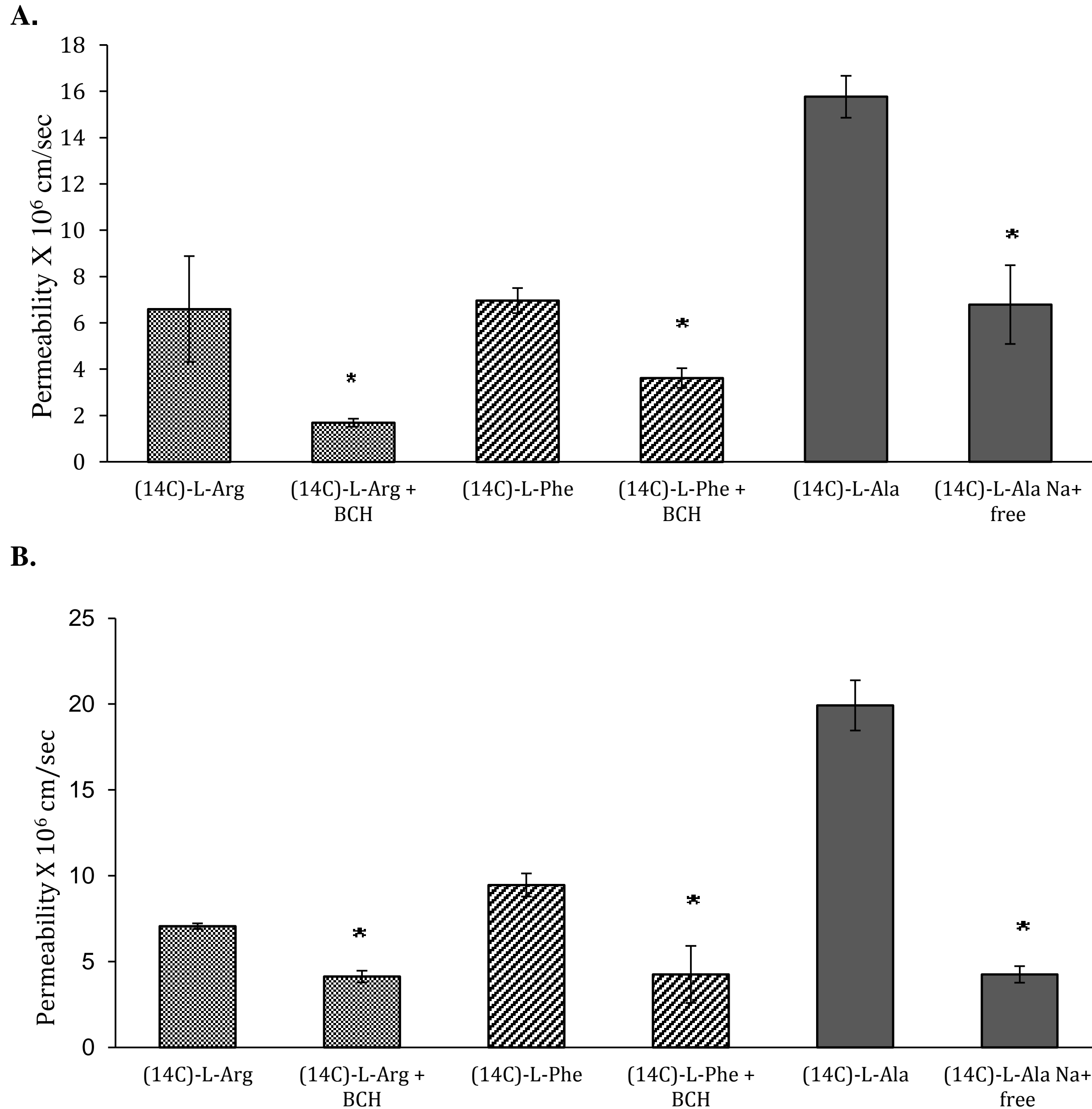

Figure 3-6: Permeability of [14C]L-Arginine (0.5 μCi/ml), [14C]L-Arginine (0.5 μCi/ml) in the presence of BCH (5mM), [14C]L-Phenylalanine(0.5 μCi/ml), [14C]L-Phenylalanine(0.5 μCi/ml) in the presence of BCH (5mM), [14C]L-Alanine (0.5 μCi/ml) and [14C]L-Alanine (0.5 μCi/ml) in Na^+ free medium, across corneas from eyes stored for 24h in A) phosphate buffered saline (PBS) and B) hanks balanced salt solution (HBSS) over wet ice, at 34°C. Results are depicted as mean ± SD (n=3). * $p<0.05$

3.4.7. Enzymatic Activity

Non-specific esterase activity was assessed using p-nitrophenyl acetate assay protocols. Esterase activity in preserved corneas was observed to be about 1.5-fold lower compared to the freshly excised corneas. There was, however, no significant difference in the esterase activity of corneas obtained from eyes preserved in PBS or HBSS (Table 3-1). Iris-ciliary tissues from eyes stored in PBS exhibited an almost 2-fold decrease in the esterase activity. However, esterase activity was preserved at normal levels in the iris-ciliary tissues when the eyes were stored in HBSS. The other ocular tissues tested did not demonstrate any significant difference between fresh or preserved eyes.

Table 3-1: Esterase activity in fresh eyes compared to eyes preserved in PBS/HBSS.

	Rate of hydrolysis of p-nitrophenol acetate [μM/(min.mg protein)]		
	Fresh Eyes	Eyes Preserved in PBS	Eyes Preserved in HBSS
Cornea	2.78 ± 0.19	*1.79 ± 0.11	*1.79 ± 0.11
Aqueous Humor	2.59 ± 0.19	3.09 ± 0.39	2.78 ± 0.32
Iris-Ciliary Body	14.01 ± 0.11	*8.46 ± 0.11	13.77 ± 0.11
Vitreous Humor	4.26 ± 0.19	5.06 ± 0.11	4.63 ± 0.19
Retina-Choroid	3.33 ± 0.19	4.51 ± 0.11	4.51 ± 0.11

3.6. DISCUSSION

Drug molecules can permeate across the cornea by passive or active transport mechanisms. Passive diffusion across the cornea involves the paracellular or transcellular pathway. In transcellular diffusion the drug molecules partition into the lipoidal cell membrane, but permeability depends on the molecular size, charge and lipophilicity (2). Paracellular diffusion, wherein the drug molecules move through the intercellular spaces, is limited by the tight junctions present on the superficial epithelial cells (15). Several carrier systems, including the amino acid transporters ASCT1, LAT1 and $B^{o,+}$, have been reported on the corneal epithelium of rabbits (98, 100, 107). These transporters are responsible for nutrient transport, but are being frequently targeted for drug delivery across biological barriers (107, 108). Thus, when transcorneal permeability of a therapeutic candidate is being evaluated *in vitro*, it is vital that these passive and active transport mechanisms remain unaltered in the model.

The objective of this study was to examine the utility of corneas isolated from preserved rabbit eyes for transcorneal permeability studies and to identify a media which would be economical, simple to prepare and would maintain the barrier and transport mechanisms of the cornea intact. A lot of work has been carried out in the field of preserving human corneas for transplantation. The cornea is either stored in organ culture media at physiological temperatures (31-37°C) or stored in serum free media at hypothermic temperatures (4°C)(109). Organ culture media are expensive and calls for complex preparation steps. Moreover, the corneas have to be de-swelled in dextran before use. This would be a laborious process for preserving corneas for *in vitro* transport experiments. Hypothermic storage media are commercially available but are extremely expensive.

Spencer *et. al.* compared storage of isolated corneas in balanced salt solution(BSS) to McCarey Kaufman (MK) media for a period of four days in the presence or absence of the steroid hydrocortisone (110). They reported that there was no significant difference if the eyes were stored in BSS or M-K media at 4°C. Also there was no significant difference on storage even in the presence of hydrocortisone. That study, however, was limited to the examination of autolysis using the marker enzyme, acid phosphatase.

In view of the earlier reports, HBSS was chosen as one of the storage media. Since the eyes were to be stored in this study for a relatively short duration (not more than 24 hours), PBS, the most common storage medium was also selected. Both HBSS and PBS buffers are easy to prepare, very economical and use chemicals readily available in most laboratories.

Currently, two methods are used to transport corneal tissues. Either the whole eyeball is transported in a moist chamber, or, the cornea is isolated *in situ* and transported in a solution. *In situ* isolation has been reported to be better at preserving the cornea compared to transporting the whole eye globes (111). The primary focus in preserving human corneas is to maintain endothelial cell viability and to prevent autolytic changes after death which may occur due to contact with stagnant aqueous humor. Thus, the cornea needs to be separated from the rest of the globe. However, for the purpose of drug permeation experiments the endothelium is a very weak barrier. Rather, an intact epithelium is essential for *in vitro* experiments.

TEER is an electrophysiological technique to measure the integrity of the paracellular pathway (112). Although the TEER values of corneas excised from preserved eyes were observed to be higher compared to fresh corneas, the difference was not statistically significant, indicating no change in the epithelial barrier properties. However, TEER values alone cannot be used as a measure for assessing structural integrity. Permeability of [^{14}C]mannitol, a paracellular

marker, was found to be three times higher in corneas from preserved eyes compared to freshly excised corneas. However, there was no significant difference in permeability of acyclovir, another hydrophilic molecule also known to permeate through the paracellular route (113). The slightly lower TEER values and higher mannitol permeability of corneas from preserved eyes, compared to fresh corneas, indicates that the tight junctions are disrupted to an extent that small linear molecules like mannitol can pass through in greater quantities. A more branched molecular structure like acyclovir, however, shows no difference in corneal permeability in all models.

A significant alteration of the tight junction integrity would also have an impact on the permeability of lipophilic molecules which diffuse transcellularly. These lipophilic agents can diffuse through the paracellular route to a greater extent than in the presence of intact tight junctions. Lack of any changes in the transcorneal permeation values of the lipophilic, transcellular markers, diazepam and quinidine, indicates that the integrity of the junction proteins is not significantly disrupted, and permeability of only very small molecules, such as mannitol, is affected. Moreover, the observed transcellular permeability values for these lipophilic agents were statistically similar in the corneal tissues obtained from both fresh and preserved rabbit eyes.

Transporter targeted prodrugs have been designed to enhance transcorneal permeation of the parent moiety using a piggy-banking approach, where the prodrug is translocated by the carrier system. *In-vitro* transport experiments are thus often carried out to determine specific transporter involvement in the translocation process of a new therapeutic agent or to optimize a transporter targeted prodrug design approach. Preservation of the active transport mechanisms is thus essential for accurate permeation predictions or for the purpose of ranking various candidates. In this study we evaluated the permeability characteristics of three amino acid

transporters reported on the rabbit corneal epithelium. Results indicate that the amino acid transporters, LAT1, ASCT1 and $B^{0,+}$, remain functionally active in the corneas extracted from the eyes preserved in PBS or HBSS as the presence of BCH or lack of sodium significantly decrease the transport of their substrates. However, for corneas extracted from whole globes preserved in PBS, the permeability of L-Phenylalanine was significantly lower compared to that across corneas from eyes that were freshly excised or were preserved in HBSS. Thus, corneas from rabbit eyes stored in HBSS exhibited functional activity of all three amino acid transporters at levels similar to that in freshly excised corneas. Both PBS and HBSS are isotonic but HBSS has added glucose and metal ions. Thus, even at low temperatures where metabolic activity is minimal some form of nutrition needs to be added to the medium for amino acid transporters to remain fully functional.

Esterase activity of corneas from eyes preserved in PBS and HBSS was observed to be lower compared to freshly excised cornea. The esterase activity in the other tissues, from eyes stored in HBSS, was not significantly different. The lower esterase activity in the cornea, compared to other tissues, can probably be explained by the fact that the cornea is exposed to the storage conditions (solution and period) to a greater extent in comparison to the other tissues which remain relatively protected inside the ocular globe, deriving nutrition from the vitreous humor. The reduced enzyme activity may impact bioreversion and thus permeability rates. However, considering the extremely rapid enzymatic degradation rates observed with most ester prodrugs and the low drug concentrations used in general, the impact of this 1.5-fold reduction in enzymatic hydrolysis rate may not be very significant with respect to relative permeability evaluation.

In conclusion, the results from this study indicates that storage of the extracted whole rabbit eye in HBSS, over wet ice, for a period of up to 24 hours, preserves the active and passive transport mechanisms in the cornea. Eyes shipped from abattoirs in this manner, within this timeframe, would serve as an excellent *in vitro* model for transcorneal permeation studies and would significantly decrease the need for laboratory animal sacrifice. Further studies evaluating means to extend the period of storage are currently underway.

3.7. ACKNOWLEDGEMENTS

This project was supported by NIH Grant Number EY018426-02 from the National Eye Institute and P20RR021929 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Eye Institute or the National Center for Research Resources.

CHAPTER 4

EFFECT OF ION-PAIRING ON *IN VITRO* TRANSCORNEAL PERMEABILITY OF A Δ^9- TETRAHYDROCANNABINOL PRODRUG: POTENTIAL IN GLAUCOMA THERAPY.

4.1. ABSTRACT

The aim of the present study was to evaluate and improve the *in vitro* transcorneal permeability characteristics of Δ^9-tetrahydrocannabinol (THC) through prodrug derivatization and formulation approaches. *In vitro* corneal permeability of THC and its hemisuccinate (THC-HS) and hemiglutarate (THC-HG) ester prodrugs and WIN 55-212-2 (WIN), a synthetic cannabinoid, was determined using isolated rabbit cornea. The formulations studied included hydroxypropyl beta cyclodextrin (HPβCD) or random methylated beta cyclodextrin (RMβCD), as well as prodrug/ion-pair complexes with l-arginine or tromethamine. Corneal permeability of WIN was found to be two-fold higher than THC in the presence of HPβCD. THC-HS and THC-HG exhibited pH dependent permeability. In the presence of HPβCD, at pH 5 (donor solution pH), both prodrugs exhibited six-fold higher permeability compared to THC. However, permeability of the prodrugs was about three-fold lower than that of THC at pH 7.4. RMβCD, at pH 7.4, led to a significant improvement in permeability. Formation of ion-pair complexes markedly improved the solubility and permeability of THC-HG (7-fold and 3-fold greater permeability compared to THC and WIN, respectively) at pH 7.4. The *in vitro* results demonstrate that the use of an ion-pair complex of THC-HG could be an effective strategy for topical delivery of THC.

4.2. INTRODUCTION

In 1971 Hepler and Frank published a report that linked marijuana smoking to a significant drop in intraocular pressure (IOP) (114). Due to its implications in the treatment of glaucoma, this report stimulated intense research towards identification of the constituents responsible for this pharmacological action. It was established that Δ^9-tetrahydrocannabinol (THC, Fig. 3-1A), a primary active constituent of marijuana, is one of the components responsible for the IOP lowering effects (29). During the course of further investigations, a reduction in IOP was observed when THC was administered either orally or intravenously but not when applied topically (31, 115). This lack of topical activity, although some reports did demonstrate that topical delivery of THC significantly lowered IOP (116), led researchers to conclude that the IOP lowering mechanism of THC was probably due to its centrally acting hypotensive effect and not due to activation of local ocular receptors.

However, recent studies suggest that THC can lower IOP and act as a neuroprotective agent by binding to the cannabinoid receptors expressed in the ocular tissues. In the 1990`s two cannabinoid receptors, CB1 and CB2, were identified and cloned (117). THC acts as an agonist for both CB1 and CB2 receptors. Affinity values for the CB1 and CB2 receptors are 5.05 and 3.13 nM respectively (118) while the EC_{50} values for the CB1 and CB2 receptors are 6 nM and 0.4 nM, respectively (119). Although the distribution of cannabinoid receptors in the body, since their identification, has been largely delineated, only recently have cannabinoid receptors been identified in the ocular tissues. CB1 receptors are expressed in the trabecular meshwork, iris, ciliary body and the retina (39-41, 46) while CB2 receptors have been found on the retina and trabecular meshwork (47). These locally expressed cannabinoid receptors are now believed to be

involved in the IOP lowering and neuroprotective activity of a number of endocannabinoid and synthetic cannabinoid derivatives.

There are two major pathways for the drainage of aqueous humor from the anterior ocular segment: drainage through the Schlemm's canal or the uveoscleral route. Activation of the CB1 receptors in the ciliary muscle, by CB1 receptor agonists, induces contraction of the ciliary muscle (120). Contraction of the ciliary muscle leads to widening of the intercellular spaces in the trabecular meshwork and enhances outflow of aqueous humor (8). Recently, bimatoprost, a prostaglandin analog that enhances uveoscleral outflow, has been shown to contract the human ciliary muscle through CB1 mediated mechanism (121). Furthermore, activation of CB1 receptors leads to fragmentation and reduction of actin stress fibers in the trabecular meshwork, further enhancing outflow of aqueous humor (46). That THC can reduce IOP through the local CB1/CB2 receptors can also be inferred from a previous clinical study. Merritt *et al.* demonstrated that 0.1% THC in mineral oil when given topically led to a 5.4 mm drop in IOP but was accompanied with a 12 mm drop in systolic blood pressure (116). A 10 mm drop in systolic blood pressure, following systemic THC administration, should be associated with less than a 1 mm drop in IOP (122). Also, 0.05% THC in mineral oil, topically administered, led to a 4.8 mm drop in IOP with no systemic hypotensive effect. Thus, these data suggest that topical THC is probably acting locally through the ocular cannabinoid receptors to reduce the IOP, and not through the systemic pathway.

In glaucoma, a reduction in IOP is often not enough to prevent or arrest the development or progression of glaucoma related optic neuropathy (21, 22). Vision loss continues even after significant IOP reduction has been achieved. It has been suggested that neuroglial cell cytotoxicity in the optic nerve and retina leads to visual field loss in glaucoma (23). Neuroglial

cell toxicity impairs macroglial glutamate metabolism and causes microglia to release inflammatory cytokines following ischemia due to compression or vascular occlusion. The released glutamate acts on receptors, including the NMDA subtype, on the retinal ganglion cells to induce calcium influx and the release of toxic reactive oxygen species, leading to apoptosis (23). Recent studies have demonstrated that CB2 or non-specific CB1/CB2 agonists were able to protect retinal cells from oxidative stress, but specific CB1 agonists had no effect (123). Intravitreally administered THC has been demonstrated to act as a neuroprotective in a rat model of glaucoma (23, 24). Hampson *et al*. also reported that the neuroprotective activity of THC could also be independent of CB1 receptor activation (124). Although the mechanism of neuroprotection of THC is not yet clearly understood, it could be due to the activation of CB2 receptors, its antioxidant effect or some other mechanism (124-126). The current evidence clearly suggests that THC possesses both IOP lowering and neuroprotectant activity, which are independent of each other.

While significant efforts have been directed towards understanding the pharmacology of THC, design of effective topical delivery strategies for THC has not seen much activity. In 1977 Green *et al.* published a paper comparing corneal penetration of THC from different oils and found that light mineral oil was the best of the four vehicles tested (50). The study reported a twenty percent drop in IOP of normotensive rabbits when a 50 µL dose (0.1% w/w THC) was administered topically. However plasma drug concentrations were not reported. All further pharmacological studies, with THC administered topically, were carried out using light mineral oil as the vehicle. However, THC being a highly lipophilic molecule, with an aqueous solubility of only 1-2 µg/mL and a logP of 6.3 (127), its effective partitioning from the oily vehicle into the tear film would be suspect. Kearse *et al.* compared *in vitro* corneal permeability of THC from

different vehicles and observed that the permeability of THC from light mineral oil was only 1.86 X 10^{-8} cm/s (51). Thus, the lack of topical activity observed in the earlier *in vivo* reports could be due to the ineffective delivery of THC to the target ocular tissues rather than absence of local pharmacological activity. When higher doses were administered, to increase the amount of THC permeating across the cornea, systemic side effects were observed. Thus, development of a formulation that can effectively deliver THC across the cornea is needed prior to its evaluation for therapeutic activity.

The aim of the current project was to improve the aqueous solubility and *in vitro* permeability of THC employing complex formation and prodrug derivatization strategies. Dicarboxylic acid esters are commonly used promoieties in prodrug derivatization approaches. These ester prodrugs exhibit higher aqueous solubility since they are ionized at physiological pH values. The hemisuccinate ester (THC-HS, Fig. 3-1B) and hemiglutarate ester (THC-HG, Fig. 3-1C) prodrugs were synthesized and evaluated for transcorneal permeability. The effect of cyclodextrins and counterion adduct/complex formation on the solubility and corneal permeability of THC and/or the two prodrugs were studied.

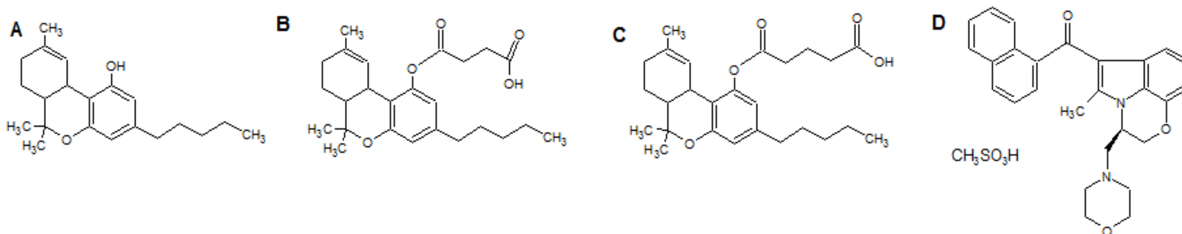

Table 4-1: Chemical structures of A) Δ9-Tetrahydrocannabinol (THC), B) Δ9-Tetrahydrocannbinol Hemisuccinate (THC-HS) and C) Δ9-Tetrahydrocannbinol Hemiglutarate (THC-HG) D) WIN 55-212-2 (WIN).

4.3. EXPERIMENTAL SECTION

4.3.1 Chemicals

Hydroxypropyl beta cyclodextrin (HPβCD), randomly methylated beta cyclodextrin (RMβCD), 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic-acid (BCH), l-arginine and Sigmacote® were obtained from Sigma (St. Louis, MO). WIN-55-212-2 (WIN) was purchased from Tocris Bioscience (Ellisville, MO). All other chemicals were obtained from Fisher Scientific (St. Louis, MO). All solvents used for analysis were of HPLC grade.

4.3.2. Animal Tissues

Whole eye globes of albino New Zealand White rabbits were obtained from Pel Freez Biologicals (Rogers, AR). Eyes were shipped overnight in Hanks balanced salt solution over wet ice and used immediately on receipt.

4.4. METHODS

4.4.1. Preparation of Dicarboxylic acid ester prodrugs

Dicarboxylic acid prodrugs (THC-HS and THC-HG) were synthesized and characterized according to previously published procedures (128).

4.4.2. Solubility of the Prodrugs

4.4.2.1. Solubility in Buffers: Since THC binds to plastic, all experiments were carried out in plastic vials/tubes coated with Sigmacote® or borosilicate glass vials were used (129). Specific measured quantities of stock solutions of THC-HS or THC-HG were transferred to the borosilicate glass or coated plastic vials and the organic solvent was evaporated using a stream of nitrogen gas. Dulbecco's Phosphate Buffered Saline (DPBS) or Isotonic Phosphate Buffered Saline (IPBS) was then added to the vials and the resulting mixture was sonicated for 10 min to dislodge the drug sticking to container walls and allowed to equilibrate for 24 hours at 25 °C in a shaking water bath at 75 shakes per minute. The resulting suspension was centrifuged at 16000 x g in a Fisher Scientific acuSpin micro17R centrifuge for 10 min using silicon coated centrifuge tubes. The supernatant was collected and analyzed by HPLC.

4.4.2.2. Solubility in Cyclodextrins: Solubility of THC-HS and THC-HG in a 2.5% solution of HPβCD or RMβCD in DPBS or IPBS was determined using methods described under solubility of THC-HS/THC-HG in buffer solutions.

4.4.2.3. Solubility in Presence of l-arginine/tromethamine: Formation of an ion-pair complex with a hydrophilic counter ion could lead to an improvement in the solubility of the drug. Aliquots of stock solutions of THC-HS and THC-HG were transferred to glass vials and the organic solvent was evaporated using a stream of nitrogen gas. l-arginine or tromethamine in IPBS was then added to reach specific concentrations of the prodrug and the counterion. The prodrug THC-HS/THC-HG and the counterion l-arginine/tromethamine were added in increasing concentrations, keeping the ratio of drug:counterion constant (1:2). The combinations were then

processed for solubility determination following the same methods as described under solubility of the prodrugs in buffer solutions.

4.4.3. *In Vitro* Transcorneal Permeability Studies

Excess THC, THC-HS, THC-HG and WIN were equilibrated in DPBS containing 2.5% HPβCD or RMβCD (pH adjusted to 5, 6 and 7.4) for 24 hours at 25 °C in a shaking water bath. The supernatant containing the drug-cyclodextrin complex was used in the transport studies. Donor solutions were analyzed for drug content at the beginning and after completion of the *in vitro* permeability studies. Receiver solution for all permeability studies with cyclodextrin formulations consisted of 2.5% HPβCD solution in DPBS with pH adjusted to 7.4.

Transcorneal permeability of the ion-pair complexes of THC-HG with l-arginine (THC-HG-ARG) and tromethamine (THC-HG-TRIS) was also studied. THC-HG (1 mM) was equilibrated with l-arginine (2 mM) or tromethamine (2 mM) in IPBS at 25 °C for 24 hours in a shaking water bath. The supernatants were collected, analyzed and used as the donor solution. For the studies investigating permeability of the ion-pair formulations, receiver medium consisted of 2.5% HPβCD solution in IPBS with pH adjusted to 7.4.

Eyes were used immediately upon receipt. Corneas were excised, following previously published protocols(101). Briefly an incision was made about 2 mm from the corneal-scleral junction and the cornea was excised by cutting radially along the sclera. The 2 mm scleral portions help in easy mounting of the cornea. The excised corneas were immediately mounted between standard, 9 mm, side-by-side diffusion cells (PermeGear Inc., Hellertown, PA). The half-cell facing the epithelial layer was termed as the donor compartment. A circulating water bath was used to maintain the temperature at 34 °C during the transport studies. The volume of the receiver solution (3.2 mL) was maintained slightly higher than that of the donor solution (3

mL drug solution) to maintain the natural curvature of the cornea. Both chambers were stirred continuously using magnetic stirrers. Aliquots, 600 µL, were withdrawn every thirty minutes for three hours and immediately replaced with an equal volume of the receiver solution. Samples were analyzed following the method described in the analytical methods section.

4.4.3.1 Involvement of Amino Acid Transporter

Amino acid transporters have been identified on the rabbit/human cornea and are functionally active. l-arginine is a substrate for amino acid transporter $B^{0,+}$, a sodium and energy dependent transporter and is specifically inhibited by BCH. The THC-HG/l-arginine adduct could be conveyed across the cornea by $B^{0,+}$. Permeability of THC-HG/l-arginine complex was thus determined in the presence of 5 mM BCH to evaluate involvement of $B^{0,+}$ in the transport process.

4.4.4. Stability in Ocular Tissue Homogenates

4.4.4.1. ***Tissue Preparation***: Aqueous and vitreous humor was used as such and without any dilution. They were centrifuged at 16000 x g for 10 min at 4 ºC and the supernatant was used. Other ocular tissues used in this study were homogenized in ice cold DPBS, on an ice bath, using TISSUEMISER (Fisher Scientific, St Louis, USA). The homogenate was then centrifuged at 16000 x g at 4 °C for 15 min. Protein content in the supernatant was determined according to the method of Bradford (101) and was standardized to 1 mg/mL.

4.4.4.2. ***Hydrolysis*** **Procedure**: The standardized homogenates were equilibrated for 30 min at 37 °C to activate the enzymes. To 1.98 mL of the supernatant, 20 µL of THC-HG (1 mg/mL) in ethanol was added and mixed. Samples were withdrawn at predetermined time intervals. Bio-conversion of THC-HG/l-arginine and THC-HG/tromethamine complex to THC in aqueous humor was also evaluated. To 0.95 mL of aqueous humor 50 µL of THC-HG-ARG or THC-HG-

TRIS complex (1:2) were added. Samples, 200 µL, were withdrawn at specific time intervals. An equal volume of ice cold methanol was added to the aliquoted samples, to arrest the reaction, and centrifuged at 16000 x g for 10 min. The supernatant was collected and taken for analysis.

4.4.5. Analytical Method

Samples were analyzed using a Waters high pressure liquid chromatography system consisting of Waters 600 pump controller, refrigerated Waters 717 plus autosampler, Waters 2487 UV detector and Agilent 3395 integrator. Primary stock solutions of THC, THC-HS and THC-HG were prepared in hexane and stored at -15 °C. For the preparation of standards a known amount of stock was taken and hexane was evaporated using nitrogen gas. Standards were reconstituted using mobile phase. Mobile phase consisted of a 85:15 mixture of methanol and 0.84 % v/v glacial acetic acid. A Phenomenex Luna PFP (2), 4.6 x 250 mm column was used. Analytes were detected at 226 nm. For quantification of THC, THC-HS and THC-HG a standard curve was constructed with a linear range of 0.1 to 5 µg/mL. Injection volume was 100 µL. Samples from the permeation studies were injected as such while the solubility study samples were diluted appropriately, in mobile phase, before being injected. The standard curve generated had co-efficient of determination values (r^2) greater than 0.9999. Retention times for the analytes were as follows: THC (10.1 min), THC-HS (13 min) and THC-HG (14.3 min). Limit of detection and quantifications were 5 ng/mL and 50 ng/mL, respectively.

4.4.6. Data Analysis

Flux was calculated from the plot of cumulative amount of drug (D_{cum}) in the receiver phase with respect to time (Eq. 1). Flux values were normalized to donor concentration (C_d) to calculate drug permeability (Eq. 2).

$$\text{Flux } (J) = dD_{Cum}/dt \qquad (1)$$

$$\text{Permeability (P)} = \text{Flux} / C_d \quad (2)$$

All experiments were carried out atleast in triplicate. Data obtained was subjected to statistical analysis using ANOVA. Variance between the groups was checked using Levenes' test. Statistical difference between groups was checked using Tukeys HSD. A p-value ≤ 0.05 was considered to signify statistically significant difference.

Table 4-2: Solubility of THC-HS and THC-HG in IPBS and DPBS as such or IPBS and DPBS containing 2.5% HPβCD or RMβCD at 25 ^{0}C. Results are depicted as mean ± SD (n=3).

Prodrug	Buffer	Cyclodextrin (2.5%)	Solubility (µg/mL)
THC-HS	IPBS	-	9.8 ± 0.9
		HPβCD	144.9 ± 23.4
		RMβCD	197.5 ± 57.9
	DPBS	-	5.4 ± 0.3
		HPβCD	418.9 ± 13.8
		RMβCD	430.2 ± 75.2
THC-HG	IPBS	-	18.8 ± 3.1
		HPβCD	899.9 ± 27.5
		RMβCD	857.7 ± 49.4
	DPBS	-	4.3 ± 0.2
		HPβCD	678.5 ± 84.2
		RMβCD	910.6 ± 62.8

4.5. RESULTS

4.5.1. Solubility

4.5.1.1. Solubility in Buffers: Solubility of the prodrugs, THC-HS and THC-HG, in IPBS or DPBS (Table 4-1) was found to be markedly higher than the reported solubility of THC (1-2 µg/mL) in water (127). THC-HS and THC-HG demonstrated significantly higher solubility in IPBS (9.8 ± 0.9 and 18.8 ± 3.1 µg/mL, respectively) compared to that in DPBS (5.4 ± 0.3 and 4.3 ± 0.2 µg/mL, respectively).

Table 4-3: Solubility of THC-HG in IPBS, and the resulting solution pH, as a function of increasing concentrations of THC-HG and l-arginine/tromethamine while maintaining the ratio of drug: counter-ion constant (1:2), at 25 ^{0}C. Results are depicted as mean ± SD

Counter ion	THC-HG (mM)	Counter Ion (mM)	pH	Concentration of THC-HG in solution (µg/mL)
l-Arginine	1	2	7.4	423.4 ± 52.2
	2	4	7.9	795.5 ± 51.1
	3	6	8.4	1154.9 ± 47.5
	4	8	8.9	1716.5 ± 49.1
Tromethamine	1	2	7.5	219.3 ± 16.9
	2	4	7.6	517.2 ± 46.5
	3	6	7.7	868.5 ± 80.5
	4	8	7.8	1158.4 ± 39.9

4.5.1.2. Solubility in Cyclodextrins: Solubility of the prodrugs (THC-HS and THC-HG) was significantly improved in the presence of cyclodextrins (Table 4-1). A significant difference between the solubility of THC-HS in the presence of HPβCD (144.9 ± 23.4 µg/mL) and RMβCD (197.5 ± 57.9 µg/mL) was not observed in IPBS. With both HPβCD (418.9 ± 13.8 µg/mL) and RMβCD (430.2 ± 75.2 µg/mL), THC-HS demonstrated a 2-fold higher solubility in DBPS

compared to that in IPBS. THC-HG solubility in the presence of the cyclodextrins was about 1.5-2 fold higher compared to THC-HS in DPBS containing HPβCD or RMβCD. Solubility of THC-HG in IPBS was independent of the cyclodextrin used. However in DPBS higher solubility in RMβCD was observed compared to HPβCD (Table 4-1).

4.5.1.3. Solubility in Presence of l-arginine/tromethamine: Since THC-HS and THC-HG demonstrated higher aqueous solubility in IPBS compared to DPBS (Table 4-1), ion- pairing studies with l-arginine and tromethamine were carried out in IPBS only. Preliminary studies demonstrated that THC-HS was unstable in the presence of l-arginine and tromethamine in IPBS. At the end of the 24 hour equilibration period, THC-HS was completely converted into THC (data not presented). Solubility of THC-HG in IPBS was found to increase linearly with increasing concentrations of l-arginine or tromethamine (Table 4-2). At the highest concentration studied, the solubility of THC-HG (4 mM) with l-arginine (8 mM) was found to be 1716.5 ± 49.5 μg/mL. Aqueous solubility of THC-HG (4 mM) with tromethamine (8 mM) was found to be 1158.4 ± 39.9 μg/mL. With a further increase in the concentrations of l-arginine or tromethamine the solution pH was observed to increase above pH 10 and were not investigated any further.

4.5.2. Corneal Permeation

Transcorneal permeability of THC (0.15 mM) across isolated rabbit cornea at pH 7.4 was found to be 5.57×10^{-6} cm/s (Fig. 3-2). DPBS containing 2.5% HPβCD was used to prepare the donor solution. Decrease in donor solution pH to 5 did not produce any significant effect on the corneal permeability of THC. When the concentration of HPβCD in the donor solution was increased from 2.5% to 30%, keeping the drug concentration constant, THC permeating into the receiver chamber was found to be below the detection limit (<0.05 μg/mL). WIN, a synthetic CB1/CB2 agonist, which has been demonstrated to reduce IOP when applied topically, exhibited 2-fold

Table 4-4: Donor concentrations and flux of THC, THC-HS and THC-HG (in terms of total THC) and WIN in different vehicles at 34 °C across isolated rabbit cornea. Results are depicted as mean ± SD (n=3).

Drug	Donor pH	Donor Vehicle	Donor Concentration (µg/mL)	Flux (µg/cm^2/min x 10^{-2})	Predicted Maximum Flux* (µg/cm^2/min x 10^{-2})
THC	5	2.5 % HPβCD in DPBS	40.7 ± 0.7	1.7 ± 0.2	-
	7.4	2.5 % HPβCD in DPBS	46.8 ± 2.9	1.6 ± 0.1	-
	7.4	30 % HPβCD in DPBS	40.1 ± 0.7	No drug detected	-
	7.4	2.5 % RMβCD in DPBS	19.9 ± 0.9	1.5 ± 0.2	-
WIN	5	2.5 % HPβCD in DPBS	73.3 ± 2.8	5.4 ± 0.3	-
	7.4	2.5 % HPβCD in DPBS	86.3 ± 12.4	5.7 ± 0.6	-
	7.4	30 % HPβCD in DPBS	97.2 ± 0.4	0.8 ± 0.1	-
	7.4	2.5 % RMβCD in DPBS	238.3 ± 17.5	13.2 ± 0.6	-
THC-HS	5	2.5 % HPβCD in DPBS	64.3 ± 4.3	14.9 ± 0.9	-
	6	2.5 % HPβCD in DPBS	29.8 ± 2.9	1.5 ± 0.1	-
	7.4	2.5 % HPβCD in DPBS	73.1 ± 0.7	0.9 ± 0.5	5.1 ± 3.7
	7.4	2.5 % RMβCD in DPBS	103.5 ± 12.9	3.5 ± 0.1	14.7 ± 0.5
THC-HG	5	2.5 % HPβCD in DPBS	53.8 ± 3.1	11.4 ± 1.1	-
	6	2.5 % HPβCD in DPBS	30.1 ± 1.5	1.7 ± 0.2	-
	7.4	2.5 % HPβCD in DPBS	30.9 ± 1.9	0.3 ± 0.1	6.5 ± 2.1
	7.4	2.5 % RMβCD in DPBS	30.1 ± 0.4	1.9 ± 0.2	56.3 ± 6.1
	7.6	2 mM l-arginine in IPBS	78.9 ± 8.1	14.9 ± 0.9	79.9 ± 11.9
	7.6	2 mM l-arginine + BCH in IPBS	71.6 ± 6.5	13.4 + 0.9	-
	7.6	2 mM Tromethamine in IPBS	71.9 ± 2.2	17.1 ± 2.6	51.9 ± 9.1

*Maximum flux values were calculated theoretically by multiplying observed permeability with maximum solubility values depicted in Table 4-1 and Table 4-2. Maximum values have not been presented for formulations wherein maximum solubility was not calculated.

higher corneal permeability compared to THC. Permeability of WIN across the cornea, like THC, was also found to be pH independent. When the concentration of HPβCD in the donor solution was increased from 2.5% to 30%, keeping the concentration of WIN constant, a 10-fold decrease in corneal permeability of WIN was observed. Replacing HPβCD with RMβCD (2.5%) in the donor solution led to a 2-fold improvement in the *in vitro* corneal permeability of THC but did not affect the permeability of WIN. Expectedly, THC-HS and THC-HG demonstrated pH dependent permeability across the cornea. At pH 5 (donor solution pH) permeability of THC-HS and THC-HG was about 6-fold higher compared to the permeability of THC in 2.5% HPβCD in DPBS (Fig. 3-3). An increase in the donor solution pH significantly decreased the permeability of THC-HS and THC-HG. Compared to the permeability of THC in 2.5% HPβCD (pH 7.4), permeability of THC-HS and THC-HG was about 2-fold higher at pH 6 and about three fold lower at pH 7.4 (Fig. 3-3). This is probably because the prodrugs are ionized at physiological pH values (pK_a of THC-HG is 3.6 ± 0.4). For all permeability studies involving cyclodextrin formulations, DPBS containing 2.5% HPβCD was used as the receiver solution.

To shield the negative charge on the prodrugs, permeability of THC-HS and THC-HG was evaluated in the presence of l-arginine and tromethamine since they are positively charged at physiological pH values. At physiological pH (pH 7.4) THC-HG-ARG and THC-HG-TRIS were found to be almost 7-fold more permeable compared to the permeability of THC in 2.5 % HPβCD. This was about 3-fold higher compared to the corneal permeability of WIN at pH 7.4. IPBS containing 2.5% HPβCD was used as the receiver solution in these studies for determining the permeability of the ion-pairs. Donor concentrations (in terms of THC) and flux values for all the formulations have been reported in Table 4-3.

Analysis of the donor solution samples collected at the start and end of the permeation studies indicated that THC-HS and THC-HG, in all formulations studied, remained intact for the duration of the *in vitro* permeability studies.

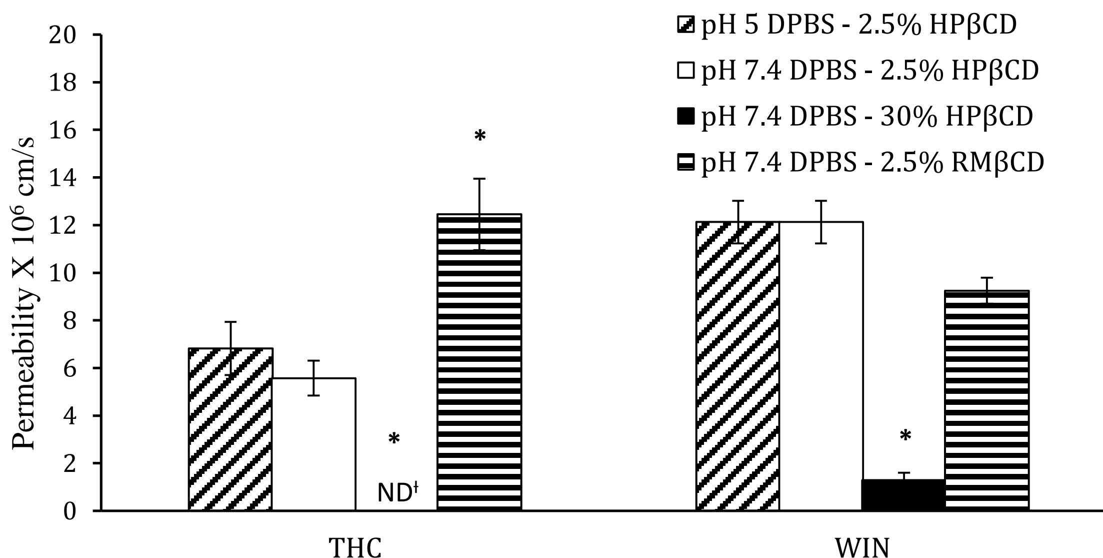

Figure 4-1: Permeability of THC and WIN at 34 °C across isolated rabbit cornea. The legends indicate the donor solution pH and composition. Receiver solution used in these studies was DPBS containing 2.5% HPβCD (pH 7.4). Results are depicted as a mean ± SD (n=3). *$p < 0.05$. †ND – THC concentrations could not be detected in the presence of 30% HPβCD.

4.5.2.1. Involvement of amino acid transporter

BCH is an L-amino acid transporter inhibitor. There was no significant difference in the permeability of the THC-HG-ARG complex in the presence of 5 mM BCH.

4.5.3. Stability in corneal homogenates

Apparent first order enzyme mediated degradation rate constants (after adjusting for buffer mediated hydrolysis) and half-life of THC-HG in aqueous humor, vitreous humor and other ocular tissue homogenates (1 mg/mL) have been depicted in Table 4-4. THC-HG was rapidly converted to THC in the aqueous humor ($t_{1/2}$, 25 ± 2.1 min) and the retina choroid ($t_{1/2}$,

36.7 ± 1.2 min). Bio-conversion of THC-HG in aqueous humor was not affected by the presence of l-arginine or tromethamine.

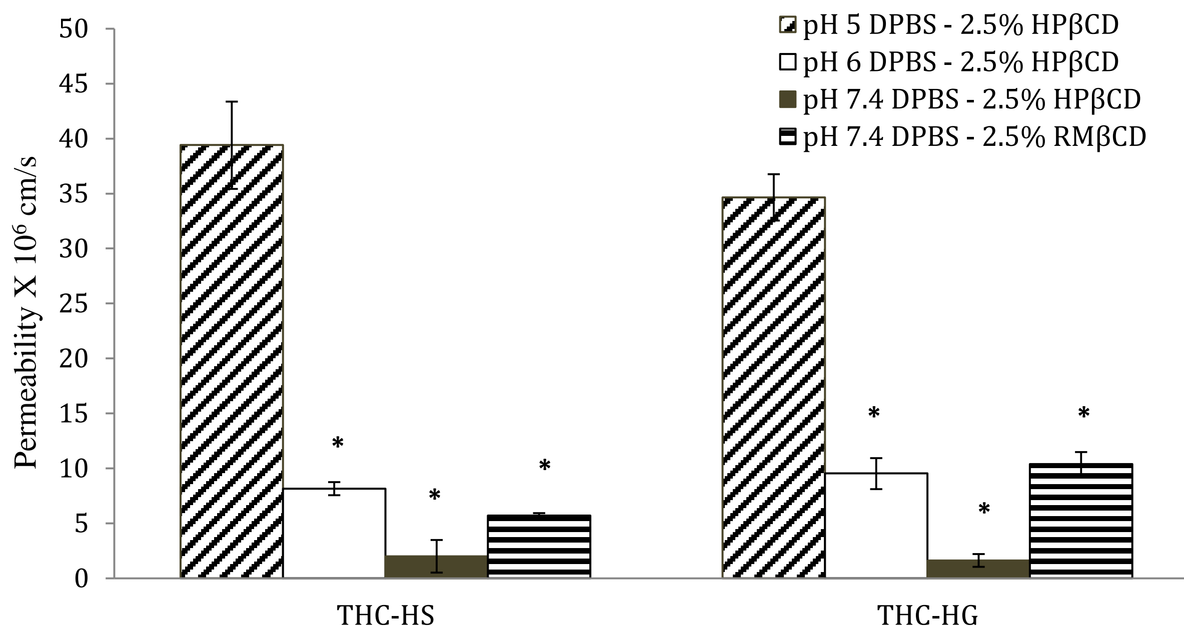

Figure 4-2: Permeability of THC-HS and THC-HG (in terms of total THC) at 34 °C across isolated cornea. The legends indicate the donor solution pH and composition. Receiver solution used in these studies was DPBS containing 2.5% HPβCD (pH 7.4). Results are depicted as a mean ± SD (n=3). *$p < 0.05$.

4.6. DISCUSSION

Establishing effective delivery of a drug to the target tissues is a prerequisite to clinical studies evaluating pharmacological response. However, all previous clinical studies evaluating topical effectiveness of THC in glaucoma used light mineral oil based formulations, a vehicle from which *in vitro* corneal permeability of THC is reported to be 1.86 x 10^{-8} cm/s (51). Thus, inefficient delivery of THC to the target ocular tissues could be responsible for the sporadic

evidence with respect to the efficacy of topically administered THC on IOP. In the present investigation, prodrug and formulation approaches to improve the aqueous solubility and transcorneal permeability of THC were studied.

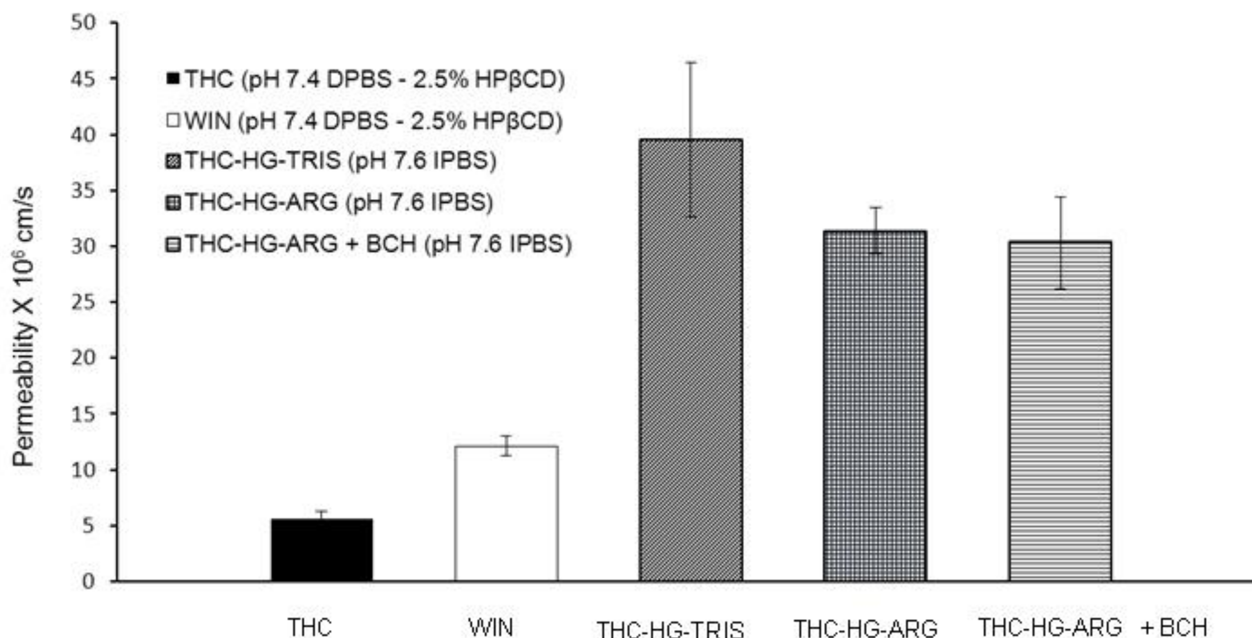

Figure 4-3: Comparative permeability (in terms of total THC) of THC, WIN, THC-HG-ARG complex, THC-HG-ARG complex + BCH and THC-HG-TRIS complex at 34 °C across isolated rabbit corneas. The legends indicate the donor solution pH and composition. Receiver medium was 2.5 % HPβCD in DPBS (pH 7.4) for THC and WIN, while for the ion-pair complexes the receiver solution was IPBS containing 2.5% HPβCD (pH 7.4). Results are depicted as a mean ± SD (n=3). *$p < 0.05$.

Cyclodextrins have been widely used as complex forming agents to improve the solubility and permeability of many hydrophobic drug candidates (130). Since their discovery, cyclodextrins have been used in numerous marketed products. Of these, HPβCD when used up to 12.5% w/v has been shown to be safe and well tolerated when administered topically to the eye (131). It has also been demonstrated that HPβCD only transports the drug molecule to the surface

of the membrane and does not itself permeate through the membrane in any significant quantities (131).

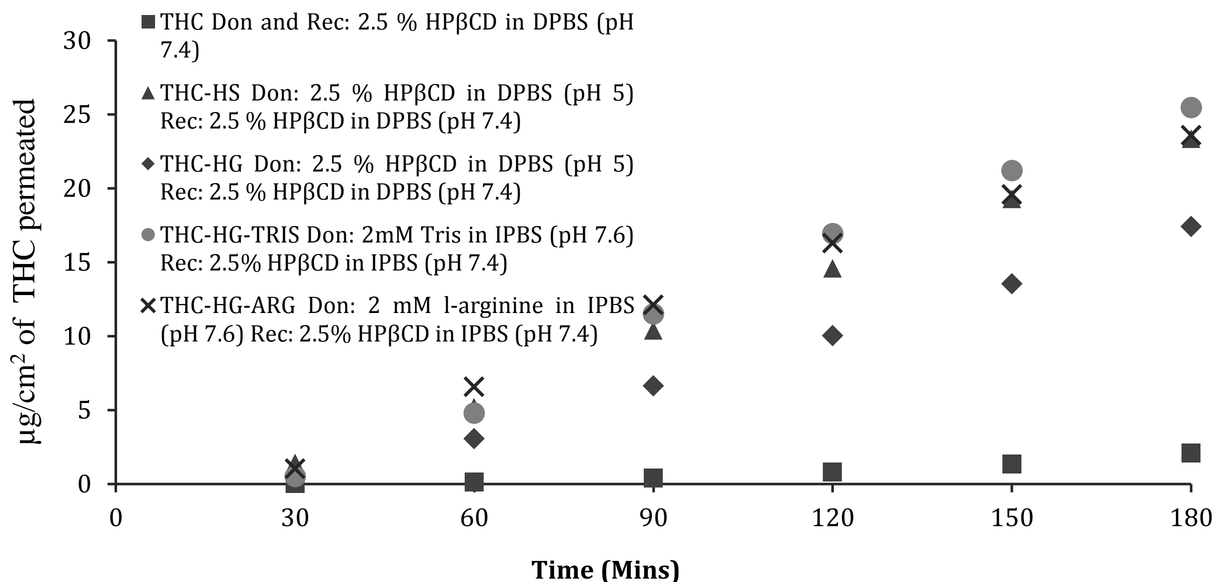

Figure 4-4: Cumulative transport of THC, THC-HS and THC-HG (in terms of total THC) from cyclodextrin and ion-pair based formulations across isolated rabbit corneas at 34 °C, as a function of time. The donor solution (Don) and receiver medium (Rec) pH and composition is indicated in the legends. Results are depicted as a mean ± SD (n=3). *$p < 0.05$.

RMβCD has also been used by researchers in ophthalmic drug delivery (132). When used at high concentrations both HPβCD and RMβCD have been shown to affect tight junction integrity (133, 134). However, *in vitro* corneal permeability studies demonstrate that use of HPβCD up to concentrations of 5 % w/v does not affect the tight junctions (134). Concentration dependent effect of RMβCD on corneal tight junctions has not been reported as yet. Thus, some of the observed enhancement in corneal permeability in the presence of the cyclodextrins could have resulted from the interaction of the cyclodextrins with the corneal tight-junctions also, besides enhanced solubility and greater availability of the drug at the corneal surface.

Permeability of THC from 2.5% HPβCD solutions in DPBS was found to be about 300-fold higher compared to the permeability of THC from light mineral oil based formulations. Incidentally, when Keith and Green used 30% HPβCD as the vehicle, transcorneal permeability of THC was observed to be only 3.3 x 10^{-8} cm/s (51). Thus, permeability of THC from the 2.5% HPβCD drug saturated solution in this study was about 200 times higher compared to the permeability value reported by Green *et al.*(51).

Table 4-5: Apparent first order rate constants (k*) and half-lives (t1/2) of THC-HG in ocular tissue homogenates. Results are depicted as mean ± SD (n=3).

		Cornea	Aqueous Humor	Iris-Ciliary Body	Vitreous Humor	Retina Choroid
THC-HG	$k^* \times 10^3$ (min^{-1})	9.7 ± 0.6	28.1± 0.1	14.7 ± 1.9	4.8 ± 0.2	18.9 ± 0.7
	$t_{1/2}$ (min)	71 ± 1.2	25 ± 2.1	47.5 ± 5.1	144.7 ± 5.3	36.7 ± 1.2

Consistent with this observation, when the concentration of cyclodextrin was increased to 30%, from 2.5% in the current study, while keeping the drug concentration constant, a dramatic decrease in corneal permeability was observed (Fig. 4-2). Our results thus demonstrate that the presence of excess amounts of free cyclodextrins (use of unsaturated drug cyclodextrin solutions) results in decreased permeability. WIN also demonstrated a similar phenomenon (Fig. 4-2). Thus, the presence of excess cyclodextrin probably leads to a decrease in the free drug concentration available for permeation. Interestingly, the use of RMβCD improved permeability of THC by almost 2-fold. However, even after such dramatic improvements, corneal permeability of THC only equaled the permeability of WIN, which is about five times more

potent than THC. Thus, the relatively hydrophilic THC-HS and THC-HG prodrugs were evaluated as a means to further improve transcorneal permeability.

Aqueous solubilities of THC-HS and THC-HG were found to be significantly higher in IPBS than in DPBS (Table 4-1). This could be due to the higher ionic strength of DPBS, compared to IPBS, which could inhibit ionization of the prodrugs and thus aqueous solubility. Use of cyclodextrins significantly improved the aqueous solubility of THC-HS and THC-HG in both IPBS and DPBS (Table 4-1). However, aqueous solubility of THC-HS in HPβCD or RMβCD in DPBS was found to be higher than that in IPBS. The increased solubility in DBPS could be due to the presence of higher concentrations of unionized THC-HS in DPBS which may demonstrate higher affinity for the cyclodextrins. Alternatively, stability of THC-HS in DPBS could be higher compared to that in IPBS resulting in higher solubility. Generally, THC-HG demonstrated higher solubility compared to THC-HS. This is probably because THC-HG is significantly more stable than THC-HS, especially at higher pH values. The solubility studies involved a 24 hour equilibration period and would thus be exposed to significant hydrolysis (128).

THC-HS and THC-HG demonstrated pH dependent corneal permeability. At pH 5 (donor solution pH) the permeability of the prodrugs was about 5-fold higher compared to THC, in HPβCD. Corneal permeability decreased 3-fold when the donor solution pH was 7.4, probably due to ionization of the prodrugs. Dicarboxylic acids are commonly used as promoieties in prodrug derivatization approaches to increase the solubility of water insoluble drugs, but their acidic pKa values keep them in the ionized state at physiological pH values. When orally administered, the gastro intestinal tract (GIT) possesses a large surface area and allows prolonged contact time. Also, endogenous ligands could form complexes with ionized drugs

when given orally. Thus, even ionized drugs can be absorbed on oral administration over a period of time. In contrast, when a drug is instilled topically it has to overcome the limited surface area available for absorption and also faces an extremely short contact time. Moreover, the mucus lining the corneal epithelial cells is negatively charged and would thus repel the negatively charged prodrugs, leading to decreased permeability. A soluble, unionized and highly permeable molecule is thus most favorable for ocular delivery.

Ion-pairing agents have been used to neutralize the charge on ionic drugs and prodrugs and to improve ocular permeability (135, 136). l-Arginine carries a positive charge at physiological pH and was thus chosen to form an ion-pair complex. Tromethamine, also known as tris, is a primary amine and is positively charged at pH 7.4. Tromethamine has been successfully used as a counterion for preparing ophthalmic ketorolac formulations (Acular®, Allergan Inc.).

THC-HS was found to be highly unstable in the presence of the cationic counterions. At the end of the 24 hour equilibration period with l-arginine and tris the prodrug was completely converted to THC at all concentrations studied. Further studies of THC-HS with counter-ions were discontinued. The maximum aqueous solubility values reported for THC-HG are 539.56 μg/mL at pH 8 and 411.3 μg/mL at pH 9 (128). Improved aqueous solubility of THC-HG with l-arginine (1.7 mg/mL at pH 8.9, Table 4-2) and tromethamine (1.2 mg/mL, pH 7.8, Table 4-2) in IPBS suggests formation of ion-pairs. All further studies with counter-ions were carried out with THC-HG.

At physiological pH values, THC-HG-ARG and THC-HG-TRIS ion-pair complexes in IPBS were 7-fold more permeable compared to THC in 2.5% HPβCD in DPBS. The ion-pair

complexes demonstrated almost a 1000-fold improvement over the reported permeability of THC from light mineral oil based formulations. Three amino acid transporters LAT1, ASCT1 and $B^{0,+}$ have been shown to be present and functionally active on the corneal epithelium (98, 99, 107). l-Arginine utilizes the $B^{0,+}$ system to permeate though the cornea. $B^{0,+}$ transports amino acids which may be neutral or positively charged. Although both L and D amino acids are transported, L is more preferred (101). The amino acid transporter $B^{0,+}$ is known to accept a wide range of substrates and possibly could tolerate significant structural modifications to their substrates. The increased corneal permeability of THC-HG-ARG could be because of the involvement of the $B^{0,+}$ system. However, permeability of THC from the THC-HG-ARG complex was not inhibited by BCH, indicating that the improvement in physicochemical properties was responsible for the observed improvement in permeability. Alternatively, it could be speculated that the improvement in permeability could also be due to the positively charged counter ions neutralizing the negative charge on the mucous layer covering the corneal epithelium.

Although prodrug derivatization and complex formation improves solubility and corneal permeability, the prodrug has to revert back to the parent drug once it reaches the site of action in order to elicit pharmacological response. Our results demonstrate that THC-HG undergoes rapid bioconversion to THC in the aqueous humor, retina and iris ciliary (Table 4-4).

The present study has thus lead to the development of a topical drug delivery system which improved the solubility and permeability of THC. The THC prodrugs, especially THC-HG can thus be formulated as 0.05% topical solutions for future preclinical studies exploring their utility in glaucoma. Considering the promising data obtained with the cyclodextrins in this

study, the inclusion complex formed between THC-HG and HPβCD / RMβCD will be characterized in the near future, in a manner similar to our previous studies with THC-HS (137).

4.7. ACKNOWLEDGEMENTS

This publication was partially supported by grants (1R41EY020042 & 2R42GM067304-02) from the National Institutes of Health to ElSohly Laboratories, Incorporated. The project was also partially supported by grant number 5P20RR021929 form the National Center for Research Resources/NIH. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CHAPTER 5

OCULAR DELIVERY AND DISPOSITION OF THE HEMIGLUTARATE ESTER PRODRUG OF Δ^9-TETRAHYDROCANNABINOL

5.1. ABSTRACT

The overall goal of this project is to enhance ocular delivery of Δ^9-Tetrahydrocannabinol (THC) through the topical route. In this study a relatively water soluble hemiglutarate ester prodrug of THC (THC-HG) was evaluated with respect to *in vitro* and *in vivo* ocular penetration from various ophthalmic formulations. Solubility, stability and *in vitro* transcorneal permeability of THC-HG was studied in the presence of surfactants. The solutions were characterized with respect to micelle size, zeta potential and solution viscosity. Effect of BAK and EDTA on *in vitro* transcorneal permeability from the micellar solutions was also studied. *In vivo* studies were carried out with the most promising formulations in New Zealand albino rabbits. A previously reported promising THC-HG ion-pair formulation was also studied in vivo. Total THC concentrations produced in the ocular tissues 1h or 3h following topical application was determined. Additionally, ocular penetration of THC from mineral oil and emulsion formulations was also evaluated. Solubility, stability and *in vitro* transcorneal permeability of the ester prodrug was enhanced significantly in the presence of surfactants. BAK and EDTA led to further increase in corneal permeability. THC levels in the ocular tissues (except cornea) were found to be below detection limits when THC was formulated in mineral oil, surfactant or emulsion based formulations. In contrast, THC-HG micellar and ion pair formulations produced significantly higher total THC concentrations in the anterior ocular chamber. *In vivo* results suggest that the lack of activity noted in many of the earlier studies investigating topical THC application could be due to inefficient delivery to the targeted ocular tissues. Rational prodrug design and formulation development may significantly enhance the utility of this promising compound in the management of glaucoma.

5.2. INTRODUCTION

The visual information in the eye is collected in the retina and transmitted to the brain via the optic nerve. The amacrine cells and the bipolar cells collect the visual information from the rods and the cones in the retina and pass it to the retinal ganglion cells (RGC). The RGC then transmits the visual information from the retina to the brain. Glaucoma is an ocular disease characterized by progressive and irreversible neurodegeneration of the RGC leading to visual field loss(138, 139).

A number of hypotheses have been presented on what triggers RGC cell apoptosis but the exact mechanism is as yet unknown(140). An increase in intraocular pressure (IOP) is a significant risk factor in the development and progression of the disease(141). Achieving a reduction in intraocular pressure (IOP) has been the mainstay for glaucoma therapy(142). However the visual field loss continues even after significant IOP reduction (143, 144). Protection of the optic nerve by a mechanism independent of IOP reduction would greatly benefit the treatment and management of glaucoma(145). A number of mechanisms such as excessive glutamate excitotoxicity, oxidative stress, mitochondrial dysfunction, protein misfolding and neurotropin withdrawal have been identified as plausible factors for RGC apoptosis(140). Memantine, a NMDA receptor blocker, the only drug, to our knowledge, that has been studied as a neuroprotectant for glaucoma in phase 3 human clinical trials, failed to meet clinical endpoints(146, 147).

Δ^9-Tetrahydrocannabinol (THC) is the primary active ingredient of the plant *Cannabis Sativa*. It has shown promise in the treatment of glaucoma due to its IOP lowering and neuroprotective effects (23, 24). THC may have an advantage over current class of anti-glaucoma agents in that in addition to its IOP lowering activity it could act as a neuroprotectant through independent mechanisms (124, 148). THC is a partial agonist of both CB1 and CB2

receptors present in the eye and these might be involved in its IOP lowering and neuroprotective activity (44, 47). However, blocking of cannabinoid receptors have failed to completely abolish the neuroprotective activity of THC indicating multiple mechanisms might be involved (24, 149, 150). Evidence now suggests that PPARγ could be an additional target for THC(151). PPARγ have been found to be expressed and functionally active in the retina(152). THC could also induce vasorelaxation of retinal arteries as well as reduction in ROS activity.

Literature demonstrates that THC has failed to exhibit consistent IOP lowering and neuroprotective activity following topical application(31, 115). The observed inconsistency could be due to poor and variable delivery of THC to the target ocular tissues. Formulation of THC as an ophthalmic solution is especially challenging due to its low aqueous solubility (1-2 µg/mL) and high lipophilicity (logP 6.4). Thus, most of the earlier *in vivo* studies investigating the effect of THC through the topical route used mineral oil or emulsion systems as the vehicle.

In order to improve the physicochemical properties of THC, the relatively water soluble hemiglutarate ester prodrug (THC-HG) was synthesized. The goal of this project is to study the effect of surfactants on the solubility, stability, *in vitro* permeability and *in vivo* bioavailability of THC-HG. Effect of benzalkonium chloride (BAK), a preservative, and ethylenediaminetetraaceticacid (EDTA), a preservative aid, on *in vitro* transcorneal permeability was also studied. In a previous study, the effect of ion pair formation of THC-HG with tromethamine and l-arginine on *in vitro* transcorneal permeability was reported (148). Both l-arginine and tromethamine significantly improved transcorneal THC delivery. Thus, the ion-pair formulation was also included in this study to compare its in vivo distribution with that of the surfactant formulations. Since l-arginine is a biologically active molecule, tromethamine was selected as the preferred ion pairing agent.

Previous studies exploring the utility of topical THC application in glaucoma, in preclinical and clinical studies, have used light mineral oil as the vehicle (31, 50, 116). Some studies have also used a submicron emulsion to deliver THC topically (116). Considering that the mixed data with respect to the IOP lowering characteristics of topical THC (31, 50, 116) could be due to inefficient ocular delivery from these formulations, in the current study we also examined the THC levels achieved in the ocular tissues from these formulations. The studies thus provide a comparison between the *in vivo* bioavailability of THC formulated in light mineral oil/emulsion/surfactant formulations and THC-HG formulated in micellar or ion-pair solutions.

5.3. METHODS

5.3.1. Materials

Super-refined polysorbate 80 was received as a gift sample from Croda Inc (Mill Hall, PA). Cremophor RH 40, poloxamer 188 and poloxamer 407 were received as gift samples from BASF (Chattanoga, TN) and lipoid E 80 was received as a gift sample from Lipoid (Ludwigshafen, Germany). Propofol, sigmacote, hydroxypropyl beta cyclodextrin and tyloxapol were purchased from Sigma (St. Louis, MO). All other chemicals were purchased from Fisher Scientific (St. Louis, MO). Solvents used for analysis were of HPLC grade.

5.3.2. Animal Tissues

Whole eye globes of New Zealand Albino rabbits were purchased from Pel Freez Biologicals (Rogers, AK). Eyes were shipped overnight over wet ice in Hanks Balanced Salt Solution. Corneas were isolated from whole eye globes and used immediately on receipt. We have previously reported that active and passive transport processes in corneas obtained from

ocular globes stored in Hanks Balanced Salt Solution are equivalent to freshly excised rabbit corneas(153).

5.3.3. Animals

Male New Zealand White Albino Rabbits were procured from Harlan Labs (Indianapolis, IN). All of the animal experiments conformed to the tenets of the Association for Research in Vision and Ophthalmology statement on the Use of Animals in Ophthalmic and Vision Research and followed the University of Mississippi Institutional Animal Care and Use committee approved protocols.

5.3.4. Mineral Oil and Emulsion Formulations containing THC

An accurately weighed amount of THC was dissolved in light mineral oil, NF, to prepare the mineral oil based formulation. Emulsion formulations were prepared according to previously published protocols (154, 155). However, instead of crude phospholipids, Lipoid E 80 was used. The emulsion formulation consisted of super refined soybean oil (14 % w/v), oleic acid (6% w/v), glycerin (2.25% w/v), poloxamer 188 (2% w/v), Δ^9-tetrahydrocannabinol (1 % w/v), lipoid E 80 (1 % w/v), α-tocopherol (0.02% w/v) and deionized water to prepare 20 mL. Briefly, Δ^9-Tetrahydrocannabinol, α-tocopherol and oleic acid were dissolved in super refined soyabean oil. Poloxamer and glycerin were dissolved in deionized water. Lipoid E 80 was dispersed in the aqueous phase. Both phases were heated to 70 °C. The aqueous phase was added to the oily phase and a coarse emulsion was formed by using a high speed homogenizer, Ultra Turrax T25 (IKA®, Wilmington, NC). The coarse emulsion was then passed through a high pressure homogenizer Emulsiflex C5 (Avestin®, Ottawa, Canada). The pH of the final emulsion was adjusted to pH 7.4 using 1% w/v sodium hydroxide and it was filtered through a 0.8 μM membrane filter. The drug loading in the final emulsion was determined by HPLC analysis.

5.3.5. Preparation of THC-HG

THC-HG was synthesized as per previously reported procedures(128).

5.3.6. THC-HG Ion pair formulation

The formulation was prepared as described in our earlier report(148). Briefly, THC-HG (11 mg) was taken from a stock solution (in hexane) in a glass vial and the solvent was evaporated under a stream of nitrogen gas. Tromethamine solution (8 mM) prepared in IPBS was added to the glass vial. The vial was sonicated for 10 minutes. HPMC (0.5% w/v) was added to the formulation and stirred for one hour.

5.3.7. Solubility of THC-HG in Surfactants

Excess THC-HG was taken (stock solution 50 mg/mL of THC-HG in hexane) in a glass vial. Hexane was evaporated using a stream of nitrogen gas. Isotonic phosphate buffered saline (IPBS) containing various types and concentrations of surfactants was then added to the glass vials and sonicated for 10 mins. The vials were then placed in a reciprocating water bath (100 shakes per minute) at 25 °C for a period of 24 hours. The solution was subsequently transferred to silicone coated plastic microcentrifuge tubes and centrifuged at 16,000 g for 60 mins (Fisher Scientific acuSpin 17R). The supernatant was diluted in mobile phase and taken for analysis.

Osmolarity of the final solutions was measured using Osmette S (model 4002, Precision Systems Inc., Natick, MA) using the freezing point depression method. The instrument was calibrated using 100 mOsm and 500 mOsm standards.

5.3.8. Determination of Micellar Particle Size, Zeta Potential and Viscosity

A dynamic light scattering instrument, Zetasizer Nano ZS (Malvern Instruments Inc., Westbrough, MA) was used for measurements at 25 °C. A high concentration zeta cell was used to measure both mean particle size (z averaged) and zeta potential of the micelles. Viscosity measurements were carried out at 25 °C (temperature was maintained using a circulating water bath) using a Brookfield DV II + Pro cone and plate viscometer. Rheocalc was used as the data

acquisition software. Micellar solutions were prepared as described under the solubility studies section. Blank vehicles (no drug) were also evaluated. All measurements were carried out in triplicate.

5.3.9. Stability of THC-HG in the Micellar Solutions

THC-HG loaded micellar solutions were prepared as described under the solubility studies section. Stability studies were carried out at 4 °C, 25 °C and 40 °C in glass vials for a period of two months. Aliquots taken at predetermined time points were diluted in mobile phase and taken for analysis. Apparent degradation rate constants were calculated from the inverse slope of a semi logarithmic plot of percentage drug remaining vs time.

5.2.10. Corneal Permeability Studies

Corneas were extracted from whole ocular globes by making an incision just below the corneal scleral limbus and cutting radially around sclera. It was then mounted between side by side permeation cells (Permgear Inc.). Temperature was maintained at 34 °C by circulating water through the jacket of the permeation cells. Donor solutions (3 mL), prepared as described under solubility studies, was added to the epithelial side (donor cell) and 3.2 mL of 2.5% hydroxypropyl beta cyclodextrin solution in IPBS was added to the endothelial side (receiver side). The difference in volumes between the donor and the receiver chamber helped maintain the natural curvature of the cornea. Aliquots (0.6 mL) were withdrawn every 30 min and immediately replaced with donor solution. Samples were taken for analysis.

5.3.11. *In Vivo* Bioavailability Studies

Male New Zealand albino rabbits weighing between 2-2.5 Kg were used for determining *in vivo* bioavailability of THC from the mineral oil/emulsion/micellar formulations and also total THC from the prodrug (THC-HG) loaded ion pair and micellar formulations. Rabbits were anesthetized using a combination of ketamine (35 mg/kg) and xylazine (3.5 mg/kg) injected

intramuscularly and maintained under anesthesia throughout the experiment. Topical formulations were prepared as described under the solubility section. Fifty microliters of the formulations were placed in the cul de sac of the right eye of the anesthetized rabbits. At the end of one hour after topical application the rabbits were euthanized by an overdose of pentobarbital injected through the marginal ear vein. The eye was washed with ice cold IPBS and immediately enucleated and washed again. The ocular tissues were separated, weighed and placed at -80 °C until further analysis. All experiments were carried out in triplicate.

5.3.12. Analytical Procedure for *In Vitro* Samples

A Waters HPLC system comprising of 600 E pump controller, 717 plus autosampler and 2487 UV detector was used. Data handling was carried out using an Agilent 3395 integrator. THC and THC-HG stock solutions were prepared in hexane and stored at -15 °C. An 85:15 mixture of methanol and 0.84% glacial acetic acid was used as a mobile phase on a Phenomenex Luna PFP(2) 4.6 x 250 mm column at a flow rate of 1.2 mL/min. Detection was carried out at 226 nM. Retention time for THC and THC-HG was 10.1 mins and 14.3 mins.

5.3.13. Bioanalytical Method

THC-HG was hydrolyzed to THC under alkaline conditions and total THC was determined. The hydrolysis process was validated to ensure complete conversion of the prodrug to THC. Separate studies have established that THC-HG is rapidly converted to THC in the ocular tissue matrix (148). Nevertheless, the additional deconjugation steps were employed to ensure conversion of any intact THC-HG into THC.

Stock solutions of THC, THC-HG and internal standard (propofol) were prepared in acetonitrile. THC/THC-HG was spiked in blank ocular tissues and allowed to stand for 15 minutes before protein precipitation procedure. Standard curves were prepared for THC/THC-HG in aqueous humor (10 ng – 200 ng), vitreous humor (20 ng-200 ng), cornea (20ng-200ng),

iris ciliary body (10ng-200ng), retina choroid (10-200 ng) and sclera (20-200 ng). For THC-HG the standard curve was plotted in terms of total calculated THC. Sample preparation for the various ocular tissues is described as follows.

5.3.13.1. ***Aqueous and vitreous humor sample preparation***: To 100 µL of aqueous humor or 400 µL of vitreous humor 20 µL of drug (10-200 ng) and 20 µL of internal standard (200 ng) prepared in acetonitrile was added. To the aqueous humor and vitreous humor samples 50 µL and 100 µL of 1N sodium hydroxide was added, respectively, and placed at 25 °C. At the end of two hours, 50 and 100 µL of 1N HCl (to neutralize the sodium hydroxide) and 200 µL and 400 µL of ice cold acetonitrile (to precipitate the proteins) was added to the aqueous humor and vitreous humor samples, respectively. All samples were centrifuged at 16,000 g and taken for analysis.

5.3.13.2. ***Preparation of other ocular tissue samples***: To weighed amount of the cornea/iris ciliary body/RPE choroid or sclera, 20 µL of drug (20-200 ng) and 20 µL of internal standard (200 ng) in acetonitrile was added followed by 600 µL of ice cold acetonitrile and 100 µL of 1 N NaOH. The samples were placed at 25 °C and at end of two hours 1N HCl was added. The samples were centrifuged at 16000 g for 30 mins and taken for analysis.

A previously published analytical method using fluorescence detection was modified and used(156). For HPLC analysis, a Phenomenex Luna PFP(2) 4.6 x 250 mm column was used. The mobile phase consisted of 70% Acetonitrile:30% water containing 5.05% v/v of o-phosphoric acid at a flow rate of 1 mL/min. A Waters 2475 detector was set at an excitation wavelength of 220 nm and THC was detected at emission wavelength of 305 nm. EUFS was set at 150 and gain was set at 50. Injection volume was 50 µL. Retention time for propofol and THC were 6.9 mins and 11.9 mins respectively. Total runtime was for the method was 20 mins.

All standard curves generated had R^2 values greater than 0.98. Recovery value of THC from the ocular tissues was determined at three concentrations (low, medium and high). Average recovery values were determined in cornea (99.4 %), aqueous humor (93.2 %), vitreous humor (91.2%), iris ciliary body (93.2%), retina (102%) and sclera (91.6%). Limit of detection of THC in various ocular tissues were determined in aqueous humor (5ng), vitreous humor (10 ng), cornea (10 ng), iris ciliary body (5 ng), retina choroid (5 ng) and sclera (10 ng).

5.3.14. Data Analysis

All experiments were carried out at least in triplicate. Flux was obtained from a linear regression analysis of the cumulative amount of THC-HG in the receiver chamber versus time. Permeability was calculated by normalizing flux values to donor concentrations.

Unpaired student's t-test was used to compare between two groups. Statistical analysis between multiple groups was carried out by one way analysis of variance. Levenes test was used to find out variation between the groups. Tukey's Honestly Significant test was used to differentiate between the groups. $p < 0.05$ was considered to be statistically significant.

5.4. RESULTS

5.4.1. Solubility and Osmolarity in Surfactant Solutions

Use of surfactants led to a significant improvement in the aqueous solubility of THC-HG (Table 5-1). At 0.1% w/v, Polysorbate 80 led to the greatest increase in solubility (36-fold, 678.2 ± 23.4 µg/mL) followed by Poloxamer 407 (29-fold, 507.4 ± 6.6 µg/mL), Tyloxapol (24-fold, 467.6 ± 27.9 µg/mL) and Cremophor RH 40 (22-fold, 424.9 ± 60.5 µg/mL) respectively. Poloxamer 188 led to only a 6-fold improvement in solubility and was thus not studied any further. Solubility of THC-HG was further studied at higher concentration of Cremophor RH 40 and Poloxamer 407 (maximum concentrations approved for ophthalmic formulations). Solubility

of THC-HG in 0.25% w/v and 0.5% w/v of Cremophor RH 40 was found to be 890.7 ± 166.2 µg/mL and 2136.2 ± 112.7 µg/mL, respectively. Solubility of THC-HG at 0.16% Poloxamer 407 was found to be 621.9 ± 77.1 µg/mL.

Inclusion of surfactants and THC or THC-HG in the formulations did not change the osmolarity significantly at the concentrations studied.

Table 5-1: Solubility and Osmolarity of THC-HG in Cremophor RH 40, Poloxamer 188, Poloxamer 407, Polysorbate 80 and Tyloxapol in IPBS at 25 °C. Results are depicted as mean ± SD (n=3).

Excipient	**Concentration (% w/v)**	**THC-HG**	
		Solubility (µg/mL)	**Osmolarity (mOsm)**
Plain IPBS	-	18.8 ± 3.1	281.6 ± 1.7
Cremophor RH 40	0.1	424.9 ± 60.5	282.6 ± 0.5
	0.25	890.7 ± 166.2	284.3 ± 0.5
	0.5	2136.2 ± 112.7	292.3 ± 1.2
Poloxamer 188	0.1	106.8 ± 23.4	282 ± 2.2
Poloxamer 407	0.1	507.4 ± 6.6	282.9 ± 1.2
	0.16	621.9 ± 77.1	283.6 ± 0.5
Polysorbate 80	0.1	678.2 ± 23.4	282.6 ± 0.5
Tyloxapol	0.1	467.6 ± 27.9	283 ± 0.8

Table 5-2: Hydrodynamic Radius, D90 and Zeta Potential of blank and THC-HG loaded (DL) micelles prepared in IPBS at 25 °C. Results are depicted as mean ± SD (n=3).

Surfactant	Concentration (% w/v)		Hydrodynamic Radius (nm)	D_{90} (nm)	PDI	Zeta Potential (mV)	Viscosity (Cp)
Cremophor RH 40	0.1	Blank	9.4	41.8 ± 3.7	0.259	1.0	1.2 ± 0.01
		DL	16.4	68.4 ± 6.9	0.220	-6.4	1.26 ± 0.01
	0.25	Blank	7.9	27.5 ± 0.6	0.135	0.4	1.23 ± 0.03
		DL	11.3	41.2 ± 1.6	0.164	-6.4	1.25 ± 0.01
	0.5	Blank	7.6	27.2 ± 0.8	0.081	-1.2	1.25 ± 0.02
		DL	14	69.3 ± 3.1	0.322	-6.6	1.28 ± 0.02
Poloxamer 407	0.16	Blank	8.1	30.7 ± 2.9	0.162	-2.3	1.22 ± 0.02
		DL	15.9	61.7 ± 7.4	0.264	-5.5	1.24 ± 0.01
Polysorbate 80	0.1	Blank	11.1	36.6 ± 6	0.152	-1.4	1.22 ± 0.01
		DL	-	-	-	-11	1.26 ± 0.01
Tyloxapol	0.1	Blank	6.8	25.1 ± 1.5	0.154	0.1	1.20 ± 0.01
		DL	14.3	65.1 ± 8.3	0.237	-15.8	1.24 ± 0.01

5.4.2. Particle Size and Viscosity Measurements

The hydrodynamic radius, D_{90} and zeta potential of the blank and THC-HG loaded surfactant formulations are presented in Table 5-2.

5.4.3. Stability in Presence of Surfactants

The hydrolysis of THC-HG followed first order degradation. Apparent first order hydrolysis rate constants and half-life of THC-HG in the presence of surfactants is presented in Table 5-3. Stability of THC-HG was enhanced significantly in the presence of surfactants and was found to be temperature dependent. Energy of activation was calculated from the Arrhenius Plot constructed with the data. Formulations containing Poloxamer 407 had the lowest energy of activation (984 cal/mol). Energy of activation of THC-HG in the presence of Polysorbate 80 (2161 cal/mole), Tyloxapol (2354 cal/mol) and Cremophor RH 40 (2330 cal/mol) was not significantly different.

5.4.4. Permeability of THC-HG in the presence of surfactants

Permeability of THC-HG in the presence of 0.1% w/v Cremophor RH 40 was found to be 14 x 10^{-6} cm/s. Increasing the concentration of Cremophor RH 40 to 0.25 % w/v (10.5 x 10^{-6} cm/s) and 0.5 % w/v (10.9 x 10^{-6} cm/s) did not affect the permeability significantly. Permeability of THC-HG in the presence of 0.16 % w/v Poloxamer 407 was found to be slightly higher (21.4 x 10^{-6} cm/s compared to 0.1 % w/v (15 x 10^{-6} cm/sec). In the presence of 0.1 % Polysorbate 80 and Tyloxapol permeability was found to be 7.3 x 10^{-6} cm/s and 12.3 x 10^{-6} cm/s, respectively. The above data have been illustrated in Fig. 5-1.

Since Cremophor RH 40 and Poloxamer 407 containing formulations demonstrated the highest permeability, the effect of inclusion of BAK and EDTA on permeability was also studied. However, Poloxamer 407 interacted with BAK and led to the formation of a precipitate. Hence

the effect of BAK on the permeability of poloxamer 407 based formulations could not be studied. Permeability of THC-HG in 0.01% w/v BAK and 0.1% w/v of Cremophor RH-40 was significantly increased to 19.9 x 10^{-6} cm/s (Fig. 5-2). Use of 0.02% BAK in 0.1 % w/v Cremophor RH 40 led to a two-fold enhancement in permeability of THC-HG. Addition of 0.1% w/v disodium EDTA to the 0.1% Cremophor RH 40 formulation also led to a significant increase in the permeability of THC-HG (18.6 x 10^{-6} cm/s). A combination of 0.01% BAK with 0.1% disodium EDTA in 0.1% Cremophor RH 40 yielded a permeability value of 24.2 x 10^{-6} cm/s.

Table 5-3: Apparent first order rate constants (k* x 10^4, h^{-1}) and half lives (t1/2, days) of THC-HG in various surfactant solutions in IPBS at 4 °C, 25 °C and 40 °C. Results are depicted as mean ± SD (n=3).

Formulation	Concentration (% w/v)		4 °C	25 °C	40 °C
IPBS	0.001	k	161 ± 34.5	1755.7 ± 84.3	2556.7 ± 97.4
		$t_{1/2}$	45.5 ± 11.3	3.9 ± 0.2	2.7 ± 0.1
Cremophor RH 40	0.1	k	1.9 ± 0.4	5.3 ± 0.1	13.6 ± 0.4
		$t_{1/2}$	155.3 ± 34.4	54.2 ± 0.5	21.1 ± 0.6
Poloxamer 407	0.1	k	4.4 ± 1.8	6.8 ± 0.4	17.5 ± 0.6
		$t_{1/2}$	77.1 ± 30.1	42.2 ± 2.3	16.4 ± 0.6
Polysorbate 80	0.1	k	1.7 ± 0.2	4.3 ± 0.1	10.7 ± 0.5
		$t_{1/2}$	167.7 ± 15.6	67.7 ± 2	27.1 ± 1.2
Tyloxapol	0.1	k	1.8 ± 0.5	4.5 ± 0.1	13 ± 3.5
		$t_{1/2}$	174.9 ± 52.4	64.7 ± 1.8	23.7 ± 5.4

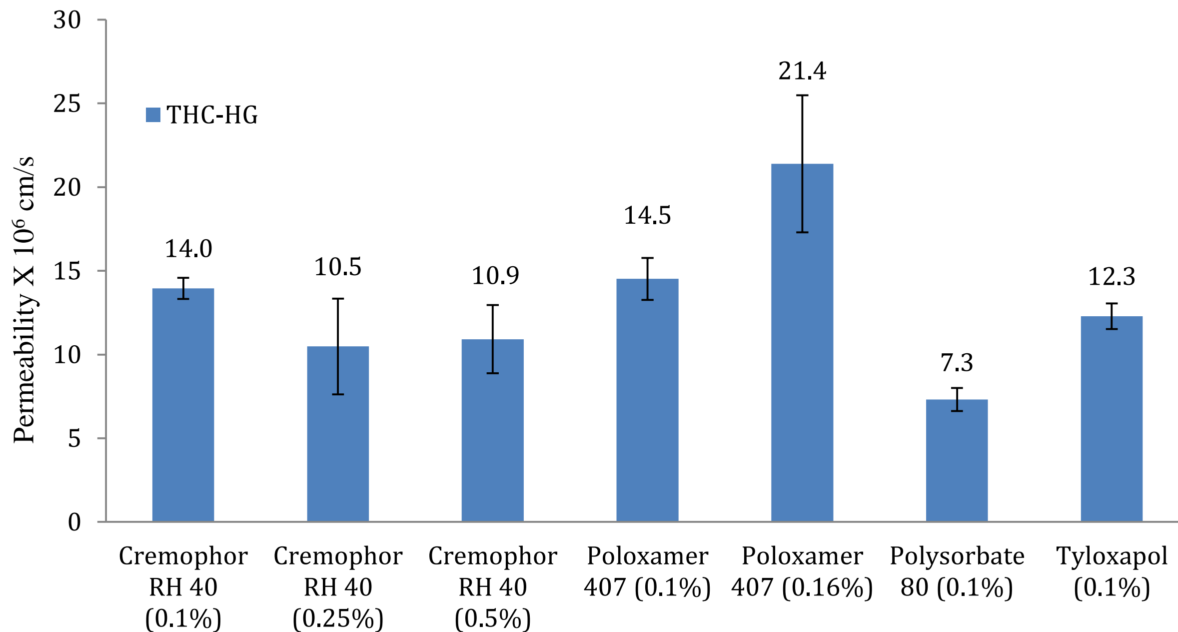

Figure 5-1: Permeability of THC-HG from various surfactant solutions across isolated rabbit cornea at 34 °C. Receiver solution consisted of IPBS containing 2.5% HPβCD (pH 7.4). Results are depicted as mean ± SD (n=4). *p<0.05.

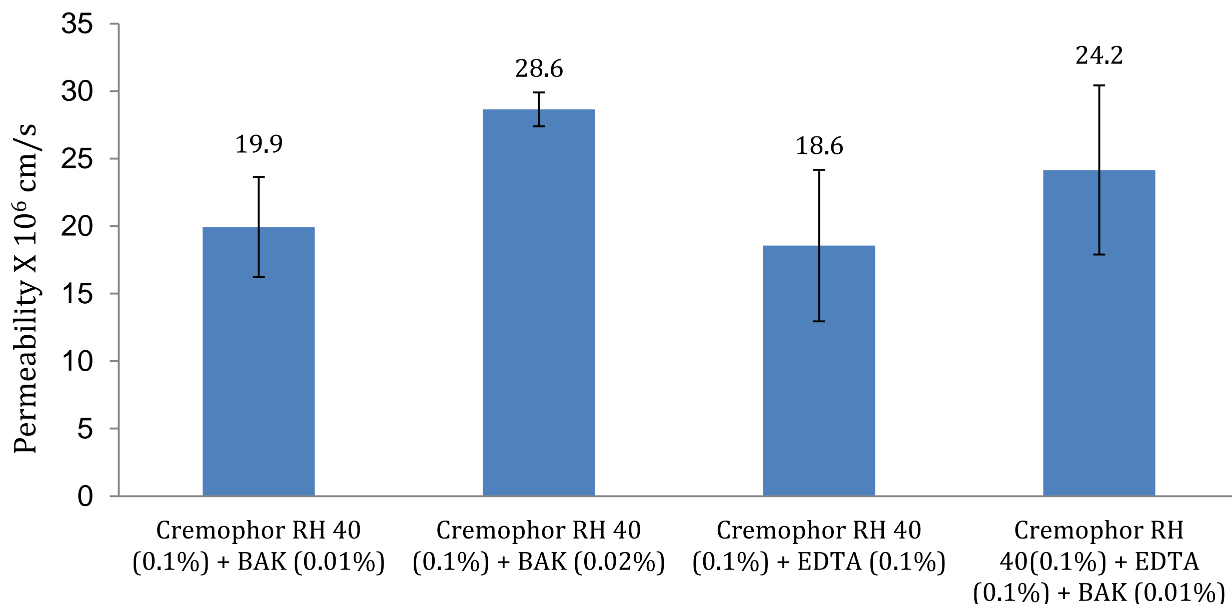

Figure 5-2: Permeability of THC-HG from donor solutions containing Cremophor RH 40, BAK and/or EDTA across isolated rabbit cornea at 34 °C. Receiver solution used was IPBS containing 2.5% HPβCD (pH 7.4). Results are depicted as mean ± SD (n=4). *p<0.05.

5.4.5. *In Vivo* Bioavailability

The THC or THC equivalent (for the THC-HG formulation) content in the formulations are provided in Table 5-4 and Table 5-5. THC formulated in mineral oil (0.1%) or as emulsions (0.4%) did not produce any detectable THC levels in the aqueous humor or the iris ciliary body. On the other hand, THC-HG formulation (0.18% THC equivalent) consisting of 0.5% Cremophor RH 40 + 0.02 % BAK + 0.1 % EDTA + 0.5 % HPMC delivered 32.1 ± 12.6 ng/100 µL to the aqueous humor and 35.6 ± 12.5 ng/50 mg to the iris ciliary body. THC-HG ion pair formulation (0.16% THC equivalent) was able to deliver 52.2 ± 18.7 ng/100 µL to the aqueous humor and 93.1 ± 41.4 ng/50 mg to the iris ciliary body. THC concentrations achieved in the cornea from the THC-HG formulation in cremophor (2451.6 ± 645.6 ng/50 mg) and ion pair formulations (2245.7 ± 240.2 ng/50 mg) were several fold greater than that obtained from the THC loaded mineral oil (68.8 ± 14.5 ng/50 mg) and emulsion (300.6 ± 79.6 ng/50 mg) formulations. THC-HG containing micellar solution (354.7 ± 86.4 ng/250 mg) as well as ion pair formulation (2258.1 ± 1331.8 ng/250 mg) also delivered significantly higher concentration to the sclera compared to light mineral oil (104.1 ± 36.1 ng/250 mg) and emulsion (171.1 ± 66.6 ng/250 mg) THC formulations.

Intraocular tissue concentrations 3h post instillation of the ion pair formulations were also determined since significantly higher concentrations of THC were detected in the iris ciliary body from this formulation when compared to the surfactant formulation. THC concentrations in the cornea (1382.7 ± 109.8 ng/50 mg), aqueous humor (27.3 ± 11.3 ng/100 µL), iris ciliary (67.1 ± 55.6 ng/50 mg) and sclera (166.1 ± 49.3) were still detected at the end of three hours.

When THC, rather than THC-HG, was incorporated into the Cremophor based micellar solution (0.125% w/v THC, 0.5% Cremophor RH 40, 0.02% BAK, 0.1% EDTA, 0.5% HPMC)

THC was detected in the cornea (553.9 ± 87.4 ng/ 50 mg tissue) and sclera (354.7 ± 86.4 ng/250 mg tissue but not in the aqueous humor or iris ciliary body. the retina choroid.

However, none of the formulations studied produced THC concentrations in the vitreous humor (LOD 5ng/400 µL) or the retina choroid (LOD 10 ng/ 50 mg of tissue). The above results have been presented in Tables 5-4 and 5-5.

Table 5-4: Total THC concentrations observed in rabbit ocular tissues 1 h post topical administration of 50 µL of THC in Light Mineral Oil (0.1% w/v), Emulsion (0.4% w/v) or micellar solutions (0.125 % w/v THC, 05% Cremophor RH 40 + 0.1% EDTA + 0.02% BAK + 0.5% HPMC). Results are depicted as mean ± SD (n=3).

Tissue	THC		
	Light Mineral Oil	Emulsion	0.5% Cremophor RH 40 + 0.1% EDTA + 0.02% BAK + 0.5% HPMC
Drug Concentration in terms of THC (% w/v)	0.1	0.37	0.125
Cornea (ng/50 mg Tissue)	68.8 ± 14.5	300.6 ± 79.6	553.9 ± 87.4
Aqueous Humor (ng/100 µL)	ND*	ND*	ND*
Iris-Cilliary Body (ng/50 mg Tissue)	ND*	ND*	ND*
Vitreous Humor (ng/mL)	ND*	ND*	ND*
Retina-Choroid (ng/50 mg Tissue)	ND*	ND*	ND*
Sclera (ng/250 mg Tissue)	104.1 ± 36.1	171.1 ± 66.6	439.3 ± 280.2

Table 5-5: Total THC concentrations observed in rabbit ocular tissues 1 h post topical administration of 50 µL of THC-HG in or THC-HG (0.2 % w/v THC equivalent) formulated in, 05% Cremophor RH 40 + 0.1% EDTA + 0.02% BAK + 0.5% HPMC or ion pair formulation (8 mM tromethamine + 0.5% HPMC). Results are depicted as mean ± SD (n=3).

Tissue	THC-HG		
	0.5% Cremophor RH 40 + 0.1% EDTA+0.02% BAK + 0.5% HPMC	Ion Pair Formulation	
	1 Hour	1 Hour	3 Hours
Drug Concentration in terms of THC (% w/v)	0.18	0.16	0.16
Cornea (ng/50 mg Tissue)	2451.6 ± 645.6	2245.7 ± 240.2	1382.7 ± 109.8
Aqueous Humor (ng/100 µL)	32.1 ± 12.6	52.2 ± 18.7	27.3 ± 11.3
Iris-Cilliary Body (ng/50 mg Tissue)	35.6 ± 12.5	93.1 ± 41.4	67.1 ± 55.6
Vitreous Humor (ng/mL)	ND*	ND*	ND*
Retina-Choroid (ng/50 mg Tissue)	ND*	ND*	ND*
Sclera (ng/250 mg Tissue)	354.7 ± 86.4	2258.1 ± 1331.1	166.1 ± 49.4

5.5. DISCUSSION

Helper and Frank in 1971 observed that smoking marijuana leads to a drop in IOP and subsequently THC was identified as one of the constituents responsible(157). Several preclinical

and clinical studies were initiated to determine whether THC could lower IOP when applied topically. However the results were ambiguous. One of the reasons behind the observed variability could be lack of an effective ophthalmic THC formulation in these earlier studies. The ocular tissues presents significant physiological barriers to the permeation of external moieties e.g. tear flow, corneal ultrastructure and aqueous humor outflow (158). Delivery to the posterior segment of the eye is even more challenging (159). On top of these factors, THC is a highly lipophilic agent and will not efficiently partition into the aqueous precorneal environment from lipophilic formulations to be available for ocular absorption. In this study, the *in vitro* transcorneal permeability and *in vivo* bioavailability of THC has been improved using a combination of hydrophilic prodrug derivatization and formulation approaches. Ocular bioavailability using the prodrug, through the topical route, has also been compared to that of THC from mineral oil and emulsion based formulations, similar to that used in the earlier in vivo studies.

In order to increase the aqueous solubility, stability and permeability of THC-HG, surfactants commonly used in ophthalmic formulations were used. The maximum surfactant concentrations to be used in these studies were determined from the FDA database of inactive excipients.

Use of surfactants led to a significant improvement in the aqueous solubility of THC-HG. Moreover, the ester hydrolysis rate constant of THC-HG was significantly reduced in the presence of the surfactants leading to a marked improvement in the stability of THC-HG in aqueous solutions.

Amongst the surfactants studied, THC-HG demonstrated highest in vitro transcorneal permeability in the presence of poloxamer 407 and this formulation was thus selected for further

evaluation. Although the extent of permeation enhancement achieved with Cremophor RH 40 was less marked than poloxamer 407, Cremophor RH 40 has been used at concentrations up to 0.5% in ophthalmic eyedrops, in contrast to only 0.16% for poloxamer, and can thus provide advantages with respect to higher donor concentrations and flux. Thus, both poloxamer 407 and Cremophor RH 40 were investigated further.

BAK, a cationic surfactant, is commonly used as a preservative in ophthalmic formulations. Preservative action is primarily dependent on free BAK concentration present in the formulation. BAK is known to interact with some surfactants, leading to loss of antimicrobial activity (160). THC-HG being a weak acid is negatively charged at physiological pH and may also interact with positively charged BAK. Moreover, BAK is also known to act as a permeability enhancing agent (113). Thus, the effect of inclusion of BAK and EDTA in the promising THC-HG surfactant formulations was also investigated.

Surfactant micelles present in dilute solutions usually form spherical/ellipsoid micelles. At higher surfactant concentrations micelles undergo transformation from spherical/ellipsoid to cylindrical shapes which is usually associated with a significant increase in viscosity(161). Since the viscosity of the drug loaded micellar formulations did not change significantly it probably indicates that the drug loaded micelles were mostly spherical/ellipsoid in shape. Literature reports also suggest that surfactants listed in Table 5-2 form spherical/ellipsoid shaped micelles in dilute solutions (162-166).

Poloxamer 407 demonstrated the highest *in vitro* permeability but addition of 0.02 % BAK to the formulation led to the formation of a precipitate. Formation of a precipitate was however not observed with the blank (no drug added) vehicle. Thus, the observed precipitation could be due to an interaction of BAK with THC-HG. Poloxamer 407 was thus not studied any

further with respect to THC-HG formulation. In contrast to the results with poloxamer 407, a clear solution could be prepared with BAK and Cremophor RH 40 formulation. This could be due to better shielding of the HG promoiety of THC-HG in the Cremophor RH 40 micelles or packing characteristics of the Cremophor RH 40 micelles.

Based on the *in vitro* permeability and solubility data the Cremophor RH 40 based formulation was selected for *in vivo* evaluation in rabbits. Since, permeability of THC-HG in Cremophor RH 40 was found to be independent of the surfactant concentration (within the range studied), 0.5% Cremophor RH 40 concentration, which allowed 2.1 mg/mL of THC-HG loading, was selected for the *in vivo* studies. BAK (0.02%) and EDTA (0.1%) were also included in the formulation in view of their permeability enhancing effect and their common role as preservatives in ophthalmic formulations(160). To increase the corneal residence time 0.5% hydroxyl propyl methyl cellulose (HPMC) was added to the formulation as a viscosity enhancer.

In an earlier study Green *et al* reported intraocular tissue concentration of radiolabelled THC (0.1% w/v) formulated in light mineral oil. Approximate THC concentrations have been reported in the cornea (100 ng/50 mg of tissue), iris-ciliary body (15 ng/50 mg) and the aqueous humor (4 ng/100 µL) at the end of one hour(50).

In the current study, topical administration of 0.1% THC in mineral oil produced similar THC concentrations (68.8 ng ± 14.5 ng/50mg) in the cornea. However, THC levels in the aqueous humor and the iris ciliary body were found to be below the detection limits of the analytical method used in this study. The 0.4% w/v THC emulsion formulation was also not able to deliver THC to the intraocular tissues. These results suggest that, in the earlier studies, THC was probably not achieving significant concentrations in the targeted ocular tissues with the mineral oil and emulsion formulations.

Use of the relatively hydrophilic HG ester prodrug of THC and incorporation into a micellar solution markedly improved the delivery of THC to the aqueous humor) and the iris ciliary body. This indicates that the prodrug was able to partition from the vehicle into the ocular tissues more efficiently. To delineate the effect of the prodrug from the surfactant system, THC (0.125% w/v) was applied topically in the same vehicle. In this case THC levels were not detected in the aqueous humor, iris ciliary body, vitreous humor and the retina choroid. This strongly suggests that, chemical modification of THC into THC-HG led to a significant improvement in the physicochemical properties allowing better partitioning into the aqueous tear fluid and from there into the cornea and other intraocular tissues. However, THC levels in the back-of-the eye tissues were still not observed.

Ion pair formulation of THC-HG with tromethamine delivered significantly higher concentrations to the iris ciliary body compared to micellar formulation Although rapidly cleared from the back-of-the eye ocular tissues, significant THC concentrations were still detectable in the aqueous humor and iris ciliary body at the end of three hours. However, even the ion pair formulation was unable to deliver THC to the back of the eye tissues. Thus, to utilize the neuroprotective potential of THC, further improvement in the topical delivery system is needed.

5.6. ACKNOWLEDGEMENTS

This publication was partially supported by grants (1R41EY020042 & 2R42GM067304-02) from the National Institutes of Health to ElSohly Laboratories, Incorporated. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CHAPTER 6

EVALUATION OF AMINO ACID AND AMINO ACID-DICARBOXYLIC ACID CONJUGATED Δ^9- TETRAHYDROCANNABINOL PRODRUGS FOR TOPICAL DELIVERY

6.1. ABSTRACT

The aim of the present study was to determine the preformulation characteristics of Δ^9-Tetrahydrocannabinol (THC) prodrugs, and to study their ocular disposition following topical instillation. Synthesis and preformulation characterization was carried out for amino acid based Δ^9-Tetrahydrocannabinol (THC) prodrugs. An amino acid-dicarboxylic acid conjugate prodrug of THC, THC-Valine-Hemisuccinate (THC-Val-HS), was also synthesized. *In vitro* transcorneal permeability of the prodrugs in the presence of solubilizing agents was studied across isolated rabbit cornea using a side by side diffusion apparatus. Promising formulations were selected for *in vivo* ocular bioavailability studies in the anesthetized rabbit model. Amino acid based THC prodrugs, THC-Val and THC-Val-Val, revealed poor solubility characteristics at physiological pH and were thus not studied any further. THC-Val-HS, however, demonstrated much improved solubility and also *in vitro* transcorneal permeability at pH 7.4, compared to THC. Formulation of THC-Val-HS as an aqueous eye drop was most effective in delivering THC to the anterior chamber of the eye. However, THC was below quantifiable levels in the vitreous humor and the retina-choroid tissue from this formulation. THC-Val-HS in a polymeric delivery system produced significant THC concentrations in both anterior and posterior ocular chambers. A combination of prodrug derivatization and formulation development approaches significantly improved delivery of THC into the anterior and posterior segments of the eye.

6.2. INTRODUCTION

Δ^9-Tetrahydrocannabinol (THC), an active ingredient of the plant *cannabis sativa*, could potentially be a dual acting antiglaucoma agent(23, 24, 124). In addition to intraocular pressure (IOP) lowering effect, it could also demonstrate an independent neuroprotective activity(148). Since 1970`s a lot of studies have been carried out to elucidate the pharmacological activity but the outcome has been inconclusive due to mixed results (31, 167, 168). We have previously studied dicarboxylic acid prodrugs of THC to improve ocular bioavailability(148). These prodrugs were ionized at physiological pH values and various formulation approaches were adopted to improve transcorneal permeation. In this study we synthesized and evaluated a series of amino acid prodrugs including valine (THC-Val) and valine-valine (THC-Val-Val) as well as an amino acid dicarboxylic acid conjugate, valine hemisccinate (THC-Val-HS), ester prodrugs of THC for ocular bioavailability.

Topical eye drops are the most preferred means of drug delivery to the eye. But delivery of THC as an eye drop is especially challenging due to its low aqueous solubility (1-2 µg/mL) and high logP (6.42)(127). To improve solubility, THC has been formulated in lipophilic vehicles, complexed with cyclodextrins and incorporated in submicron emulsions(51, 127, 169). These approaches have helped improve the solubility of THC but due to its high lipophilicity most of the drug gets entrapped in the corneal epithelium and does not reach the targeted ocular tissues (iris ciliary body and retina). The dose of the topically applied THC has to be increased to improve flux, which leads to increased systemic exposure. THC being a psychoactive drug, increased plasma drug load may lead to undesirable side effects.

Tear formation in the precorneal area leads to rapid dilution and drainage of topically applied drug(170). Since volume of the eye drop administered is relatively constant, higher drug

solubility helps increase drug concentration in the precorneal area and thus increases transcorneal flux. Another significant barrier to drug permeability is the presence of intercellular tight junctions (zona ocludens) on the corneal epithelium which restricts transcellular permeability(171). High lipophilicity improves transepithelial permeability through the transcellular route. However, high lipophilicity and low aqueous solubility prevents effective partitioning in the stroma and thus lowers intraocular drug bioavailability. Thus, desired physicochemical properties for topically applied drugs are high aqueous solubility and logP values between 2-3(172). Since formulation approaches failed to deliver sufficient THC concentrations to the intraocular tissues, THC prodrugs were synthesized. Prodrug approach in drug delivery is commonly utilized to improve the physicochemical properties of the parent compound (173). Prodrugs, in addition to improving physicochemical properties, can also be used to target amino acid and peptide transporters that can help improve transcorneal permeability by piggybacking drug through nutrient barriers(101).

Valine ester prodrugs of acyclovir and gancyclovir (valcyclovir and valgancyclovir) have demonstrated significant improvement in aqueous solubility and transcorneal permeability(97). Thus, the amino acid based hydrophilic prodrug derivatization approach was adopted in this study to evaluate its effect on the disposition of topically administered THC into the deeper ocular tissues.

6.3. METHODS

6.3.1. Materials

6.3.1.1. Chemicals

Hydroxypropyl beta cyclodextrin (HPβCD), randomly methylated beta cyclodextrin (RMβCD), hydroxypropyl methyl cellulose (HPMC, 3000 cps) and tyloxapol were purchased

from Sigma (St. Louis, MO). Super refined polysorbate 80 was received as a gift sample from Croda Inc. (Mill Hall, PA). Poloxamer 188, Poloxamer 407 and Cremophor RH 40 were received as a gift sample from BASF (Florham Park, NJ). Polyethyleneoxide (PolyOx N10) was received as a gift sample from Dow Chemical Company (Midland, MI). All other chemicals and solvents were purchased from Fisher Scientific (St. Louis, MO).

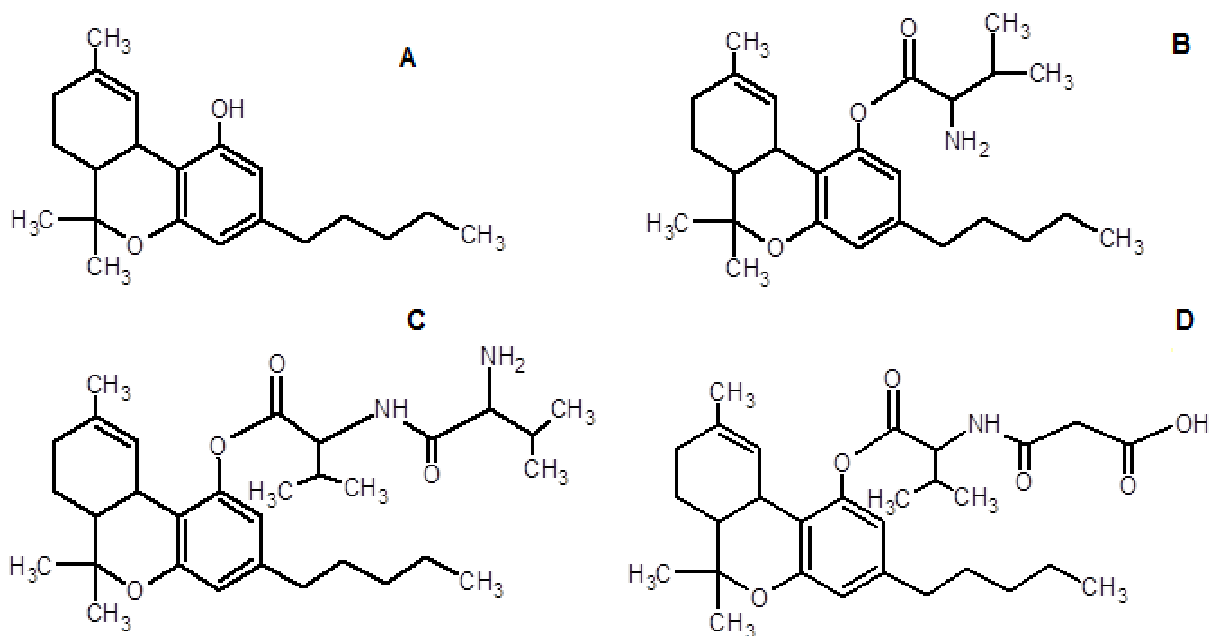

Figure 6-1: Chemical structures of A) Δ^9-Tetrahydrocannabinol (THC) B) Δ^9-Tetrahydrocannabinol Valine (THC-Val) C) Δ^9-Tetrahydrocannabinol-Valine-Valine (THC-Val-Val) and D) Δ^9-Tetrahydrocannabinol Valine Hemisuccinate (THC-Val-HS).

6.3.1.2. Animal Tissues

Earlier studies in the laboratory have reported the effect of storage on active and passive diffusion processes across the cornea(153). Whole eye globes of New Zealand Albino rabbits, purchased from Pel Freez Biologicals (Rogers, AK), were shipped overnight over wet ice in

Hanks Balanced Salt Solution. Corneas isolated from whole eye globes were used for *in vitro* permeability studies immediately on receipt.

6.3.1.3. Animals

Male New Zealand Albino Rabbits (2-2.5 kg) were procured from Harlan Labs (Indianapolis, IN). All of the animal experiments conformed to the tenets of the Association for Research in Vision and Ophthalmology statement on the Use of Animals in Ophthalmic and Vision Research and followed the University of Mississippi Institutional Animal Care and Use committee approved protocols.

6.3.2. Synthesis of Δ^9-Tetrahydrocannabinol Prodrugs

The prodrugs were synthesized by Dr. Waseem Gul of ElSohly Laboratories. Structures of THC, THC-Val, THC-Val-Val and THC-Val-HS are depicted in Fig. 6-1.

6.3.3. Solubility Studies

Standard shake flask method was used for the determination of prodrug solubility in aqueous solutions. For pH dependent solubility, buffers at pH 3, pH 5, pH 7 and pH 9 were prepared according to USP XXX. Surfactant solutions at various concentrations were prepared in Isotonic Phosphate Buffered Saline (IPBS). Excess drug from an acetonitrile stock solution was added to a glass vial and the organic solvent was evaporated in a vacuum chamber. Buffers or surfactant solutions were added to the glass vials and the solutions were equilibrated at 25 °C in a shaking water bath for 24 hours. The solutions were transferred to silicon coated tubes and centrifuged at 16000g in a Fisher Scientific acuSpin Micro 17R for 30 mins. The supernatant was diluted in mobile phase and analyzed by HPLC. All experiments were carried out in triplicate.

6.3.4. Preparation of Polymeric Film

Hot melt cast method was utilized to prepare the polymeric film. Polyethyleneoxide (MW 100000 Daltons) was used as the matrix forming material. THC-Val-HS was dissolved in acetonitrile and dispersed in polyethylene oxide with adequate mixing. The mixture was placed in a vacuum oven to evaporate the organic solvent. A 13 mm die was placed over a brass plate and the brass plate was heated to 70 °C using a hot plate. The drug-polymer mixture was placed in the center of the die, compressed and was heated further for 2-3 mins. Following cooling 4 mm x 2 mm film segments were cut from the extrudate. To determine the content, the film was placed in 10 mL of acetonitrile and sonicated for 15 mins. The film dissolved completely in acetonitrile. The solution was then analyzed by HPLC.

6.3.5 Stability of THC-Val-HS in the presence of surfactants and cyclodextrins

THC-Val-HS loaded micellar solutions and cyclodextrin complexes were prepared as described under the solubility studies section. Stability studies were carried out at 4 °C, 25 °C and 40 °C in glass vials for a period of two weeks. Aliquots taken at predetermined time points were diluted in mobile phase and taken for analysis. Apparent degradation rate constants were calculated from the inverse slope of a semi logarithmic plot of percentage drug remaining vs time.

6.3.6. Corneal Permeability Studies

Method to isolate corneas from whole ocular globes has been described earlier(153). Isolated corneas were placed between jacketed side by side diffusion cells (PermeGear Inc, Hellertown, PA) maintained at 34 °C using a circulating water bath. Donor solution (3mL) was added to the side of cornea facing the epithelium and 3.2 mL of 2.5% HPβCD in IPBS was used

as the receiver chamber. Aliquots (0.6 mL) were taken every 30 mins for 3 hours and replaced with an equal volume of fresh buffer. Samples were analyzed by HPLC.

6.3.7. *In Vivo* Bioavailability Studies

In vivo bioavailability of formulations was determined in Male New Zealand albino rabbits weighing between 2-2.5 Kg. Rabbits were administered ketamine (35 mg/kg) and xylazine (3.5 mg/kg) intramuscularly and maintained under anesthesia throughout the experiment. The rabbits were placed on one side and 50 µL of the formulations was placed in the cul de sac. The polymeric film was placed 2 mm below the corneoscleral junction. No ocular irritation was observed in all of the formulations applied. Rabbits were sacrificed at 1 or 3 hours after dosing with an overdose of pentobarbital. The eyes were washed thoroughly with ice cold IPBS and immediately enucleated. The various ocular tissues were separated and placed at -80 °C until further analysis. All experiments were carried out in triplicate. A previously published method to determine THC concentration in ocular tissues was adopted after necessary validation.

6.3.8. Analytical Method for I*n Vitro* Samples

A HPLC system consisting of a Waters 717 plus autosampler, 600E pump controller, 2487 UV detector and an Agilent 3395 integrator was used for analysis. An analytical method was developed for the determination of THC-Val and THC-Val-Val. Stock solutions of the prodrugs were prepared in acetonitrile and used immediately. A Luna PFP(2) column (4.6 mm x 250 mm) was used for analysis. Mobile phase developed was 10 mM phosphate buffer at pH 7.35 and acetonitrile in the ratio of 1:3 at a flow rate of 1.2 mL/min. Standard curves were generated from 0.2 µg- 10 µg/mL with an R^2 value greater than 0.9999. Retention time was 8.5 min for THC, 11.5 min for THC-Val and 13.5 min for THC-Val-Val.

Determination of THC in ocular tissues was carried out as described previously. A protein precipitation method was used for isolation of the prodrug and the drug from the ocular tissue homogenates. All of THC-Val-HS and other intermediates were converted to THC by carrying out alkaline hydrolysis at room temperature for 1 hour. The homogenate was neutralized by the addition of hydrochloric acid and centrifuged. The supernatant was separated and analyzed by HPLC-fluorescence method previously described.

6.3.9. Data Analysis

Flux and *in vitro* permeability was determined as previously reported(153). Difference in variance between the groups was checked with Levenes test before carrying out ANOVA. Statistical significant difference among multiple groups was checked using one way ANOVA. Tukey's Honestly Significant test was carried out to differentiate between the groups. A p values less than 0.05 was considered to be statistically significant.

6.4. RESULTS

6.4.1. Predicted Physicochemical Properties

Unionized drug in solution is preferred since permeation of unionized drug is greater compared to ionized drug. The predicted pKa values (ACD ILab 2.0) for mono- and di- amino acid THC prodrugs were found to be close to the physiological pH values. Thus, a greater fraction of the drug would remain in the unionized state at physiological pH values. On the other hand, THC-Val-HS is a weak acid with a predicted pKa value of 4.7 ± 0.1. Although logP values did not change significantly for the prodrugs synthesized, logD (pH 7.4) values were lower. The predicted values are presented in Table 6-1. .

6.4.2. Solubility studies

Aqueous solubility of THC-Val and THC-Val-Val was below the detection limit of the analytical method used (<1 μg/mL). Some of the other amino acid THC prodrugs, THC-glutamine, THC-sarcosine and THC-serine, demonstrated significantly higher aqueous solubility but they had extremely short half-lives in aqueous solutions. On the other hand, aqueous solubility and stability of THC-Val-HS was found to be significantly higher in the physiological pH range, compared to THC and all the other prodrugs tested (Table 6-2).

In view of the higher stability of THC-Val, THC-Val-Val and THC-Val-HS solubility was tested further as a function of pH. THC-Val and THC-Val-Val solubility in the buffers was found to be below detection limits (<1 μg/mL) within the pH range studied. pH dependent solubility of THC-Val-HS was also studied at pH 3 (Not detected, < 1μg/mL), pH 5 (1.3 ± 0.1 μg/mL), pH 7 (76.8 ± 12.9 μg/mL) and pH 9 (141.8 ± 32.9 μg/mL).

Table 6-1: Predicted Physicochemical Properties of Amino Acid Prodrugs using ACD-I Lab 2.0.

Pro-Drug	ACD I-Lab Predicted Values					
	Molecular Weight	pKa	logP	logD (pH 7.4)	Solubility (μg/mL)	Polar Surface Area
THC	314.2	9.8 ± 0.6	6.8 ± 0.4	7.1	1.0	29.5
THC-Val	413.6	7.5 ± 0.3	6.7 ± 0.4	6.3	0.83	61.6
THC-Val-Val	512.7	8.2 ± 0.3 13.4 ± 0.5	7.4 ± 0.6	5.9	0.79	90.7
THC-Valine-HS	513.6	4.7 ± 0.1 14.3 ± 0.5	6.3 ± 0.5	3.9	0.96	101.9

6.4.3. Solubility in the presence of surfactants

Solubility of THC-Val, THC-Val-Val and THC-Val-HS in the presence of various solubilizing agents has been presented in Table 6-3. THC-Val-HS demonstrated significantly higher solubility compared to the other prodrugs tested. With cyclodextrins, THC-Val-HS demonstrated better solubility in RMβCD (4266.1 ± 188.8 µg/mL) compared to HPβCD (2973.5 ± 108.1 µg/mL). Solubility of THC-Val-HS was studied in the presence of 0.1% (2721.2 ± 198.9 µg/mL), 0.25% (3371 ± 603.3 µg/mL) and 0.5% Cremophor RH 40 (4755 ± 355.4 µg/mL). Use of 0.1% poloxamer 407 did not lead to a significant improvement in solubility (677.8 ± 51.7 µg/mL). THC-Val-HS solubility was also studied in the presence of 0.1% polysorbate 80 (3163.3 ± 248.02 µg/mL) and 0.1% tyloxapol (3358.7 ± 117.3).

Table 6-2: pH Dependent solubility of THC-Val-HS at pH 3, 5, 7 and 9. Results are depicted as mean ± SD (n=3).

pH	Solubility (µg/mL)
Water	37.6 ± 6.6
IPBS	97.3 ± 1.7
3	ND*
5	1.3 ± 0.1
7	76.8 ± 12.9
9	141.8 ± 32.9

6.4.4. Stability in the presence of Surfactants and Cyclodextrins

The hydrolysis of THC-Val-HS followed first order degradation. Apparent first order hydrolysis rate constants and half-life of THC-HG in the presence of surfactants is presented in Table 6-4. No degradation was observed in the presence of HPβCD at 4 °C and 25 °C.

6.4.5. Corneal Permeability Studies

In vitro permeability studies for THC-Val and THC-Val-Val were carried out in the presence of RMβCD 2.5%, Cremophor RH 40 0.5%, Polysorbate 80 0.1% and Tyloxapol 0.1%. Permeability of THC-Val in the presence of 2.5% RMβCD was found to be 4.75 x 10^{-6} cm/s. No drug was detected in the receiver chamber from the other THC-Val formulations. THC-Val-Val was not detected in the receiver chamber with any of the formulations studied.

Since THC-Val-HS demonstrated significantly higher solubility, lower concentrations of the solubilizers were used in the *in vitro* studies. Permeability of THC-Val-HS (Fig. 6-2) was found to be highest in 1.5% HPβCD (14.3 x 10^{-6} cm/s). Transcorneal permeability was significantly lower in 1.5% RMβCD (6.5 x 10^{-6} cm/s) and in the presence of surfactants. However, no significant difference in the permeability of THC-Val-HS was found between 0.05% polysorbate 80 (4.06 x 10^{-6} cm/s), 0.05% tyloxapol (3.1 x 10^{-6} cm/s) and 0.05% Cremophor RH 40 (3.16 x 10-6 cm/s).

BAK (0.02%) and EDTA (0.1%) are commonly used preservatives/preservative aids in ophthalmic formulations. Thus, permeability of 0.05% Cremophor RH 40 with 0.02% BAK and 0.1% EDTA was also studied.

Table 6-3: Solubility of THC-Val, THC-Val-Val and THC-Val-HS in different surfactant solutions at 25 °C. Results are depicted as mean ± SD (n=3).

Excipient	Concentration % w/v	Solubility (µg/mL)		
		THC-Val	THC-Val-Val	THC-Val-HS
Plain IPBS	-	< 1 µg/mL	< 1 µg/mL	96.3 ± 1.1
HPβCD	2.5	32.2 ± 2.5	32.3 ± 1.1	2973.5 ± 108.1
RMβCD	2.5	191.7 ± 1.1	179.1 ± 1.9	4266.1 ± 188.8
Cremophor RH 40	0.1	181.6 ± 11.5	178.1 ± 7.5	2721.2 ± 198.9
	0.25	443.6 ± 7.9	393.2 ± 10.1	3371 ± 603.3
	0.5	676.5 ± 38.8	632.1 ± 32.2	4755 ± 355.4
Poloxamer 188	0.1	0.85 ± 0.1	1.4 ± 0.1	-
Poloxamer 407	0.1	26.4 ± 5.7	4.9 ± 1.1	677.8 ± 51.7
Polysorbate 80	0.1	105.4 ± 17.9	179.4 ± 4.1	3163.3 ± 248.02
Tyloxapol	0.1	67.4 ± 3.3	130.4 ± 4.5	3358.7 ± 177.3

Table 6-4: Apparent first order rate constants ($k^* \times 10^5$, h^{-1}) and half lives ($t_{1/2}$, days) of THC-Val-HS in hydroxylpropyl beta cyclodextrin (HPβCD) and Cremophor RH 40 in IPBS at 4 °C, 25 °C and 40 °C. Results are depicted as mean ± SD (n=3).

Formulation	Concentration (%w/v)		4 °C	25 °C	40 °C
HPβCD	2.5	k	ND*	ND*	196.4 ± 3.6
		$t_{1/2}$			14.7 ± 0.3
	5	k	ND*	ND*	58.4 ± 3.4
		$t_{1/2}$			49.6 ± 2.9
Cremophor RH 40	0.1	k	49.6 ± 0.2	45.8 ± 9.2	51.3 ± 11.5
		$t_{1/2}$	58.3 ± 0.2	65.7 ± 13.2	59.1 ± 12.8

ND* - No degradation observed for 2 weeks

6.4.6. *In Vivo* Bioavailability Studies

Based on the *in vitro* data, a cyclodextrin and a surfactant formulation were selected for the *in vivo* bioavailability studies. HPMC (0.5%) was added to the selected formulations to increase precorneal contact time. *In vivo* bioavailability studies were carried out for a period of one hour post topical instillation. The better formulation was then selected for a three hour time period study. Ocular penetration of THC-Val-HS from a polymeric film formulation was also studied.

THC-Val-HS when formulated in 2.5% HPβCD and 0.5% HPMC led to significant increase in the amount of THC reaching the aqueous humor (69.4 ± 16.7 ng/100 μL) and iris ciliary body (65.8 ± 15.9 ng/50 mg). When the HPβCD in the formulation was increased to 5% at double the prodrug concentration, a significant decrease in THC concentrations was observed in the aqueous humor (31.3 ± 13.4 ng/100 μL) but not in the iris ciliary body (50.2 ± 9.9 μg/mL). However, THC concentrations were observed to be below detection levels in the vitreous humor and the retina choroid in both cases.

In vitro permeability data suggested a five-fold lower permeability of THC-Val-HS in the presence of Cremophor RH 40 formulation compared to HPβCD formulation. However, similar *in vivo* tissue THC concentrations were obtained in the aqueous humor (62.1 ± 12.6 ng/100 μL) and the iris ciliary body (51.4 ± 19.5 μg/50 mg). In this case also THC was not detected in the back of the eye tissues.

Since stability of THC-Val-HS in the cyclodextrin formulation was markedly better compared to the cremophor formulation (Table 6-4) a three hour time period study, post instillation, was carried out using the cyclodextrin formulation. THC concentrations at 3 hours

decreased significantly in the aqueous humor (38.3 ± 10.2 ng/100 µL) but were maintained in the iris ciliary body (57.9 ± 16.1 ng/50 mg).

In contrast to the earlier studies, the polymeric film was able to deliver THC to the aqueous humor (61.3 ± 32.1 ng/100 µL), iris ciliary body (86.03 ± 38.2 ng/50 mg) and retina choroid (355.5 ± 155.2 ng/50 mg). However, THC levels were below detection limits in the vitreous humor. At the end of 3 hours, THC concentrations decreased significantly in the aqueous humor (29.1 ± 14.2 nd/100µL) and retina choroid (11.9 ± 4.9 ng/50 mg) but remained constant in the iris ciliary body (104.2 ± 41.2) (Table 6-5).

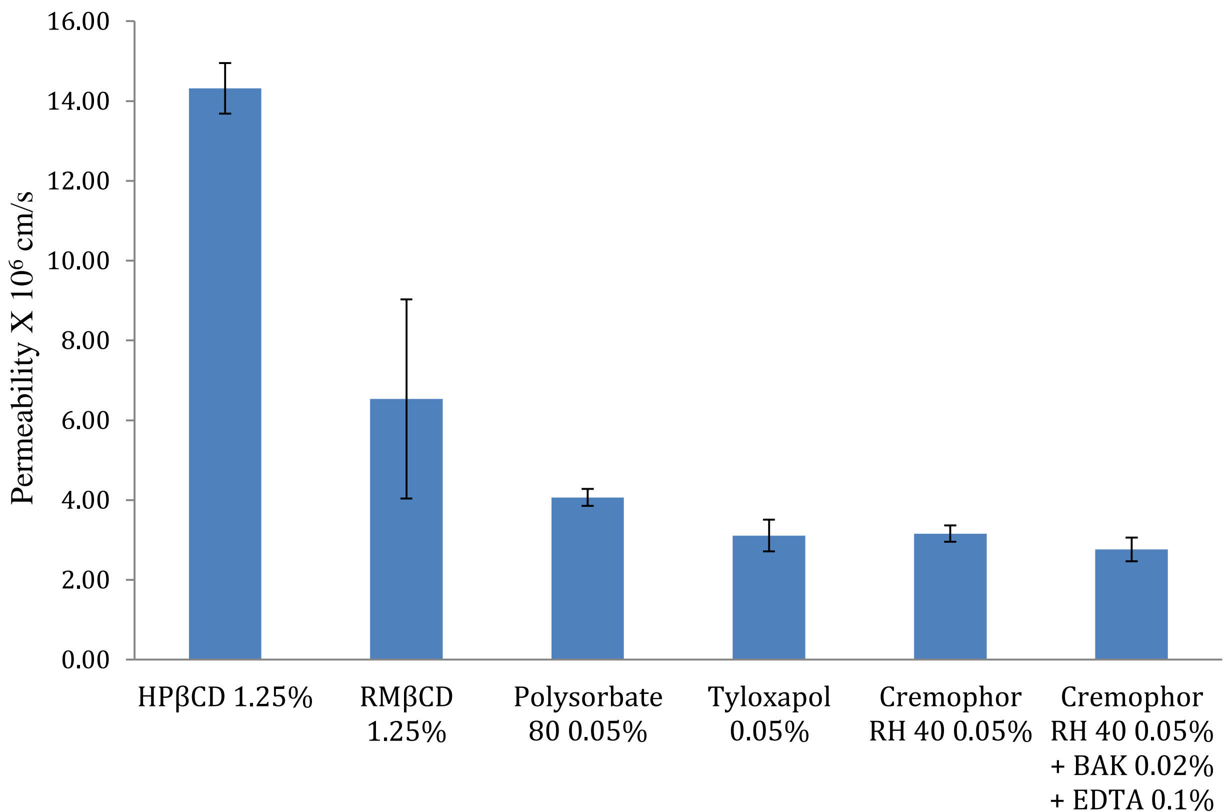

Figure 6-2: Permeability of THC-Val-HS from various surfactant solutions across isolated rabbit cornea at 34 °C. Receiver solution consisted of IPBS containing 2.5% HPβCD (pH 7.4). Results are depicted as mean ± SD (n=3-4). *p<0.05.

Table 6-5: Total THC concentrations observed in rabbit ocular tissues post topical administration of 50 µL of THC-Val-HS in 2.5% HPβCD + 0.5% HPMC (0.1% w/v), 5% RMβCD (0.4% w/v), 0.1% Cremophor RH 40 + 0.1% EDTA + 0.5% HPMC or formulated in an hot melt extruded film. Results are depicted as mean ± SD (n=3).

Tissue	2.5 % HPβCD + 0.5% HPMC		5% HPβCD + 0.5% HPMC	0.1% Cremophor RH 40+ 0.02% BAK + 0.1% EDTA + 0.5% HPMC	Ocular Film	
	1 Hour	3 Hours	1 Hour	1 Hour	1 Hour	3 Hours
Drug Concentration (% w/v)	0.26		0.5	0.25	0.78	
Cornea (ng/50 mg Tissue)	1677.1 ± 172.1	1142.3 ± 415.9	443.5 ± 152.2	1191. 7 ± 231.1	1634.5 ± 756.5	1043.4 ± 614.4
Aqueous Humor (ng/100 µL)	69.4 ± 16.7	38.3 ± 10.2	31.3 ± 13.5	62.1 ± 12.6	61.3 ± 32.1	29.1 ± 14.2
Iris-Cilliary Body (ng/50 mg Tissue)	65.8 ± 15.9	57.9 ± 16.1	50.2 ± 9.9	51.44 ± 19.5	86.03 ± 38.2	104.2 ± 41.2
Vitreous Humor (ng/mL)	ND*	ND*	ND*	ND*	ND*	ND*
Retina-Choroid (ng/50 mg Tissue)	ND*	ND*	ND*	ND*	355.5 ± 155.2	11.9 ± 4.9
Sclera (ng/250 mg Tissue)	882.2 ± 185.8	241.8 ± 106.6	191.5 ± 50.1	913.4 ± 432.9	378.2 ± 154.3	162.5 ± 100

ND*- Not Detected

6.5. DISCUSSION

Glaucoma is a neurodegenerative disease characterized by a progressive and irreversible loss of retinal ganglion cells (RGC) which leads to vision loss(140). Control of intraocular pressure, although a significant risk factor, is insufficient to completely halt the progression of the disease. Neuroprotection of retinal ganglion cells against apoptosis could lead to a new generation of antiglaucoma agents that could prevent/reduce vision loss. Δ^9-Tetrahydrocannabinol is one such molecule that could demonstrate neuroprotective activity independent of intraocular pressure lowering properties.

There are however significant barriers to the development of a topical neuroprotectant for the treatment of glaucoma. The posterior segment of the eye is well protected by a number of anatomical and physiological barriers(174, 175). Access to the retina and vitreous humor is restricted for topically applied drugs as well as drugs administered through the systemic circulation. A number of strategies have been used with limited success to target the back of the eye tissues(176). Current strategy for drug delivery to the retina is invasive intravitreal administration of a solution/suspension or polymeric insert in the vitreous humor(159, 177). Non-invasive delivery to the back of the eye tissues forms a significant barrier to the delivery of any potentially neuroprotective agent. In addition there are no clinical models/clinically monitored endpoints available to determine neuroprotective effect of a drug molecule for the treatment of glaucoma(178).

Following topical application, the physicochemical characteristics of the drug determine its permeability across the cornea. In the present study synthesis of THC prodrugs was carried out to improve hydrophilicity with an aim to increase intraocular delivery. Amongst all the

prodrugs tested, the solubility, stability and permeability values obtained with THC-Val-HS were markedly better than that observed with any other prodrug tested, including THC-HG which was evaluated in our earlier studies(148). Topically administered THC-Val-HS, as ocular eye drops, was able to deliver THC to the anterior segment tissues (aqueous humor and the iris ciliary body) but not to the retina and the vitreous humor. The results thus underline the difficulty in delivering drug to the back-of-the eye through the topical route. The significant vascular and lymphatic drainage in the sclera and choroid and the barrier characteristics of the RPE and Bruch's membrane severely restrict the passage of the drug through the sclera into the retina and vitreous humor. Previous reports demonstrate the sclera→choroid→retina→vitreous humor is the principal pathway for the diffusion of the drug from the topical application site to the back-of-the eye.

THC-Val-HS was incorporated in a polymeric film to improve precorneal retention time, provide intimate contact between the drug and the conjunctiva/sclera and to control the release of drug in the eye. The polymeric film allowed higher drug loading in the formulation allowing administration of higher doses. Unlike the other formulations tested, significantly high THC concentrations were observed in the retina with this dosage form. THC concentrations in the retina were observed even at the end of three hours post instillation. This formulation needs further investigation to optimize the delivery of the prodrug across the ocular barriers. The present study thus effectively designed an approach to deliver THC to both the anterior and the posterior chamber of the eye. Further studies to determine therapeutic outcomes need to be carried out in the future.

6.6. ACKNOWLEDGEMENTS

This publication was partially supported by grants (1R41EY020042 & 2R42GM067304-02) from the National Institutes of Health to ElSohly Laboratories, Incorporated. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CHAPTER 7

SUMMARY OF ALL THE STUDIES

Current glaucoma therapy is based on the presumption that neurodegeneration of the retina is directly linked to increased intraocular pressure. However visual field loss occurs even after sufficient reduction of intraocular pressure has been achieved. Δ^9-Tetrahydrocannabinol is a promising candidate that could achieve both intraocular pressure reduction and neuroprotection through independent mechanisms of actions. Even though early evidence of its efficacy was discovered four decades ago, its utility in glaucoma is unknown due to poor formulation and study designs.

Δ^9-Tetrahydrocannabinol is a highly lipophilic compound with very low aqueous solubility. A model for the high throughput screening of the corneal permeability of pro-drugs and formulations was developed. To improve physicochemical properties prodrugs were designed so as to add an ionizable group to the parent molecule. Two prodrugs, Δ^9-Tetrahydrocannabinol hemisuccinate (THC-HS) and Δ^9-Tetrahydrocannabinol hemiglutarate (THC-HG) were synthesized. Being weak acids these prodrugs displayed improved aqueous solubility at physiological pH values. Additionally both prodrugs demonstrated improved inherent permeability in the unionized state. At physiological pH values, however, their permeability dropped significantly.

Four formulation approaches were chosen to improve the solubility and permeability of the prodrugs at physiological pH. Since THC-HG was more stable compared to THC-HS it was selected for further studies. Use of cyclodextrins led to improved solubility but did not shield the charge on the prodrugs leading to poor permeability values at physiological pH. An ion-pair prodrug formulation helped to shield the charge and improved permeability. Inclusion of surfactants improved solubility, solution stability and permeability. Due to constraints on the concentration of surfactants that can be used for ophthalmic formulations, solubility could not be

improved beyond a certain extent. Second generation valine, valine-valine and valine-hemisuccinate ester prodrugs of THC were synthesized to further improve physicochemical parameters. Screening of THC-Val and THC-Val-Val did not demonstrate favorable solubility and permeability characteristics and thus were not studied any further. THC-Val-HS demonstrated significant improvement in solubility and permeability.

THC-HG (ion pair and micellar formulations) and THC-Val-HS (cyclodextrin and micellar) were instilled in an anesthetized rabbit model to determine *in vivo* bioavailability. THC formulated in mineral oil, micellar and emulsion formulation was used as controls. Intraocular tissues concentration of THC was found to be below detectable levels for mineral oil, micellar and emulsion formulations. THC-HG and THC-Val-HS were able to deliver significantly higher concentrations to the anterior chamber tissues (aqueous humor, and iris ciliary body) but no drug was detected in the vitreous humor and the retina. Incorporation of THC-Val-HS, on the other hand, in a polymeric film delivered THC to the aqueous humor, iris ciliary body and the retina.

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