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Effect of Ion-Pairing on *In Vitro* Transcorneal Permeability of a ⁹-Tetrahydrocannabinol Prodrug: Potential in Glaucoma Therapy

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Abstract

The aim of the present study was to evaluate and improve the *in vitro* transcorneal permeability characteristics of 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 (HP β CD) or random methylated beta cyclodextrin (RM β CD), 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 HP β CD. THC-HS and THC-HG exhibited pH dependent permeability. In the presence of HP β CD, 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. RM β CD, 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.

INTRODUCTION

In 1971 Hepler and Frank published a report that linked marijuana smoking to a significant drop in intraocular pressure (IOP)¹. Due to its implications in the treatment of glaucoma, this report stimulated intense research towards identification of the constituents responsible

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for this pharmacological action. It was established that Δ^9 -tetrahydrocannabinol (THC, Fig. 1A), a primary active constituent of marijuana, is one of the components responsible for the IOP lowering effects². 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^{3,4}. This lack of topical activity, although some reports did demonstrate that topical delivery of THC significantly lowered IOP⁵, 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 cloned⁶. 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 respectively⁷ while the EC₅₀ values for the CB1 and CB2 receptors are 6 nM and 0.4 nM, respectively⁸. 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 retina⁹⁻¹² while CB2 receptors have been found on the retina and trabecular meshwork¹³. 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 muscle¹⁴. Contraction of the ciliary muscle leads to widening of the intercellular spaces in the trabecular meshwork and enhances outflow of aqueous humor¹⁵. Recently, bimatoprost, a prostaglandin analog that enhances uveoscleral outflow, has been shown to contract the human ciliary muscle through CB1 mediated mechanism¹⁶. Furthermore, activation of CB1 receptors leads to fragmentation and reduction of actin stress fibers in the trabecular meshwork, further enhancing outflow of aqueous humor⁹. 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 pressure⁵. A 10 mm drop in systolic blood pressure, following systemic THC administration, should be associated with less than a 1 mm drop in IOP¹⁷. 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^{18,19}. 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 glaucoma²⁰. 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 apoptosis²⁰. 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 effect²¹. Intravitreally administered THC has been demonstrated to act as a neuroprotective in a rat model of glaucoma^{20,22}. Hampson *et al.* also reported that the neuroprotective activity of THC could also be independent of CB1 receptor activation²³. 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^{23–25}. 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 tested²⁶. 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 $\mu\text{g}/\text{mL}$ and a logP of 6.3²⁷, 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 1.86×10^{-8} cm/s²⁸. 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. 1B) and hemiglutarate ester (THC-HG, Fig. 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.

MATERIALS

Chemicals

Hydroxypropyl beta cyclodextrin (HP β CD), randomly methylated beta cyclodextrin (RM β CD), 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.

Animal Tissues

Recently we have evaluated the effect of storage on corneas obtained from ocular globes preserved in Hanks balanced salt solution and found the corneas to be equivalent to freshly excised corneas²⁹. Both active and passive transport processes in the preserved corneas are intact for 24 hours. 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.

METHODS

Preparation of Dicarboxylic acid ester prodrugs

Dicarboxylic acid prodrugs (THC-HS and THC-HG) were synthesized and characterized according to previously published procedures³⁰.

Solubility of the Prodrugs

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 used³¹. 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 × g in a Fisher Scientific acuSpin micro17R centrifuge for 10 min using silicon coated centrifuge tubes. The supernatant was collected and analyzed by HPLC.

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.

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.

***In Vitro* Transcorneal Permeability Studies**

Excess THC, THC-HS, THC-HG and WIN were equilibrated in DPBS containing 2.5% HP β CD or RM β CD (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% HP β CD 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% HP β CD solution in IPBS with pH adjusted to 7.4.

Eyes were used immediately upon receipt. Corneas were excised, following previously published protocols³². 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 μ L, 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.

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.

Stability in Ocular Tissue Homogenates

Tissue Preparation—Aqueous and vitreous humor was used as such and without any dilution. They were centrifuged at 16000 \times g for 10 min at 4 °C 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 \times g at 4 °C for 15 min. Protein content in the supernatant was determined according to the method of Bradford³² and was standardized to 1 mg/mL.

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 × g for 10 min. The supernatant was collected and taken for analysis.

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 × 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.

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).

$$\text{Flux}(J)=dD_{cum}/dt \quad (1)$$

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

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.

RESULTS

Solubility

Solubility in Buffers—Solubility of the prodrugs, THC-HS and THC-HG, in IPBS or DPBS (Table I) was found to be markedly higher than the reported solubility of THC (1–2 µg/mL) in water²⁷. 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).

Solubility in Cyclodextrins—Solubility of the prodrugs (THC-HS and THC-HG) was significantly improved in the presence of cyclodextrins (Table I). 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 DPBS 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 I).

Solubility in Presence of l-arginine/tromethamine—Since THC-HS and THC-HG demonstrated higher aqueous solubility in IPBS compared to DPBS (Table I), 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 II). 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.

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. 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 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 HP β CD with RM β CD (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% HP β CD in DPBS (Fig. 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% HP β CD (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). 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% HP β CD 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% HP β CD. This was about 3-fold higher compared to the corneal permeability of WIN at pH 7.4. IPBS containing 2.5% HP β CD 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 III.

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.

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.

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 IV. 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.

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 1.86×10^{-8} cm/s²⁸. 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.

Cyclodextrins have been widely used as complex forming agents to improve the solubility and permeability of many hydrophobic drug candidates³³. Since their discovery, cyclodextrins have been used in numerous marketed products. Of these, HP β CD when used up to 12.5% w/v has been shown to be safe and well tolerated when administered topically to the eye³⁴. It has also been demonstrated that HP β CD only transports the drug molecule to the surface of the membrane and does not itself permeate through the membrane in any significant quantities³⁴. RM β CD has also been used by researchers in ophthalmic drug delivery³⁵. When used at high concentrations both HP β CD and RM β CD have been shown to affect tight junction integrity^{36,37}. However, *in vitro* corneal permeability studies demonstrate that use of HP β CD up to concentrations of 5 % w/v does not affect the tight junctions³⁷. Concentration dependent effect of RM β CD 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% HP β CD 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% HP β CD as the vehicle, transcorneal permeability of THC was observed to be only 3.3×10^{-8} cm/s²⁸. Thus, permeability of THC from the 2.5% HP β CD drug saturated solution in this study was about 200 times higher compared to the permeability value reported by Green *et al.*²⁸. 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. 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. 2). Thus, the presence of excess cyclodextrin probably leads to a decrease in the free drug concentration available for permeation. Interestingly, the use of RM β CD 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 I). 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 I). However, aqueous solubility of THC-HS in HP β CD or RM β CD in DPBS was found to be higher than that in IPBS. The increased solubility in DPBS 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 hydrolysis³⁰.

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 HP β CD. 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^{38,39}. 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 counterions were discontinued. The maximum aqueous solubility values reported for THC-HG are 539.56 $\mu\text{g/mL}$ at pH 8 and 411.3 $\mu\text{g/mL}$ at pH 9³⁰. Improved aqueous solubility of THC-HG with L-arginine (1.7 mg/mL at pH 8.9, Table II) and tromethamine (1.2 mg/mL, pH 7.8, Table II) 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% HP β CD 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 epithelium⁴⁰⁻⁴². L-Arginine utilizes the B^{0,+} system to permeate through 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 preferred³². 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 IV).

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 HP β CD / RM β CD will be characterized in the near future, in a manner similar to our previous studies with THC-HS⁴³.

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Abbreviations

BCH	2-aminobicyclo-[2,2,1]-heptane-2-carboxylic-acid
CB1	Cannabinoid Receptor Type 1
CB2	Cannabinoid Receptor Type 2
DPBS	Dulbecco's Phosphate Buffered Saline
HPβCD	Hydroxypropyl beta cyclodextrin
IOP	Intraocular Pressure
IPBS	Isotonic Phosphate Buffered Saline
RMβCD	Randomly methylated beta cyclodextrin
THC	⁹ - Tetrahydrocannabinol
THC-HS	⁹ - Tetrahydrocannabinol hemisuccinate
THC-HG	⁹ -Tetrahydrocannabinol hemiglutarate
THC-HG-ARG	Ion-pair of ⁹ - Tetrahydrocannabinol hemiglutarate and l-arginine

THC-HG-TRIS Ion-pair of ⁹- Tetrahydrocannabinol hemiglutarate and tromethamine
WIN (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

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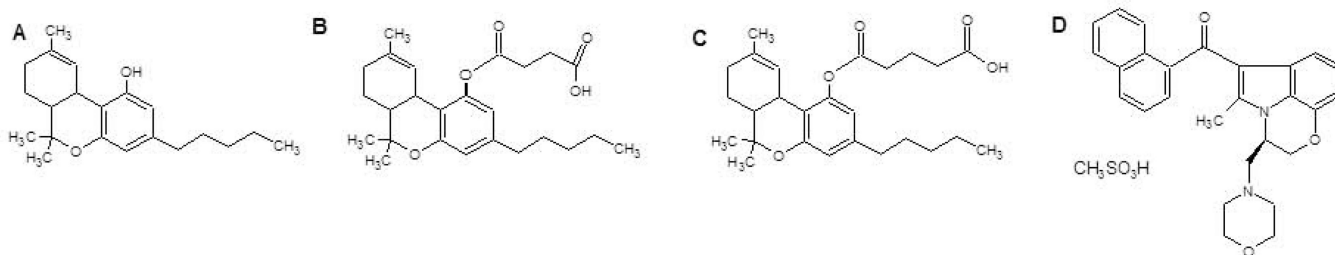


Figure 1.
Chemical structures of A) Δ^9 -Tetrahydrocannabinol (THC), B) Δ^9 -Tetrahydrocannabinol Hemisuccinate (THC-HS) and C) Δ^9 -Tetrahydrocannabinol Hemiglutarate (THC-HG) D) WIN 55-212-2 (WIN).

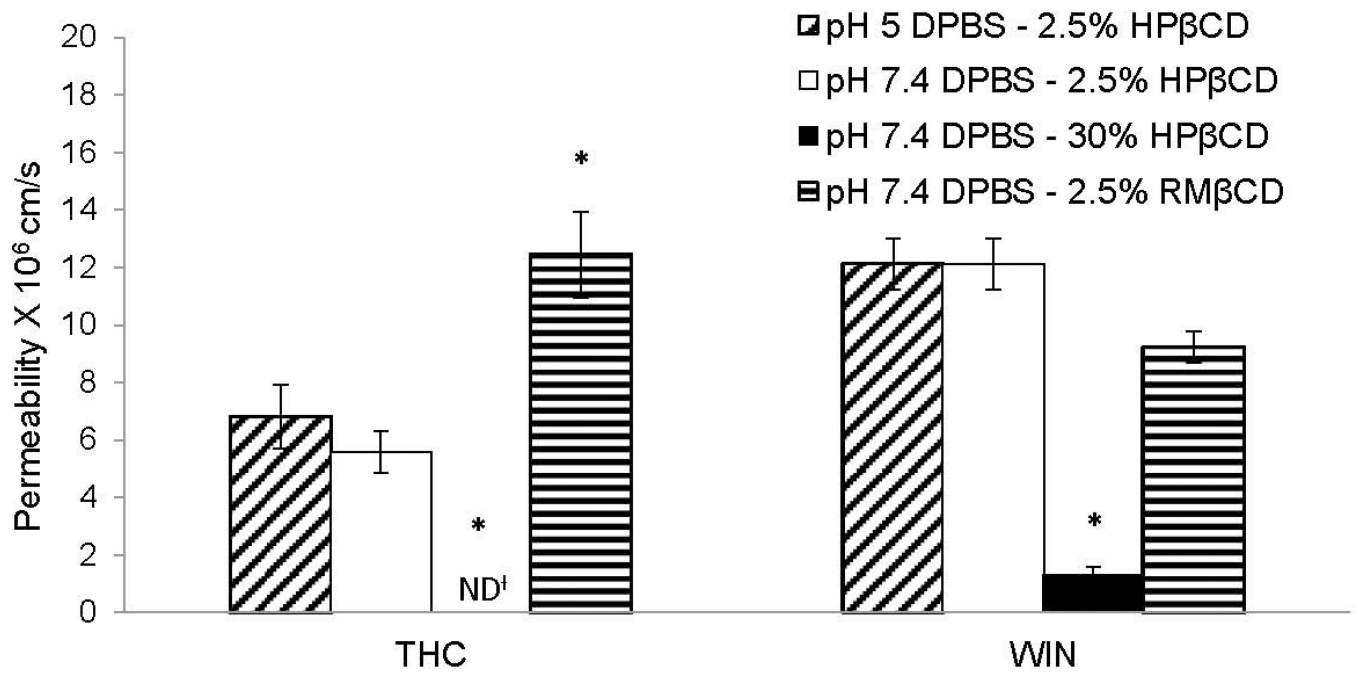


Figure 2.

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.

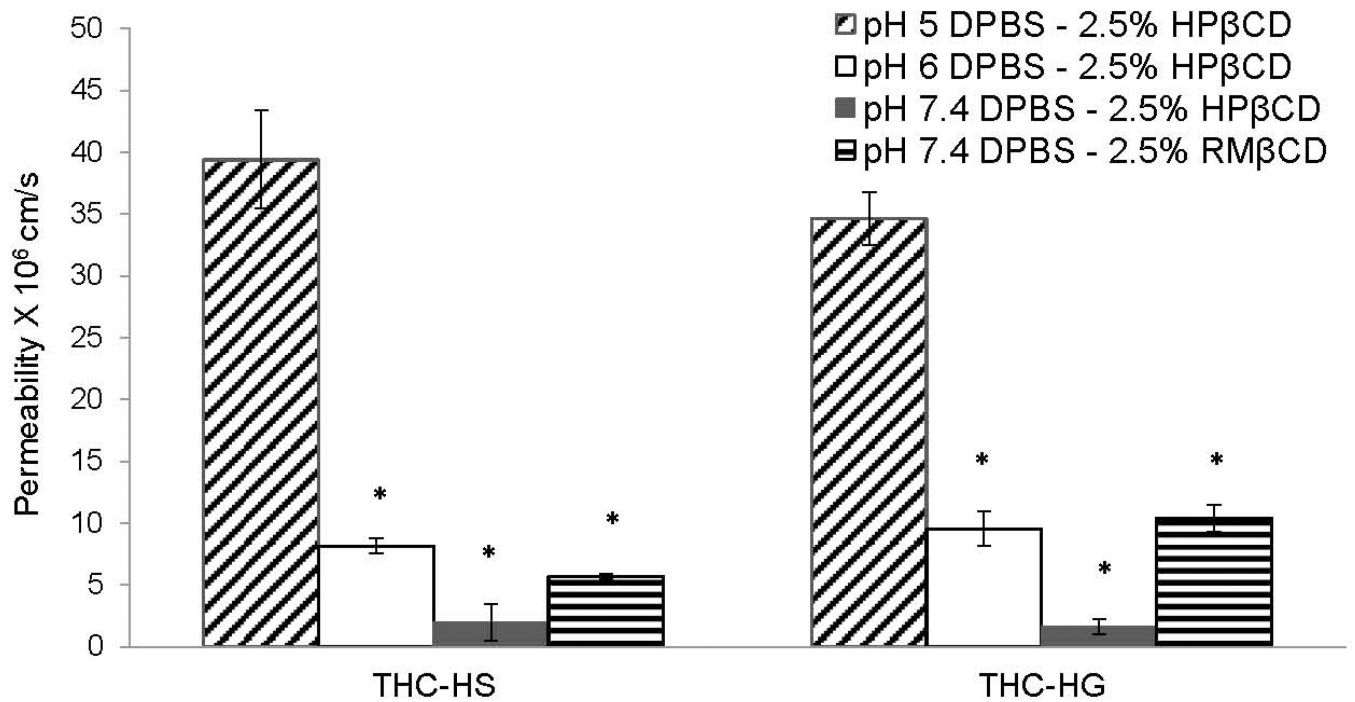


Figure 3. Permeability of THC-HS and THC-HG (in terms of total THC) at 34 °C across isolated cornea. The legends indicate the donor solution pH and composition. Receiver solution used in these studies was DPBS containing 2.5% HPβCD (pH 7.4). Results are depicted as a mean \pm SD (n=3). *p < 0.05.

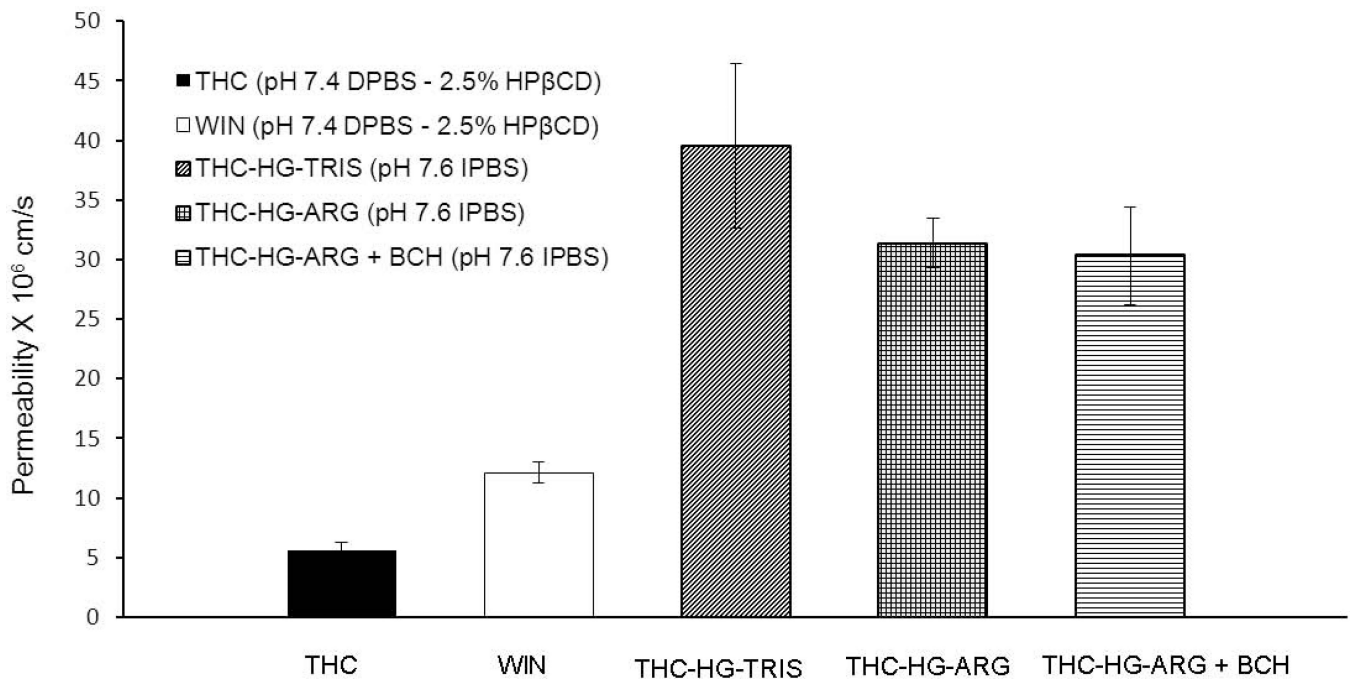


Figure 4.

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 % HP β CD in DPBS (pH 7.4) for THC and WIN, while for the ion-pair complexes the receiver solution was IPBS containing 2.5% HP β CD (pH 7.4). Results are depicted as a mean \pm SD (n=3). *p < 0.05.

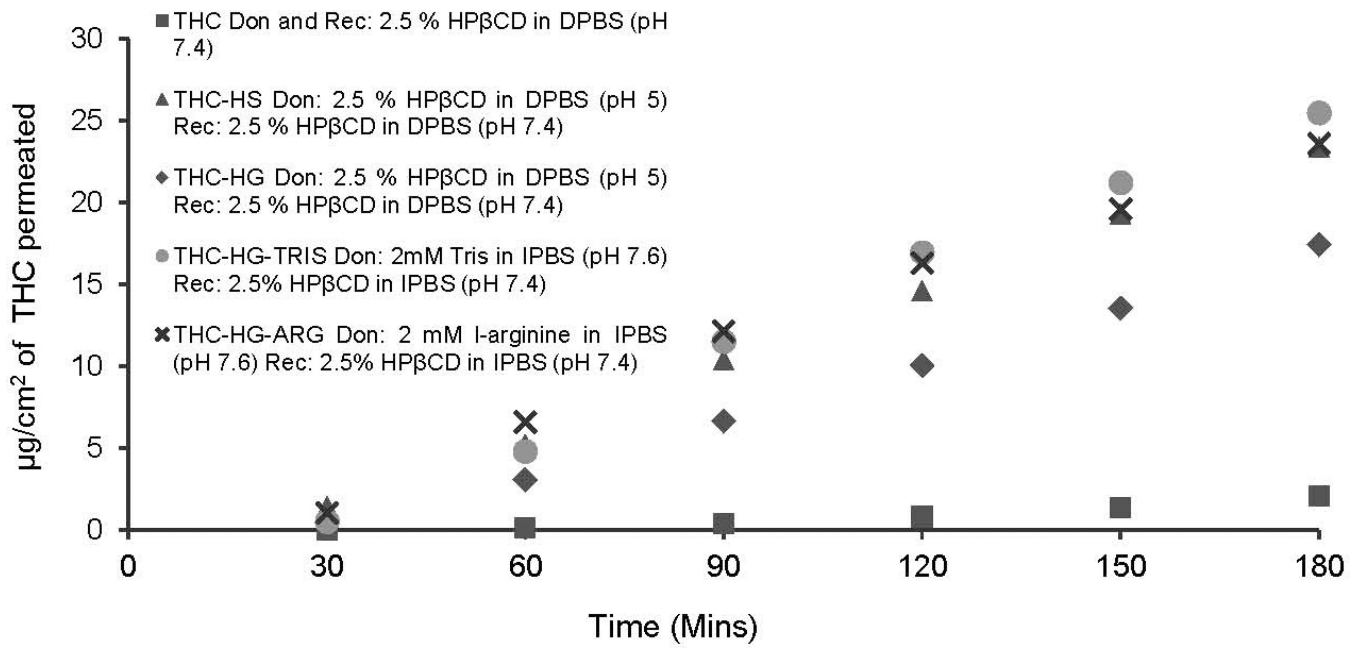


Figure 5.

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 \pm SD (n=3). *p < 0.05.

Table I

Solubility of THC-HS and THC-HG in IPBS and DPBS as such or IPBS and DPBS containing 2.5% HP β CD or RM β CD at 25 °C. Results are depicted as mean \pm SD (n=3).

Prodrug	Buffer	Cyclodextrin (2.5%)	Solubility (μ g/mL)
THC-HS	IPBS	-	9.8 \pm 0.9
		HP β CD	144.9 \pm 23.4
		RM β CD	197.5 \pm 57.9
	DPBS	-	5.4 \pm 0.3
		HP β CD	418.9 \pm 13.8
		RM β CD	430.2 \pm 75.2
THC-HG	IPBS	-	18.8 \pm 3.1
		HP β CD	899.9 \pm 27.5
		RM β CD	857.7 \pm 49.4
	DPBS	-	4.3 \pm 0.2
		HP β CD	678.5 \pm 84.2
		RM β CD	910.6 \pm 62.8

Table II

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 °C. Results are depicted as mean \pm SD (n=3).

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

Table III

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 \pm SD (n=3).

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

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

Table IV

Apparent first order rate constants (k^*) and half-lives ($t_{1/2}$) of THC-HG in ocular tissue homogenates. Results are depicted as mean \pm SD (n=3).

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