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Application of cannabidol to ocular pharmacokinetics and pharmacodynamics.

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APPLICATION OF CANNABIDIOL TO OCULAR PHARMACOKINETICS AND PHARMACODYNAMICS

By

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B.S., University of Louisville, 2014
M.S., University of Louisville, 2018

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A Dissertation approved on

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DEDICATION

This dissertation is dedicated to my family. They have all contributed meaningfully to my success, none of them have doubted me, their confidence in me gives me confidence, and this dissertation is for all of them.

My husband, Tommy Aebersold, who has provided unending support, time, patience, and love. This would not have been possible without him.

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ABSTRACT

APPLICATION OF CANNABIDIOL TO OCULAR PHARMACOKINETICS AND PHARMACODYNAMICS

Alyssa Aebersold

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Cannabidiol (CBD) is the most abundant non-psychotropic cannabinoid constituent of *Cannabis sativa*. CBD is potentially therapeutic for the eye through antioxidant, anti-inflammatory, and neuroprotective effects. However, the effect of CBD on intraocular pressure (IOP)- the major risk factor for glaucoma- is controversial and large variability exists in the literature. IOP is regulated through aqueous humor (AH) dynamics in trabecular meshwork (TM) tissues. Measurement of CBD in AH and correlation with effects on IOP would clarify what concentrations mediate changes to IOP. Therefore, the overall goal of this dissertation is to measure CBD in ocular tissues and correlate concentrations with ocular pharmacologic effects.

Effects of CBD on AH outflow were measured in porcine anterior segment explants. Effects of CBD on TM contractility and Rho/ROCK signaling were assessed *in vitro*. Porcine AH CBD concentrations were measured following

ocular topical application *ex vivo*. A cyclodextrin solution, cyclodextrin solution containing gellan gum (GG) which forms a hydrogel *in situ*, and a semifluorinated alkane (SFA) were used to apply CBD topically. Murine AH and serum were measured by LC-MS/MS, and porcine AH and cornea concentrations were measured by HPLC. IOP was assessed following intraperitoneal and topical administration *in vivo*. Corneal analgesia of topical CBD was assessed using an eye wiping test.

CBD increased AH outflow *ex vivo*, decreased TM cell contractility and inhibited TM Rho/ROCK signaling *in vitro*. Intraperitoneal administration of CBD reached an AH C_{max} of 71.55 ng/mL and decreased IOP lasting up to 4 hours.

AH concentration of CBD time dependently increased following topical application *ex vivo* when applied in cyclodextrin vehicles but was localized to the cornea when applied in SFA. Topical CBD lowered IOP when applied in cyclodextrin containing vehicles and reduced corneal pain when applied in an SFA vehicle. Addition of GG increased AH concentrations (C_{max} 1864 ng/mL) and extended IOP reduction (from 5 to 8 hours) relative to cyclodextrin-only formulations.

We have determined that low micromolar CBD concentrations alter AH outflow and IOP, and vehicle chosen can localize CBD delivery. The results highlight the importance of not only vehicle but also tissue concentrations for CBD mediated pharmacologic effects.

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LITERATURE REVIEW AND INTRODUCTION

1. Background[1]

1.1 A Brief History of Cannabis

Cannabis sativa is a plant species that includes both cannabis and hemp. It first appeared in Chinese medical texts around 2000 years ago [1]. Records from Britain indicate that cannabis was brought from Egypt by Napoleon's troops in the early 1800s [2]. Shortly thereafter, hemp was introduced to Western medicine when in 1840, a hemp tincture from ground plant matter was reported to be an effective treatment for convulsive disorders and tetanus [3]. By 1851, a cannabis extract was included in the 3rd edition of the Unites States Pharmacopoeia and readily available in American pharmacies [4,5].

In 1913, however, cannabis was made illegal in California due to a wide spread antinarcotics campaign [5]. Cannabis became federally illegal when Harry Anslinger from California introduced the Marijuana Tax Act of 1937 banning the sale and use of cannabis nationally [5,6]. A negative stigma continued to develop in the US around cannabis, then associated with narcotics, which culminated with

[1]This chapter has been published previously as part of A. Aebersold, M. Duff, L. Sloan, Z.H. Song, Cannabidiol Signaling in the Eye and Its Potential as an Ocular Therapeutic Agent, Cell Physiol Biochem, 55 (2021) 1-14, doi:10.33594/000000371.

the Controlled Substances Act (CSA) of 1970, which classified cannabis and cannabinoids as Schedule I with no recognized medical use [7].

Recently, America is witnessing a revival in the popularity of cannabis, both medically and recreationally. In 1996, California was the first state to legalize cannabis for medical use and more states have followed California in recent years [8]. To date, 16 states and Washington D.C. have legalized both medical and recreational cannabis with an additional 26 states legalizing medical cannabis at varying degrees. Moreover, the Agricultural Acts of 2014 and 2018 removed hemp from the list of controlled substances and redefined industrial hemp as cannabis containing less than 0.3% THC [9,10]. As a result of the recent wave of recreational and medical cannabis legalization, in conjunction with the end to the prohibition of hemp, cannabis research is quickly expanding.

1.2 Cannabidiol

Cannabidiol (CBD) is one of over 120 chemicals produced by the *Cannabis sativa* plant termed phytocannabinoids [11,12]. There are potentially more, as 21 previously unknown cannabinoids were recently identified [13]. The two most abundant phytocannabinoids in cannabis are psychoactive and intoxicating D9-tetrahydrocannabinol (THC) and nonintoxicating CBD.
CBD was first isolated in the 1940 and its structure and stereochemistry fully determined in 1963 [14,15]. CBD and THC are both derived from cannabigerolic acid [16]. Although the structure of CBD was discovered before THC [15,17], THC had been the major focus of research related to cannabis and cannabinoids. This focus is driven, in part, by the activity of THC at the canonical

cannabinoid receptors, CB1 and CB2. However, there are many targets for cannabinoids other than CB1 and CB2. For example, CBD has upwards of 65 known targets consisting of receptors, enzymes, ion channels and transient receptor potential (TRP) channels [18].

1.3 Cannabinoids in Pharmaceuticals

Cannabinoid containing drugs are approved for medical use in the USA and other countries. The drugs differ in their formulation and indicated uses. Dronabinol (Marinol) was the first cannabinoid-containing medicine approved by the FDA in 1985. It is a soft gel capsule containing synthetic THC [19]. Syndros is an oral solution of dronabinol [20]. Cesamet (nabilone) is the third synthetic cannabinoid drug approved by the FDA in May of 2006 [21]. All three are prescribed for anorexia associated with weight loss in AIDS patients and nausea/vomiting in cancer patients [19-22]. While plant-derived THC is a Schedule I substance, Marinol is listed under Schedule III and Cesamet and Syndros are controlled under Schedule II [19-21]. Epidiolex is an oil formulation of CBD approved by the FDA in June of 2018 for treatment of Lennox-Gastaut syndrome and Dravet syndrome, two rare and severe forms of pediatric epilepsy [23]. In July of 2020, it was approved for treating seizures in a rare genetic disease, tuberous sclerosis complex (TSC) [24]. Epidiolex is the only FDA approved drug containing a compound directly derived from cannabis. It was originally classified as schedule V, but is no longer a controlled substance as the FDA deemed it safe and effective for treatment of the aforementioned conditions [25]. Sativex is a 1:1 alcohol solution of THC and CBD administered as an

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oromucosal spray that is approved in 25 countries for the treatment of pain and spasticity in multiple sclerosis patients [26]. Despite its approval in other countries, Sativex is not yet approved by the FDA in the US.

1.4 Research on Cannabidiol

CBD, through a variety of mechanisms and targets, has numerous potential therapeutic uses for a plethora of conditions. The assertion of potential therapeutic actions of CBD is based on pre-clinical data, limited clinical data and ongoing human clinical trials. Preclinical studies show that CBD has antioxidant [27,28], anti-inflammatory [27], anti-convulsant [29,30], neuroprotective [31], and anti-cancer properties [32]. CBD also shows potential as a therapeutic agent in cardiovascular [33], neurological, and neuropsychiatric disorders [26]. The completed clinical trials involve CBD use in epilepsy and seizures disorders (21 trials), general pain and pain associated disorders (19 trials), drug abuse and use disorders (14 trials), other neurologic conditions (4 trials) and psychiatric conditions (11 trials). In addition, there are currently 85 active clinical trials in the United States containing CBD (including Epidiolex and Sativex) on clinicaltrials.gov.

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2. Cannabidiol Signaling in the Eye and its Potential as an Ocular Therapeutic Agent

Over the past two decades, multiple studies have investigated the therapeutic potentials of CBD in the eye. There are several published reviews of cannabinoids for treatment of glaucoma [34,35], and retinal disorders [36,37]. Nevertheless, there are currently no reviews that focus solely on CBD for ocular conditions. In this review, we aim to fill the gap in literature with a focus on CBD ocular pharmacology. We will discuss therapeutic potentials of CBD for ocular conditions, ocular molecular targets for CBD, and mechanisms of actions of CBD in the eye.

2.1 Therapeutic Potentials of Cannabidiol for Ocular Conditions

CBD is recognized for its antioxidant, anti-inflammatory and neuroprotective properties. In this section, we discuss the observed effects of CBD in ocular tissues and its indication for ocular disorders. Specifically, we will discuss studies of CBD in corneal inflammation and pain, endotoxin-induced inflammation, excitotoxicity, diabetic retinopathy, and intraocular pressure (Table 1 and Table 2).

2.1.1 Corneal Inflammation and Pain

The cornea is a thin and avascular tissue that is innervated by sensory nerves. When corneal damage occurs due to infection, surgery, or trauma, it can develop into corneal neuropathic pain characterized by hyperalgesia, chronic and

debilitating pain, and inflammation [38,39]. The inflammatory response to corneal damage leads to the production of proinflammatory cytokines, recruitment of leukocytes, release of pain producing neuropeptides, and neovascularization (NV) in the cornea [38,39].

In a recent study, CBD was found to be anti-nociceptive and anti-inflammatory in a mouse model of corneal hyperalgesia [39] (Table 1). Mice with silver nitrate cauterized corneas that treated with CBD showed lower pain scores in capsaicin pain challenges, indicating an antinociceptive effect of CBD. Moreover, CBD treated corneas showed less corneal neutrophil infiltration which is indicative of a CBD-induced anti-inflammatory effect. Lastly, WAY100635, a 5HT1A antagonist, blocked the effects of CBD, suggesting that the anti-inflammatory and anti-nociceptive effects are likely mediated through activation of the serotonin 5HT1A receptor [39]. This study highlights CBD as a potential therapeutic for corneal pain and inflammation.

2.1.2 Endotoxin-Induced Inflammation

The mammalian retina contains three distinct glia cells types: Müller cells, astrocytes, and microglia. Microglial cells are the resident macrophages of the retina and play important roles in retinal homeostasis [40]. Activation of microglial cells induces the release of proinflammatory cytokines, such as IL-1β and TNFα, instigating an inflammatory response. Prolonged microglial activation and chronic inflammation contribute to disease pathology and retinal degeneration [40].

The degree of microglial activation may relate to the severity of injury. *In vitro* and *in vivo* treatment with lipopolysaccharide (LPS), an endotoxin from bacteria, is used to study inflammation through activated microglia [41]. Extracellular adenosine can function as an endogenous anti-inflammatory agent suppressing immune cell responses. For example, adenosine inhibits pro-inflammatory cytokine expression such as TNFα [42]. However, the anti-inflammatory effects of adenosine are short, as it is rapidly taken up by adjacent cells. Inhibitors of adenosine uptake may enhance the adenosine signaling and endogenous activity [43]. CBD has been shown to decrease TNFα expression and inhibit equilibrative nucleoside transporter 1 (ENT1) reuptake of adenosine in LPS treated primary microglia cells and retinas from LPS treated rats [44] (Table 1). The effect of CBD is primarily mediated through the activation of the A2A receptor, the most abundant adenosine receptor in the rat retina, as a result of CBD-induced inhibition of adenosine reuptake [44]. These results suggest that CBD may be a good anti-inflammatory agent for endotoxin-induced retinal damage.

Table 1. Therapeutic Potentials of CBD for Ocular Conditions

Ref.	Disease	Model	CBD route	CBD effect	Therapeutic relevance
[39]	Corneal pain and inflammation	Silver nitrate cauterization-induced cornlea hyperalgesia in mice	topical	↓corneal hyperalgesia ↓neutrophil infiltration	CBD is anti-nociceptive and anti-inflammatory following corneal surface injury
[44]	Retinal inflammation	Endotoxin-induced inflammation in rat retina and primary retinal microglial cells	intraperitoneal	↓adenosine reuptake ↓TNFα production	CBD is anti-inflammatory in the retina via inhibiting adenosine reuptake
[51]	Retinal Neurotoxicity	Intravitreal injection of NMDA in rats	intravenous	↓nitrotyrosine formation ↓nitrite/nitrate ↓lipid peroxidation ↓apoptosis	CBD is neuroprotective against retinal excitotoxicity
[58]	Diabetic retinopathy	Streptozotocin-induced diabetic rats	intraperitoneal	↓TNFα, ICAM-1, and VEGF expression ↓p38 MAP kinase activation ↓ROS formation	CBD protects retina from diabetes related inflammation, vascular permeability, and neurotoxicity

2.1.3 Excitotoxicity

Excitotoxicity is implicated in glaucoma as a result of elevated levels of the excitatory neurotransmitter glutamate in the retina [45,46]. Over-stimulation of a glutamate receptor, such as the N-methyl-D-aspartate (NMDA) receptor, a sodium and calcium permeable channel, results in excess intracellular calcium. Increased intracellular calcium is cytotoxic, as well as induces release of more glutamate, cellular swelling, and eventually cell death [47,48]. The process of excitotoxicity also involves the activation of nitric oxide (NO) synthase and accumulation of NO and superoxide. Overproduction of these oxygen species produces oxidative stress leading to lipid peroxidation, mitochondrial dysfunction, DNA damage and eventually, cell death [46,49].

One method to measure oxidative stress is through peroxynitrite/ nitrotyrosine formation and lipid peroxidation [50]. Peroxynitrite is a product of a superoxide reaction primarily responsible for oxide- and superoxide-dependent cytotoxicity. It is highly unstable, highly reactive and difficult to measure, therefore the presence of peroxynitrite is measured by levels of nitrotyrosine which is formed by nitration of protein-bound tyrosine [50].

In a rat model of neurotoxicity, intravitreal injection of NMDA induces nitrite/nitrate accumulation, lipid peroxidation, nitrotyrosine production, apoptosis, and inner retinal neuronal loss [51]. CBD treatment decreased levels of peroxynitrite/nitrotyrosine production, prevented neurotoxicity, and lowered the amount of apoptosis (Table 1). The neuroprotective effect of CBD was dependent upon blockage of nitrotyrosine formation [51]. The retinal antioxidant

and neuroprotective effects of CBD in the rat model of retinal excitotoxicity suggest that it may be beneficial as a neuroprotectant for the treatment of ocular conditions such as glaucoma.

2.1.4 Diabetic Retinopathy

Globally, diabetic retinopathy is a major cause of vision loss. Oxidative stress, caused by reactive oxygen species, is one of the main factors in diabetic retinopathy progression [52]. The retina is particularly sensitive to reactive oxygen species because it is the most metabolically active tissue in the body and therefore easily affected by diabetes [52]. Diabetic retinopathy is characterized by retinal hypoxia, increased retinal vascular permeability, and retinal angiogenesis [52,53]. These processes cause the death of inner retinal and ganglion cells and ultimately, vision loss.

Inflammation is another important component in diabetic retinopathy. Hyperglycemia triggers the release of proinflammatory cytokines such as vascular endothelial growth factor (VEGF), Intercellular adhesion molecule-1 (ICAM-1), and Tumor necrosis factor α (TNFα) [54,55]. The elevation of these proinflammatory cytokines further facilitates pathologic changes in diabetic retinopathy as a result of neovascularization by VEGF, leukocyte adhesion and transmigration by ICAM-1 and further release of cytokines by TNFα [53-55]. Research shows that VEGF, ICAM-1, and TNFα are upstream regulators of proinflammatory and oxidative stress pathways which activate p38 MAP kinase [55]. Activation of p38 MAP kinase has been reported in diabetic retinas in high glucose conditions and is implicated in retinal ganglion cell apoptotic death [56-

58]. In addition, p38 MAP kinase activity is linked to vascular hyperpermeability in diabetic retinas [55].

One report assessed the therapeutic potential of CBD in a streptozotocin induced diabetic rat model through measurement of oxidative stress and proinflammatory cytokines [58] (Table 1). The antibiotic streptozotocin (produced by *Streptomyces achromogens*) induces type 1 diabetes through partial destruction of the pancreatic β cells after a single injection. In the streptozotocin-induced diabetic rat model there were increases in oxidative stress, retinal neuronal cell death, and vascular hyperpermeability associated with increased levels of VEGF, ICAM-1, TNFα, and activation of p38 MAP kinase [58]. Importantly, CBD decreased reactive oxygen species (ROS) formation, suppressed VEGF, ICAM-1, and TNFα expression, and prevented activation of p38 MAP kinase [58]. Taken together, these findings suggest that CBD is a potential therapeutic agent for diabetic retinopathy capable of protecting against inflammation, retinal neuronal cell death, and preservation of the blood-retinal barrier.

2.1.5 Intraocular Pressure

An estimated 3 million Americans have glaucoma, a major cause of irreversible blindness with no cure [59,60]. Even with therapeutic intervention, approximately 10% of those diagnosed still experience vision loss [59,60]. Although not always elevated, intraocular pressure (IOP) is currently the only treatable factor of the disease. Drug therapies such as prostaglandin analogs, β-adrenergic antagonists, cholinergic agonists, α2 adrenergic agonists, and

carbonic anhydrase inhibitors are used either independently or in combination, to reduce ocular pressure [61,62]. In 2018, the FDA approved Rhopressa, a Rho kinase inhibitor, as a novel IOP-lowering drug [61,62]. These IOP-lowering drugs work to decrease aqueous humor production in the ciliary body and/or increase aqueous humor drainage through the trabecular meshwork or the uveoscleral pathway [61,62]. For patients that do not respond to the above drugs or drug combinations, or patients developed tolerance to existing drugs, novel medications are needed to lower IOP and to prevent future optic nerve damage and vision loss associated with glaucoma.

THC is well documented and is consistently shown to decrease IOP [63-69]. However, the effect of CBD on IOP is much more controversial (Table 2). So far, nine independent reports have published regarding the effects of CBD on IOP: Four reports indicate that CBD has no effect on IOP [70-73], three reports demonstrate that CBD decreases IOP [74-76], and two reports show an CBD-induced increase in IOP [63,64]. A recently published study in mice showed an increase in IOP at 1 and 4 hours post topical application of CBD at a 5 mM dose [63]. Interestingly, CBD significantly decreased IOP 1 hour post treatment in CB1 knockout mice and the effect is attributed to GPR18 activation [63]. This article was cited by the American academy of ophthalmology with the headline “CBD oil may worsen glaucoma [77]. Altogether, the literature does not conclusively show whether CBD increases, decreases, or causes no change to IOP.

Table 2. The Effects of CBD on Intraocular Pressure

Ref.	Species	Route	Dose Frequency	Vehicle	Dose	Effect on IOP
[73]	Rabbit	Intravenous	1 application	2% Tween 60 and 3% Arlacel in water	1 mg/kg 10 mg/kg	No change No change
[70]	Monkey	Oral	1 application	2% Tween 60 and 3% Arlacel in water	10 mg/kg	No change
[71]	Rabbit	Intravenous	1 application	100% alcohol	0.1 mg/animal 1 mg/animal 10 mg/animal	No change No change No change
[72]	Rabbit	Intravenous	1 application	25% BSA in 95% EtOH	1 mg/ animal	No change
[75]	Rabbit	Topical	1 application	Mineral Oil Sesame Oil	0.0001% 0.001% 0.01% 01% 1% 0.1%	↓ ↓ ↓ ↓ ↓ No change
[74]	Human	Intravenous	1 application	25% human serum albumin	20 mg/ person	↓
[76]	Cat	Topical via minipump	Continuously for 9 days	Polyethylene glycol	20 mg/hour	↓
[64]	Human	Sublingual	4 sprays at 5-minute intervals	Oromucosal spray with unspecified vehicle	20 mg 40 mg	No change Transient ↑
[63]	Wild type C57B6 Mice CB1 KO mice	Topical	1 application	Tocrisolve, a soya-based solvent	5 mM 5 mM	↑ at 1 and 4 h ↓ at 1 hour

2.2 Molecular Targets and Mechanisms for CBD-Induced Ocular Effects

CBD has numerous targets in different categories such as G protein coupled receptors, enzymes, nuclear receptors, ligand-gated ion channels, transient receptor potential (TRP) channels, and potentially others [18]. Many of these CBD targets are expressed in the eye. This section of the review focuses on the effects of CBD on these molecular targets in the eye (Table 3). It is important to point out that systemic CBD administration may result in CBD metabolites that act through molecular mechanisms different from those of CBD itself.

2.2.1 Serotonin Receptor

Thapa et al. showed that CBD can reduce the pain score and neutrophil infiltration in mice after corneal cauterization and capsaicin challenge and this effect is mediated, in part, by 5-HT1A agonism [39] (Table 3). The hypoalgesic and anti-inflammatory effects of CBD seen in wild-type mice were still present in CB2 knockout mice, as well as CB2 knockout mice pretreated with AM251, a CB1 selective antagonist. These results suggest that the hypoalgesia and anti-inflammatory effects of CBD are not mediated by CB1 or CB2 receptors. Moreover, the effects of CBD were blocked in wild-type mice treated with WAY100635, a 5-HT1A receptor antagonist. These data demonstrate that the effect of CBD on corneal hyperalgesia inflammation is mediated by 5-HT1A agonism [39]. In support of the findings of Thapa et al. in the eye [39], CBD has been shown to be a 5-HT1A agonist in other tissues as well [78,79].

2.2.2 Equilibrative Nucleoside Transporter 1 and A2A Adenosine Receptor

CBD has been shown to inhibit TNF-α response to LPS stimulation by inhibiting adenosine reuptake in retinal microglia via adenosine equilibrative nucleoside transporter 1 (ENT1) [44] (Table 3). Cells that were pre-treated with CBD showed inhibition of LPS-induced release of TNF-α. The inhibition of TNF-α release was not further enhanced nor inhibited by pretreatment of NBMPR, an ENT1 selective inhibitor [44]. These results suggest that CBD competes with NBMPR for ENT1. Furthermore, CBD inhibited TNF-α in the presence of A1A adenosine antagonist CPX, whereas the effect of CBD was blocked by pretreatment with A2A adenosine receptor antagonist ZM241385. When CBD and adenosine were co-administered, TNF-α release was greatly reduced showing a synergistic effect that is greater than when either compound was administered alone [44]. In sum, Liou et al. showed that CBD inhibits adenosine reuptake through ENT1, which indirectly causes the enhanced activation of A2A adenosine receptor and reduction of TNF-α release [44]. The effects of CBD on ENT1 and adenosine receptors are corroborated by reports in rat and mouse striatal terminals [80] and in EOC-20 murine microglial cells [81].

Table 3. Molecular Targets and Mechanisms for CBD- induced Ocular Effects

Ref.	Target	Tissue/Cells	CBD route	CBD effect	CBD mechanism
[39]	5-HT_{1A}	Mouse cornea	Topical	↓ pain score	5-HT_{1A} agonist, effect blocked by 5-HT_{1A} antagonist WAY100635
[44]	$ENT1A_{21}$	Rat retinal microglia	Intraperitoneal	↓ adenosine reuptake ↓ TNFα expression	ENT1 inhibitor, reuptake inhibition blocked by ENT1 inhibitor NBMPR
[63]	GPR18 CB1	WT and CB1 KO mice	topical	↑ IOP in WT mice ↓ IOP in CB1 KO mice	CB1 negative allosteric modulator GPR18 agonist, effect blocked by GPR18 antagonist O-1918
[92]	GPR55	Mouse retinal explant cultures	Intravitreal	↓ growth cone size ↓ filopodia number ↑ chemorepulsion	GPR55 antagonist, effects absent in GPR55 KO mice
[98]	TRPV2	Porcine RPE cells	In vitro	↑ intracellular Ca^{2+}	TRPV2 agonist, effect blocked by TRPV2 blocker SKF-96365
[99]	TRPV2	ARPE-19 cells	In vitro	↑ current density ↑membrane conductance	TRPV2 agonist, effect blocked by TRPV2 blocker SKF-96365

2.2.3 CB1 and GPR18

CB1 is a well-established cannabinoid receptor and CBD has been shown to be a negative allosteric modulator of CB1 [82]. CB1 is expressed in the anterior of the eye in the ciliary and corneal epithelium and trabecular meshwork, as well as the posterior of the eye in the retina [83,84]. GPR18 is a recently identified putative cannabinoid receptor and researchers have shown that GPR18 is activated by N-arachidonoyl glycine, a carboxylic metabolite of the endocannabinoid anandamide [85]. GPR18 was further characterized in 2012 when anandamide and THC, in addition to N-arachidonoyl glycine, were shown to stimulate GPR18-mediated ERK1/2 phosphorylation [86]. Furthermore, CBD was shown to be a biased agonist for GPR18 in 2014 [87]. GPR18 is widely expressed in the ocular tissues, specifically in the ciliary and corneal epithelium, trabecular meshwork, and retina [88,89].

To date a single paper has reported on the effect of CBD at both CB1 and GPR18 receptors in the eye (Table 3). Miller et al. showed that CBD increases IOP in wild-type mice but decreases IOP in CB1 knockout mice [63]. No CBD effect on IOP was seen in CB1 knockout mice pretreated with O-1918, a GPR18 antagonist. This report highlights that CBD has independent actions both on CB1 as a negative allosteric modulator to raise IOP and on GPR18 as an agonist to lower IOP [63].

2.2.4 GPR55

GPR55 is an orphan receptor activated by lysophosphatidylinositol (LPI) [90]. GPR55 is frequently referred to as a putative cannabinoid receptor because

it is activated by phytocannabinoids, endocannabinoids, and synthetic cannabinoids [91]. CBD has been shown to be a GPR55 antagonist [91]. One group studied the involvement of GPR55 in the retina during development [92]. Growth cones are regions of developing neurites which facilitate axon growth by extending actin filaments into filopodia. Filopodia guide the growth cone in response to chemical or electrical stimulus. Cherif et al. found that GPR55 is expressed in growth cones during development, and its activity regulates morphology and growth [92]. Mouse embryonic neurons from GPR55 knockout mice showed smaller growth cones, fewer filopodia, and decreased outgrowth compared to neurons from wild type mice. Furthermore, retinal ganglion cells from wild type mice treated with GPR55 agonists LPI and O-1602 showed increased growth cone size and filopodia number and demonstrated chemoattraction. In contrast, CBD, a GPR55 antagonist, decreased growth cone size and filopodia number, and induced chemorepulsion (Table 3). GPR55 ligands had no effects in embryonic neurons from GPR55 knockout mice [92]. These data suggest that CBD inhibits growth cone activity and axonal growth in the retina in this experimental model.

2.2.5 TRPV Channels

TRP ion channels are trans-membrane proteins involved in a wide range of chemical and physical sensations including smell, taste, vision, temperature, and pressure [93]. CBD has been shown to be an agonist of TRPV 1, 2, 3, 4 and TRPA1 [94,95]. TRPV channels are implicated in the activation and desensitization of inflammatory processes and chronic pain [96,97]. Therefore,

CBD may be a desirable therapeutic for chronic pain because it can activate and desensitize the TRPV channels [94,95].

One group investigated the calcium influx activity of TRPV2 channel activity in porcine retinal pigment epithelial (RPE) cells [98]. They found that CBD strongly increased intracellular Ca2+ levels (Table 3). In the presence of TRPV2 channel inhibitor SKF96365, CBD-mediated Ca2+ intracellular increase was partially blocked [98]. These data suggest that CBD modulation of Ca2+ involves TRPV2, as well as other TRPV channels that are not blocked by SKF96365. Another study looked at TRPV2 channel regulation in ARPE-19, a human RPE cell line [99]. ARPE-19 cells preincubated with CBD demonstrated a 3-fold increase in current density, an effect that was blocked by SKF96365 (Table 3). CBD also increased membrane conductance and TRPV2 surface expression. TRPV2 are heat sensitive ion channels and heat further increased the CBD mediated increase in membrane conductance. Furthermore, the PI3 kinase inhibitor LY294002 abolished the effect of CBD on membrane conductance and surface expression. These data led to the conclusion that CBD acts through activation of TRPV2 and a PI3 kinase dependent pathway to increase cell surface expression of TRPV2 channels [99].

2.3 Challenges of Using CBD as an Ocular Therapeutic Agent

There are several challenges for practical applications of CBD as an ocular therapeutic agent. Some of these challenges include poor bioavailability, difficulty in topical delivery, and short duration of action.

2.3.1 Bioavailability

An FDA approved drug containing CBD is administered orally [23]. However, oral administration is inefficient due to poor bioavailability of CBD. Low bioavailability of CBD requires that it to be administered at high doses to achieve therapeutic effects. However, a consequence of high dosing is an increase in adverse side effects [100,101]. The potential adverse effects of CBD include drowsiness, dry mouth, reduced appetite, nausea, and gastrointestinal issues. The most notable serious adverse side effects of CBD are abnormal liver function tests (elevated liver enzymes) [102].

One major factor contributing to the poor bioavailability of CBD is its extensive first pass metabolism [100,101]. Another factor limiting CBD bioavailability is its hydrophobicity. The chemical structure of CBD contains aromatic rings and an aliphatic side chain, which make it a highly hydrophobic molecule. The hydrophobicity of CBD limits its solubility in water and makes diffusion across aqueous barriers a rate limiting step for diffusion and absorption [100,101].

2.3.2 Topical Delivery

Therapeutic treatments for ocular conditions are frequently administered orally or topically to the eye. Extensive first pass metabolism of CBD prevents a significant amount of drug reaching the eye from oral administration. Therefore, topical administration of CBD is desired.

Developing an ocular topical delivery system is a difficult task. The eye contains sophisticated protective mechanisms and physical barriers to prevent foreign material from entering, which includes the multi-layered cornea and pre-

corneal tear film. The alternating lipophilic and hydrophilic nature of the cornea makes ocular drug delivery exceptionally difficult. As a result, less than 5% of drugs applied topically enter the eye [103-105].

CBD is highly hydrophobic and insoluble in water. Studies applying CBD topically used non-aqueous vehicles for delivery [39,63,75]. Previously, CBD was topically delivered to the eye in mineral oil, sesame oil, soybean oil, and a soya oil/water emulsion, Tocrisolve [39,63,75]. In one report, CBD delivered in mineral oil produced an IOP lowering effect whilst the effect is absent in sesame oil [75]. With Tocrisolve as a vehicle, Miller et al. [63] demonstrated that a high dose of CBD increases IOP in wildtype mice, but decreases IOP in CB1 knockout mice. This indicates that at large doses CBD produces off-target effects which are detrimental. Since CBD has at least 65 targets [18], off-target effects of CBD at high doses are very likely. These results highlight a critical need for a vehicle with high ocular permeation to administer CBD in a therapeutically relevant dose.

2.3.3 Duration of Action

Another difficulty associated with CBD as an ocular therapeutic is its short duration of action, e.g., in lowering IOP. One report indicated CBD decreased IOP 1-2 hours after topical application to rabbit eyes, and IOP-lowering effects of CBD lasted for up to 5 hours after intravenous administration [75]. In another study, CBD required constant infusion via minipump to induce a decrease in IOP [76]. A short duration of action implies that CBD needs to be applied multiple times throughout the day to maintain therapeutic effects. However, patient

compliance will be worsened with frequent dosing. In contrast, greater patient compliance is observed in prescribed medications with once daily dosing [106].

3. Overall Goals of This Dissertation

Regarding CBD's effect on IOP, 11 conflicting reports are published; the five reports show no change in IOP [70-73,107], four papers demonstrate a decrease in IOP [74-76,108], and two reports show an increase in IOP [63,64]. These prior reports are not comparable due to a variety of doses, species, routes of administration, and vehicles used. Regardless, the effect of any compound on IOP is dependent on bioavailability of the compound. None of the prior reports indicated what concentration was reaching relevant tissues. Therefore, quantitation of CBD in ocular tissues is critical to determine the concentration at which CBD regulates IOP. Further, if CBD does not reach aqueous humor following topical application, it may have benefits for superficial ocular diseases.

The overall goals of this dissertation were to develop analytical methods to quantify CBD in ocular tissues, and measure effects of CBD on aqueous humor outflow, intraocular pressure, and corneal pain. Chapter 2 details a molecular mechanism regulated by CBD in trabecular meshwork cells, as well as regulation of aqueous humor outflow. Chapter 3 observes aqueous humor concentrations following intraperitoneal administration, and measurement of IOP. Chapter 4 studies aqueous humor and cornea concentrations of CBD following topical application; depending on which tissue CBD penetrated to (AH or cornea), IOP or corneal pain was assessed.

CHAPTER II

THE EFFECTS OF CANNABIDIOL ON AQUEOUS HUMOR OUTFLOW AND TRABECULAR MESHWORK CELL SIGNALING

1. Introduction[2]

Glaucoma is one of the leading causes of human blindness, with chronically elevated intraocular pressure (IOP) as the major risk factor [109-111]. IOP is regulated through a dynamic balance of aqueous humor secretion by the ciliary body and outflow via the trabecular meshwork (TM) and uveoscleral route [109-111]. Current pharmacotherapies for glaucoma include prostaglandin analogs, β-adrenoceptor antagonists, α-adrenoceptor agonists, carbonic anhydrase inhibitors, cholinergic agonists, and most recently, Rho kinase inhibitors [61,62,112-114]. However, existing drugs have side-effects and patients frequently become tolerant to the drugs over the course of their disease (available drugs no longer lower IOP). Therefore, there is an urgent need for novel drugs to lower IOP through novel mechanisms of action with fewer side effects [61,62,112].

Cannabidiol (CBD) is one of the major active constituents of cannabis [115,116]. Unlike Δ^9-tetrahydrocannabinol (Δ^9-THC), CBD is non-psychotropic

[2]This chapter has been submitted for publication as part of A. Aebersold, Z.H. Song, The effects of cannabidiol on aqueous humor outflow and trabecular meshwork cell signaling, Cells (Submitted 2022).

[115,116]. CBD has a wide range of therapeutic potentials, including the treatment of cancer [117], inflammatory diseases [118], neurodegenerative diseases [119,120], and psychiatric diseases [121]. Recently, CBD (Epidiolex) received FDA approval for treating epilepsy in children; this is the first time a constituent isolated from cannabis has been FDA approved [23].

The observation that smoked marijuana lowers IOP was first noted more than 50 years ago [122]. Since then, many papers have demonstrated that various phytocannabinoids, including Δ^9-THC and CBD, are able to lower IOP, suggesting their potentials as therapeutic agents for glaucoma [34,105,123]. However, Δ^9-THC has psychotropic side-effects which limit its clinical use for glaucoma [34,105]. Since CBD is non-psychotropic, its IOP-lowering effects have more therapeutic potential.

The Rho/Rho kinase signaling pathway is a major regulator of conventional aqueous humor outflow through the TM [124-128]. This pathway regulates phosphorylation of downstream regulators regulatory myosin light chain (MLC) and myosin phosphatase target subunit 1 (MYPT1) [129]. By preventing the phosphorylation of MLC and MYPT1, Rho kinase inhibitors such as Rhopressa (Netarsudil) and Galantec (Ripasudil), inhibit TM tissue contraction and enhance aqueous humor outflow through the TM [124-128,130-132].

Despite evidence that CBD reduces IOP, the underlying molecular mechanism has not been elucidated. One potential mechanism is that CBD enhances aqueous humor outflow in the TM, but this has not been studied. In this study we first investigated if CBD modulates aqueous humor outflow using

perfused porcine anterior segments. We then investigated whether CBD alters the contractility of cultured porcine TM cells and the involvement of the Rho/Rho kinase signaling pathways in the effects of CBD on TM cells.

2. Methods

2.1 Porcine Anterior Segment–Perfused Organ Culture Model

A previously published procedure was used for the anterior segment perfused organ culture model [133-137]. Fresh porcine eyes were obtained from a local slaughterhouse within 30 minutes of decapitation. Porcine anterior segment explants - comprised of the intact cornea, the undisturbed TM, and a 2 to 5 mm rim of sclera (with the ciliary body and iris gently removed) - were mounted in a standard perfusion culture apparatus and perfused with Dulbecco's modified Eagles medium (DMEM) using a constant perfusion head of 10 cm (~7.35 mm Hg) for 5 hours, while outflow stabilized. Only those explants that stabilized between 1.5 and 8 µL/min at 7.35 mmHg were used. Cultures were maintained at 37°C with 5% CO_2. It has been shown that in this model, outflow is through the TM, and flow rates are physiological (approximately 2.75 µL/min) [137]. After stabilization, CBD (Cayman Chemical, Ann Arbor, MI) was introduced. The anterior segments were then perfused continuously with CBD-containing medium for 5 hours, and the outflow was monitored. Medium containing no CBD was run in parallel. Aqueous humor outflow was calculated as the rate of flow of perfusate (in microliters per minute). Drug effects were evaluated in each explant as the percentage change of outflow in drug-treated eyes over pre-drug baseline outflow. Ten eyes were used for each group of treatment.

2.2 Culture of Porcine Trabecular Meshwork Cells

The TM was isolated from fresh porcine eyes by blunt dissection. Culture of TM cells was performed according to previously published methods [138,139]. The identity of TM cells was established by their morphology and their ability to take up acetylated low-density lipoprotein and to secrete tissue plasminogen activator [138,139]. TM cells were maintained in DMEM at 37°C with 5% CO_2.

2.3 Collagen Gel Contraction Assays

Collagen gel contractility assays were performed following previously published procedures [140]. The wells of 24-well cell culture plates (Corning, Corning, NY) were each coated with 1 mL 1% BSA for 1 hour at 37°C. Porcine TM cells were collected by treatment of cultures with trypsin-EDTA, washed with 1x PBS, and re-suspended in DMEM at a density of $1x10^6$ cells/mL. Rat tail collagen type I (Santa Cruz Bio, Dallas, TX), 10X PBS, sterile dH_2O, and 1N NaOH were mixed at ratios following manufacturer instructions to obtain a final concentration of 1.9 mg/mL collagen and final cell density of $2x10^5$ cells/mL. The resultant mixture was added to BSA coated wells (1% BSA, 0.5 mL/well). Collagen gels were allowed to polymerize at 37°C with 5% CO_2 for 90 minutes. Once released from the wells, serum-free DMEM (0.5 mL), with or without CBD, was added to the gels. The gels were imaged at 48 hours (n=4). The area of the collagen gels was calculated using ImageJ software (National Institutes of Health, Bethesda, MD). For normalization, the area of the collagen gel containing vehicle treated TM cells was set at 100%, and the changes in the area for CBD treatment are shown as a bar graph representing the mean±SEM (n=5).

2.4 Western Blot Analysis

Protein samples were prepared from porcine TM cells according to published procedures [141]. Samples were incubated with 2X Laemmli buffer under reducing conditions at 100^{o}C for 20 minutes and proteins were resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel using a minigel electrophoresis system (Invitrogen, Carlsbad, CA). Protein bands were transferred onto a nitrocellulose membrane. The nitrocellulose membranes were blocked with 5% nonfat dried milk in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.3% Tween 20, pH 8.0) for 1 hour and then incubated overnight at 4°C with primary anti-phospho-MLC2 (Ser19), anti-phospho-MYPT1 (Thr853), or anti-GAPDH antibody (Cell Signaling, Beverly, MA). Subsequently, the membranes were washed three times with TBS-T buffer for 5 minutes, and then incubated with horseradish peroxidase (HRP)–conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX) for 1 hour at room temperature. The membranes were then washed three times with 0.3% TBS-T buffer for 5 minutes and the antibody-recognized protein bands were visualized using Pierce™ enhanced chemiluminescence Western blotting substrate (Fisher Scientific, Waltham, MA). Experiments were performed in triplicate.

2.5 RhoA Activation Assay

Samples were collected and assays were performed according to manufacturer's (Cytoskeleton Inc Denver, CO) instructions. Cells were grown to confluence and serum starved overnight. Cells were then treated with vehicle or CBD, lysed on ice using lysis buffer (50 mM Tris, 10 mM $MgCl_2$, 0.5 M NaCl, 0.1% Triton X-100, and 0.1% SDS, pH 7.5) containing a protease inhibitor cocktail

(Sigma, St. Louis, MO), then collected into pre-chilled 1.5 mL microcentrifuge tubes. Cell lysates were then centrifuged at 10,000 x *g* at 4°C, and supernatant containing 50 µg of protein was incubated with 10 µg Rhotekin-RBD beads (Cytoskeleton, Denver, CO) in a final volume of 300 µL for 1 hour at 4°C. Following incubation, beads were washed with ice-cold wash buffer and centrifuged at 5,000 x *g*. The immunoprecipitated complex was re-suspended in 2X SDS sample buffer, boiled at 100°C for 5 minutes, and then subjected to 10% SDS- polyacrylamide gel electrophoresis, followed by Western blot analysis. The separated proteins were immunoblotted with antibody against RhoA or RhoA-GTP (Cytoskeleton Inc., Denver, CO). Experiments were performed in triplicate.

2.6 Data Analysis

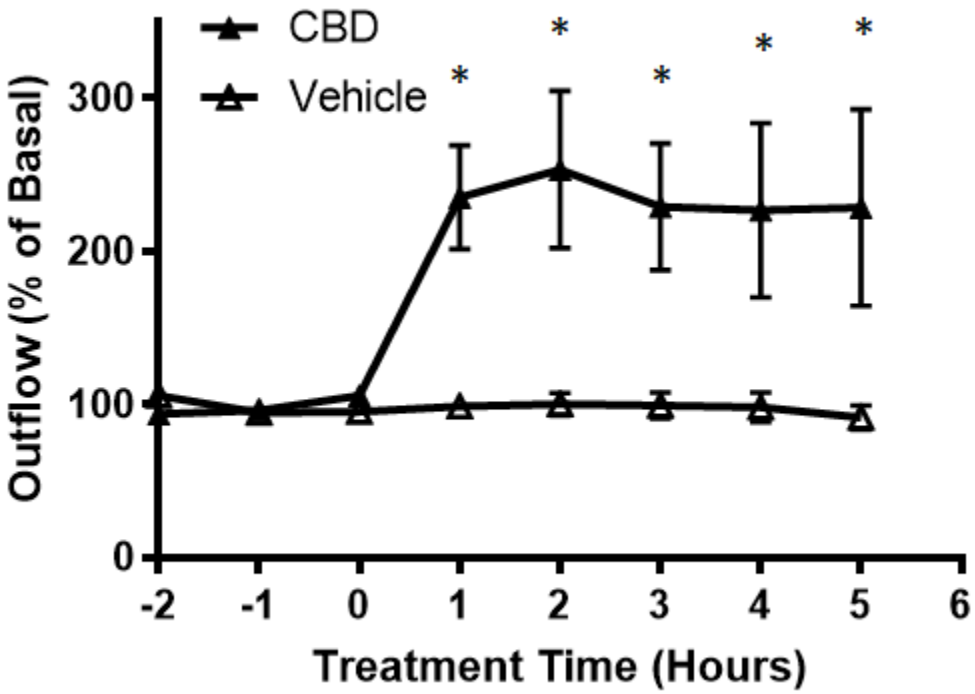
For anterior segment perfusion studies, results are presented as changes in aqueous humor outflow (% of basal). For the collagen gel contraction assay, images of gel areas were quantified with the use of ImageJ program (NIH, Bethesda, MD). For Western blot assays, the bands on x-ray films were scanned by the Epson Perfection V39 (Epson, Long Beach, CA) and were quantified with the use of ImageJ. The data are plotted with Prism software. Unpaired two-tailed Student's *t*-tests were used to compare the mean±SEM of CBD and vehicle treatment groups. The level of significance for all studies was set at $p<0.05$.

3. Results

3.1 The Effects of CBD on Aqueous Humor Outflow

Aqueous humor outflow studies were performed using the porcine anterior segment perfused organ culture model. As shown in Figure 1, the application of 1 μM CBD more than doubled aqueous humor outflow at 1 hour after treatment when compared with vehicle. This effect lasted for the measurement window of 5 hours.

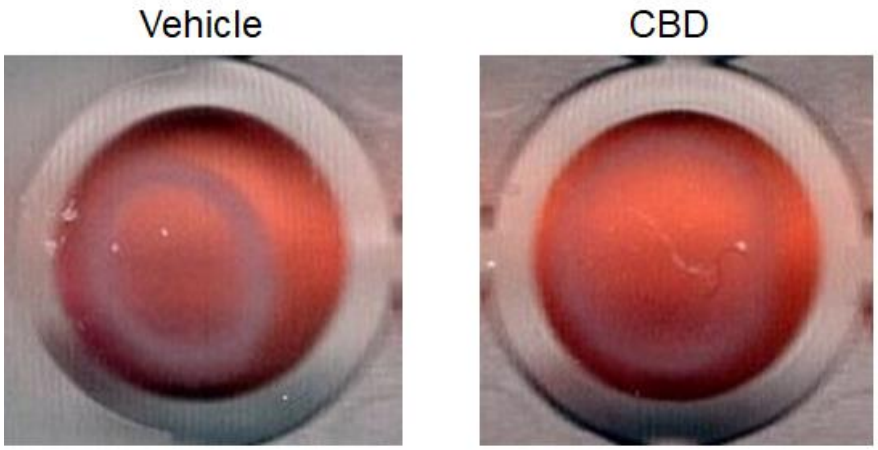
Figure 1. Effects of Cannabidiol on Aqueous Humor Outflow. Application of 1 µM CBD caused a significant increase in aqueous humor outflow lasting from 1 hour to 5 hours. Results are expressed as mean±SEM; *n*=10. *Significant differences between 1 µM CBD and vehicle determined by *t*-test, $p<0.05$.

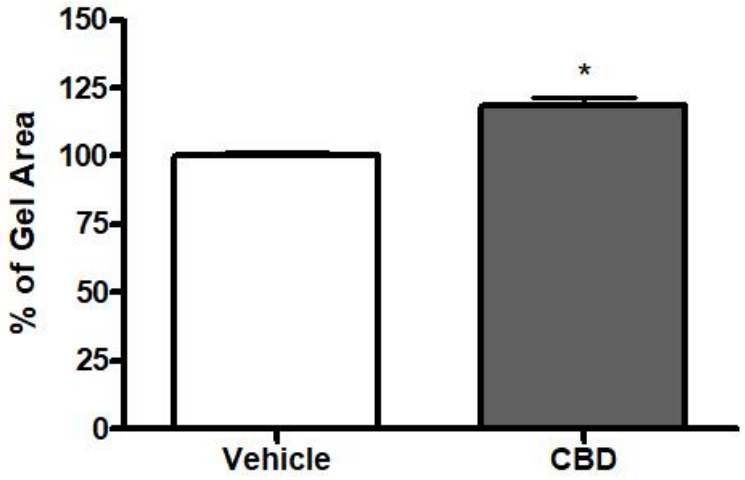


3. 2 The Effect of CBD on Collagen Gel Contraction Mediated by TM Cells

To determine if collagen contraction mediated by TM cells is altered by CBD, TM cells were cultured in a three-dimensional collagen gel, and change in area of the gel in response to DMEM (vehicle) or DMEM containing CBD (1 μM) treatment was measured. As shown in Figure 2, vehicle treated TM cells caused collagen contraction. Importantly, CBD treatment significantly opposed TM cell-mediated gel contraction (CBD gel area 118.7% ± 3.042 compared to vehicle, n=5; p<0.05). These results demonstrate that CBD relaxes TM cell contraction.

Figure 2. Effect of Cannabidiol on Collagen Gel Contraction Mediated by Trabecular Meshwork Cells. Cells grown embedded in collagen gels were treated with DMEM or DMEM containing 1 µM CBD for 48 hours. Top: Representative photographs of collagen gel cultures of TM cells incubated for 48 hours with the indicated drug treatment. Bottom: The mean±SEM (*n*=5) of results of four experiments are shown. Treatment with CBD significantly opposed the basal level of gel contraction. *Significant difference from vehicle determined by *t*-test, $p<0.05$.

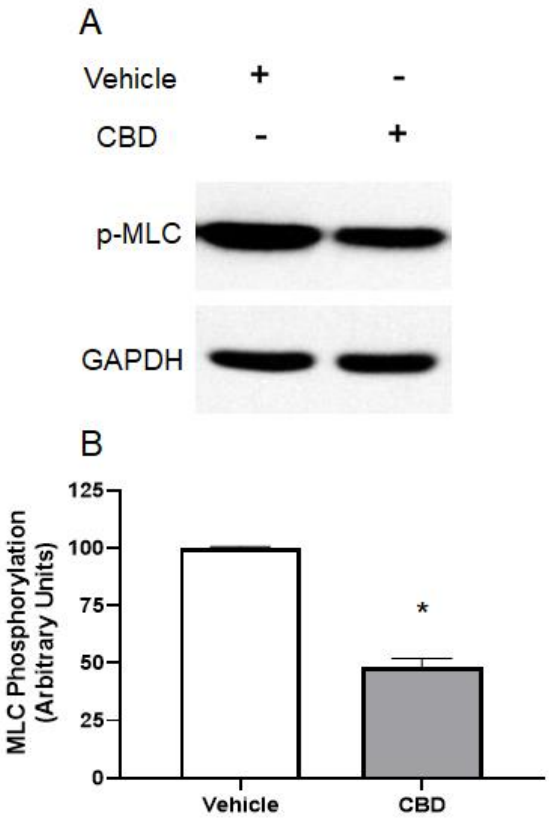




3.3 The Effects of CBD on MLC Phosphorylation in TM Cells

To determine the constitutive level of MLC phosphorylation and whether CBD stimulation would alter phosphorylation of MLC in TM cells, cells were serum starved overnight followed by treatment with vehicle, or CBD (1 μM) for 2 hours. As shown in Figure 3, MLC is constitutively phosphorylated in TM cells. Following treatment with CBD, MLC phosphorylation significantly decreased by 51.56% (mean±SEM: 48.44 ±6.268%; $p<0.05$). These results demonstrate that in TM cells CBD inhibits MLC phosphorylation.

Figure 3. Inhibition of Phosphorylation of Myosin Light Chain Protein by Cannabidiol in Trabecular Meshwork Cells. A: Western blot representative of results obtained in three experiments. Cells were serum starved overnight and treated with vehicle or 1 µM CBD for 2 hours, then phosphorylation of myosin light chain (MLC) was measured with anti-GAPDH and anti-phospho-MLC antibodies. B: densitometry quantification of Western blot data from three experiments. MLC is constitutively phosphorylated in vehicle treated TM; following treatment with CBD, MLC phosphorylation significantly decreased by 51.56%. Results are expressed as mean±SEM (*n*=3). Vehicle treated phosphorylation of MLC levels are normalized to 100%. *Significant difference from vehicle determined by *t*-test, $p<0.05$.



3.4 The Effects of CBD on MYPT1 Phosphorylation in TM Cells

To determine if MYPT1 phosphorylation is altered by CBD, TM cells were serum starved overnight followed by treatment with DMEM medium or DMEM containing CBD (1 μM) for 2 hours. As shown in Figure 4, following treatment with CBD, MYPT1 phosphorylation at Thr853 significantly decreased by 53.66% (mean±SEM: 46.34±11.95; $p<0.05$). These results demonstrate that in TM cells CBD inhibits MYPT1 phosphorylation.

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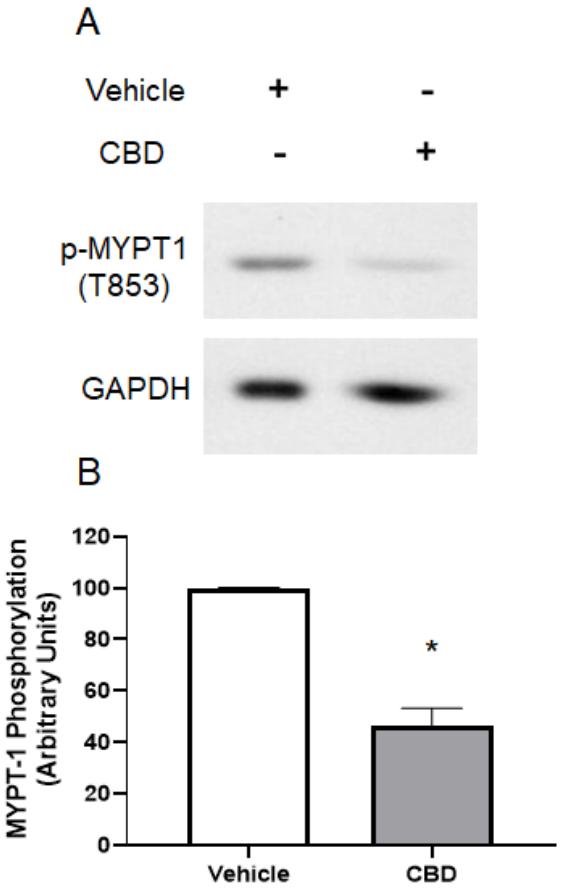
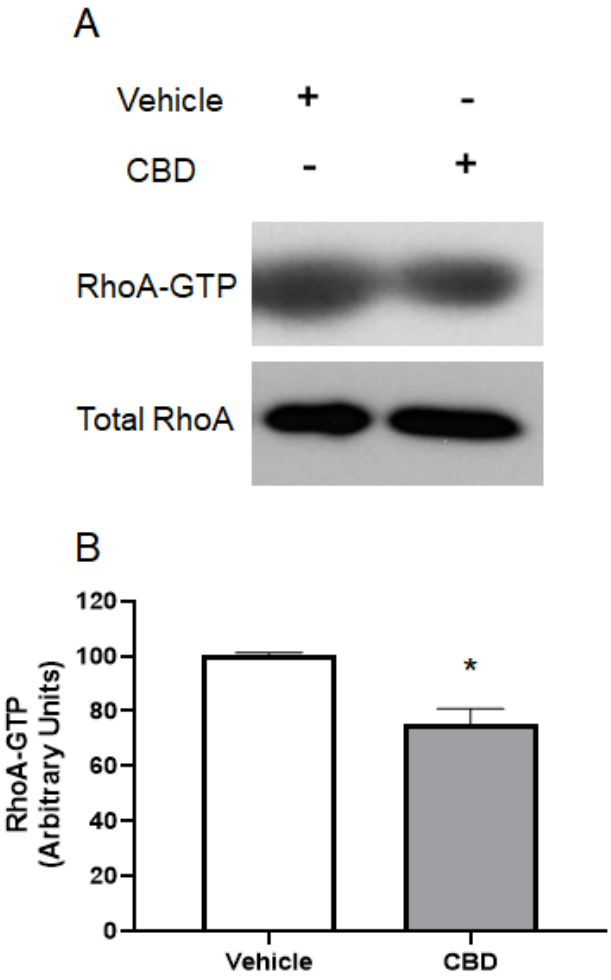
Figure 4. Effect of Cannabidiol on Myosin Phosphatase Targeting Subunit 1 (MYPT1) Phosphorylation. A: Western blot representative of phosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) at Thr853 and GAPDH for three experiments is shown. TM cells were serum starved overnight and treated with vehicle or 1 µM CBD for 2 hours, then phosphorylation of MYPT1 at Thr853 was assessed. B: densitometry quantification of phospho-MYPT1 from three experiments is shown. Following treatment with CBD, MYPT1 phosphorylation at Thr853 significantly decreased by 53.66%. Results are expressed as mean±SEM (*n*=3). Vehicle treated phosphorylation of MYPT1 levels are normalized to 100%. *Significant difference from vehicle determined by *t*-test, $p<0.05$.



3.5 The Effects of CBD on RhoA Activation in TM Cells

To determine if RhoA activation is altered by CBD, the RhoA activation assay was performed. Cells were serum starved overnight followed by treatment with DMEM or DMEM containing CBD (1 μM) for 2 hours. As shown in Figure 5, following treatment with 1 μM CBD, RhoA-GTP significantly decreased by 24.61% (mean±SEM: 75.39 ± 9.539; $p<0.05$). These results demonstrate that CBD inhibits RhoA activation in TM cells.

Figure 5. The Effect of Cannabidiol on RhoA Activation. A: representative Western blot of RhoA-GTP and Total RhoA for three experiments is shown. TM cells were serum starved overnight and treated with DMEM or DMEM containing 1 µM CBD for 2 hours, then RhoA activation was assessed. B: densitometry quantification of RhoA activation for three experiments is shown. RhoA-GTP significantly decreased by 24.61% following treatment with CBD compared to the vehicle in TM cells. Results are expressed as mean±SEM. Vehicle treated TM cell RhoA levels are normalized to 100%. *Significant difference from vehicle determined by *t*-test, $p<0.05$.



4. Discussion

IOP is maintained by balancing the production and outflow of aqueous humor [109-111]. In this study, using perfused porcine anterior segments, we demonstrated a CBD-induced significant increase in aqueous humor outflow. Therefore, our data support the notion that CBD lowers IOP through enhancing aqueous humor outflow and justifies further study. These results are consistent with previous preclinical studies that CBD was hypotensive when applied topically to cat or rabbit eyes [75,76]. Also, our data are consistent with a clinical report that CBD administered intravenously reduced IOP in human subjects [74].

In patients with primary open angle glaucoma, IOP elevation is caused by TM resistance to aqueous humor outflow associated with a loss of TM cells, excessive extracellular matrix accumulation, and an alteration of the TM cell contractility [111,112]. Previously, it has been shown that Rho/Rho kinase inhibitors enhance aqueous humor outflow through the TM route by decreasing the contractility of the TM cells [142]. In the current study, CBD was found to inhibit TM cell-mediated contraction of collagen gels. These data demonstrate that CBD was able to decrease the contractility of TM cells, supporting the role of CBD in enhancing aqueous humor outflow.

One of the signaling pathways for changing TM cell contractility is through regulation of myosin light chain (MLC) activity [124]. Previous studies have shown that aqueous humor outflow through the TM can be increased by inhibiting MLC phosphorylation [124]. In this study, administration of CBD led to an inhibition of

MLC phosphorylation in TM cells. These data support the idea that CBD inhibits TM cell MLC activity, thus causing a decrease in TM cell contractility.

Dephosphorylation of MLC is induced by myosin light chain phosphatase (MLCP), which is heterotrimeric enzyme, containing a phosphatase subunit, a subunit with undefined function, and a regulatory subunit (MYPT1) [129,143]. Rho kinase itself is capable of phosphorylating MYPT1, thereby inactivating MLCP and inhibiting its phosphatase activity, and allowing sustained contraction [129,143]. In the current study, our results showed a decrease in MYPT1 Thr853 phosphorylation in response to CBD, indicating MLCP activation and MLC dephosphorylation.

Furthermore, our data demonstrated that RhoA activation was inhibited by CBD. This finding further supports the notion that CBD inhibits Rho/Rho kinase pathway, enhances dephosphorylation of MLC, and to the same end decreases the contractility of TM cells and allows for enhanced aqueous humor outflow.

In summary, in this study we discovered, for the first time, that CBD increases aqueous humor outflow in perfused anterior segments. In addition, using cultured TM cells, we demonstrated that CBD inhibits TM cell contractility, MLC phosphorylation, MYPT1 phosphorylation and RhoA activation. Overall, our data support the notion that by altering the Rho/Rho kinase signaling to MLC, CBD was able to decrease the contractility of TM cells and enhance aqueous humor outflow via the TM route. There are many IOP-lowering drugs available to reduce aqueous humor production, but there are only limited drugs available to increase aqueous humor outflow directly through the TM route. Since our data demonstrated that

CBD enhances aqueous humor outflow, further study of the effect of CBD on IOP and subsequently glaucoma is warranted.

CHAPTER III

CANNABIDIOL PHARMACOKINETICS AND EFFECT ON INTRAOCULAR PRESSURE FOLLOWING INTRAPERITONEAL ADMINISTRATION

1. Introduction[3]

There are over 100 phytocannabinoids which can be isolated from *Cannabis sativa* [11,12]. The major psychoactive phytocannabinoid is THC, and the most abundant non-psychoactive phytocannabinoid is CBD [11,12]. Interests in CBD as a potential therapeutic agent has risen out of the desire to avoid the psychotropic effects of THC. In 2018, Epidiolex (CBD) was approved by FDA for treatment of two severe forms of pediatric epilepsy: Dravet's syndrome and Lennox-Gastaut syndrome [23]. Also, CBD is currently in numerous clinical trials for a variety of diseases, including general pain and pain associated disorders, drug abuse and use disorders, other neurologic conditions and psychiatric conditions, and COVID-19 (from clinicaltrials.gov).

Glaucoma is one of the leading causes of blindness in the world and a risk factor for glaucoma is elevation in IOP [109-111]. The potential therapeutic

[3]Content from this chapter has been submitted for publication as part of A. Aebersold, Z.H. Song, LC-MS/MS quantitation of non-psychotropic cannabinoid cannabidiol in aqueous humor, Pharmaceutical and Biomedical Analysis (Submitted 2022).

benefit of smoked cannabis on IOP was originally observed in 1971 by Frank and Hepler [122]. Since then, numerous reports have been published demonstrating the IOP lowering effects of phytocannabinoids, synthetic cannabinoids, and endocannabinoids in a variety of species [34,105,144]. Among these cannabinoids, CBD has the potential to be developed into an anti-glaucoma agent without the psychoactive effects of cannabis. However, to better understand the effects of CBD on IOP, it is extremely important to monitor their concentrations in the eye, specifically in aqueous humor.

Aqueous humor is a relevant tissue to measure CBD concentrations to correlate with IOP effects. IOP is regulated through tight regulation of aqueous humor production and outflow through the trabecular meshwork [109]. Further, measurement in aqueous humor would indicate that drugs applied topically had to penetrate the cornea, and drugs given systemically had to penetrate the blood retinal barrier, both of which are major limitations for ocular therapy.

Collection of aqueous humor is highly invasive and involves paracentesis through puncture of the cornea; this method is also used clinically to create an incision to enhance drainage of aqueous humor to lower IOP in glaucomatous emergencies [145]. The mouse eye however can be used as a model for the human eye. Though the trabecular meshwork and cells surrounding the optic nerve head are not identical in rodents and men, they function similarly. Indeed, a majority of aqueous humor outflow is mediated by the trabecular meshwork in rodents [146,147]. Additionally, hypotensive agents used in glaucoma therapy also exert hypotensive effects in mice [148-150]. There are still challenges of

using mouse eyes. Aqueous humor volumes are very small in mice, and the turnover is also very low, approximately 2.5%/min [151,152].Therefore, mice provide small samples and multiple collections from a single animal are impossible.

In order to correlate concentrations of CBD in aqueous humor with effects on IOP, a sensitive, precise and accurate method which can measure small concentrations of CBD in small aqueous humor volumes is needed.

Over the years, numerous quantitation methods for cannabinoids in different biological specimens with different instrumentations have been published [153,154]. Biological specimens studied include blood, plasma, serum, urine, oral fluid, hair, breath, sweat, dried blood spots, postmortem matrices, breast milk, meconium, and umbilical cord. Instrumentation applied includes mostly high-performance liquid chromatography- ultraviolet (HPLC-UV), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography- mass spectrometry (LC-MS), and liquid chromatography- tandem mass spectrometry (LC-MS/MS) [153,154]. Despite the abundance of methods to quantify concentrations of cannabinoids in various tissues, there are markedly few studies in the eye. Previous reports have measured cannabinoids in aqueous humor using HPLC-UV methods [155-159]. A single liquid chromatography-tandem triple quadrupole mass spectrometry (LC-MS/MS) method has been published previously for measurement of CBD in aqueous humor of rabbits [159], but CBD was not detectable following topical application. Therefore, to our knowledge, there has been no report on using a validated LC-MS/MS method, which is more

sensitive method than HPLC-UV, to measure cannabinoids in the aqueous humor.

In order to correlate the concentrations of CBD to the pharmacological effect (such as on IOP) at the anterior chamber of the eye, a sensitive, accurate and precise method must first be developed to quantify CBD in the aqueous humor and serum. However, in the literature there has been no report on a validated LC-MS/MS method which quantifies CBD in aqueous humor. To this end, in this study we have developed and validated an LC-MS/MS method for detection of CBD in aqueous humor. To test the usefulness of the method, we have applied it to the measurement of CBD concentrations in mouse aqueous humor after topical administration in porcine corneal explants *ex vivo* and intraperitoneal administration 50 mg/kg CBD *in vivo*. In addition, to correlate with the CBD pharmacokinetics in the mouse aqueous humor with its pharmacological effects, we monitored the effects of CBD on intraocular pressure (IOP).

2. Materials and Methods

2.1. Chemicals and Reagents

CBD and deuterium labeled CBD (CBD-d_3) standard solutions were purchased from Ceriliant (Round Rock, TX). CBD for corneal penetration, pharmacokinetic, and IOP experiments was purchased from Cayman Chemicals (Ann Arbor, MI). LC-MS grade water, acetonitrile, and formic acid were purchased from (Fischer Scientific; Waltham, MA). 2-hydroxypropyl-β-CD (2HPβCD) was purchased from RND Center Inc. (La Jolla, CA), and randomly methylated β CD (RAMEB) was purchased from Sigma (St. Louis, MO). Tween20 was purchased from Sigma (St. Louis, MO).

2.2. UPLC-MS/MS System and Conditions

CBD and internal standard CBD-d_3 were quantified using a Shimadzu LCMS-8050 triple quadrupole mass spectrometer coupled with an electrospray ionization (ESI) interface (Shimadzu, Tokyo, Japan). The mass spectrometer was operated in positive ion mode, with nebulizing, heating, and drying gas flow at 1.5, 15, and 5 L/min, respectively. Nitrogen gas was the nebulizing, heating, and drying gas whereas argon was the collision gas. The interface temperature was set at 260 °C, the desolvation temperature 460 °C, desolvation line temperature 150 °C, heat block temperature 400 °C. Capillary voltage was set at 4 kV. Quantitation was achieved using multiple reaction monitoring (MRM), and tuned parameters for CBD and CBD-d_3 are listed in Table 4.

Instrument control, data acquisition and data analysis were performed with LabSolutions Software (Shimadzu). An injection volume of 5 µL of processed samples were separated on a Raptor ARC-18 column (1.8 µm, 150 x 2.1 mm; Restek Corporation, Bellefonte, PA) maintained at 55°C. The autosampler tray was maintained at 20±5°C. Solvent A consisted of LC-MS grade water containing 0.1% formic acid, and solvent B consisted of LC-MS grade acetonitrile containing 0.1% formic acid. The total flow rate was 0.32 mL/min. The gradient elution program was as follows: 0-0.5 min, 5% B; 0.5-1 min, increase to 70%; 1-7 min, 70%; and finally, 7- 8 min 5% for reconditioning the column. The total chromatographic time including re-equilibrium was 8 minutes. To prevent carryover, the needle was washed in the autosampler port for 30s before each injection using a wash solution consisting of water: acetone: formic acid (water 90%: acetone 10%: formic acid 0.1%, v/v/v).

Table 4. Multiple Reaction Parameters Tuned for Detection of Daughter Ions of CBD and Deuterium Labeled CBD

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Dwell Time (msec)	Q1 Pre Bias (V)	Collision Energy (V)	Q3 Pre Bias (V)
CBD	315.25	193.15	50	-23	-20	-20
	315.25	123.05	50	-12	-40	-12
CBD-d_3	318.2	196.2	50	-23	-20	-20
	318.2	123.05	50	-12	-40	-12

2.3 Stock and Working Solutions

CBD stock solution (1 mg/mL in methanol) was diluted to the following working concentrations. In porcine aqueous humor: 0.5, 1, 4, 8, 20, 40, 200, 500, and 1000 ng/mL. In serum: 0.5, 2.5, 5, 25, 50, 250, 500, and 2500 ng/mL. All concentrations were used to generate calibration curves and conduct linearity assessments. Internal standard CBD-d_3 was prepared at a concentration of 100 ng/mL in acetonitrile for aqueous humor, and 200 ng/mL in acetonitrile for serum. All solutions were protected from light and stored at -20°C until further analysis.

2.4. Sample Preparation

Prior to use, standard working solutions and unknown samples were thawed. Internal standard was allowed to reach room temp before use. Standards and samples were vortex mixed for 10 seconds prior to addition. For aqueous humor samples, 5 µL was added to 250 µL glass inserts containing 15 µL acetonitrile. For serum samples, 10 µL was added to 250 µL glass inserts containing 35 µL acetonitrile. Subsequently, 5 µL of internal standard solution was added. The final concentration of internal standard for both tissue types was 20 ng/mL, and standards and unknown samples were diluted five times. The glass inserts were then vortex mixed briefly, centrifuged at 4000 rpm for 10 minutes at room temp, and loaded into amber HPLC vials. The vials were then loaded in the autosampler for analysis.

2.5. Method Validation

Prior to application of the developed UPLC-MS/MS to unknown samples, method validation was conducted to demonstrate reliability of the method

following FDA guidance [160]. During assay validation the following were systematically assessed: selectivity, specificity, sensitivity, standard curve range and linearity, precision and accuracy, extraction recovery, matrix effects, and carryover.

2.5.1. Selectivity and Specificity

The selectivity of the method was assessed by comparing chromatograms of six different blank aqueous humor/ serum samples to ensure no endogenous substances interfered at the retention times of either analyte. Specificity analysis was performed by comparing blank matrix samples with matrix spiked with CBD standard at the lower limit of quantitation (LLOQ; 0.5 ng/mL) and upper limit of quantitation (ULOQ; 1000 ng/mL in aqueous humor, 2500 ng/mL in serum). MRM was used to monitor two reactions to ensure the absence of interference at the retention time for each analyte.

2.5.2. Sensitivity

The sensitivity was defined as the lowest non-zero standard of the calibration curve, the LLOQ [160]. The LLOQ is set as the concentration at which a signal to noise (S/N) of 10:1 was reached with acceptable precision and accuracy (±20%). To assess sensitivity, blank aqueous humor/ serum samples were compared with samples of known concentrations to determine limit of detection (LOD). LOD was defined as the lowest concentration with S/N of 3:1.

2.5.3. Calibration Curve and Linearity

For the LLOQ to be acceptable, the lowest concentration of the calibration curve had to demonstrate acceptable precision and accuracy (±20% of nominal).

The calibration curves were constructed using calibration standards over the range of 0.5-1000 ng/mL in aqueous humor and 0.5-2500 ng/mL in serum. Linearity was assessed by plotting peak area ratio of CBD to internal standard vs. the nominal concentrations of the analyte. A weighted (1/x) least squares regression model was applied (GraphPad Prism 9, San Diego, CA). Calibration curves had to demonstrate acceptable precision and accuracy of ±15% at all concentrations except LLOQ which required precision and accuracy of ±20%. For linearity to be acceptable, a determination coefficient (R^2) of >0.98 was set.

2.5.4. Precision and Accuracy

Precision (coefficient of variance, C.V.) is defined as the percentage standard deviation divided by the mean CBD concentrations. Accuracy (% Bias) is calculated by percentage of the measured CBD concentration divided by the nominal concentration and subtracting 100. Precision and accuracy were evaluated using 4 concentrations along the calibration curve: LLOQ (0.5 ng/mL), 8 ng/mL, 500 ng/mL and ULOQ (1000 ng/mL) in aqueous humor; 2.5 ng/mL, 25 ng/mL, 500 ng/mL, and ULOQ (2500 ng/mL) in serum. For within-day assessments, samples were prepared on the same day (*n*=5); for between-day assessments, samples were prepared on five separate days (*n*=5). To be acceptable, the method had to demonstrate precision and accuracy within ±15% of the nominal value except at LLOQ that requires ±20% for both within- and between- day assessments.

2.5.5. Extraction Recovery, Matrix Effects, and Carryover

Extraction recovery was assessed by spiking blank matrix with CBD before and after protein precipitation. Samples were prepared in triplicate at four concentrations along the calibration curve. Extraction recovery was calculated with the following equation:

Recovery= (area ratio pre-extraction spike/area ratio post-extraction spike)*100

Matrix effects were assessed by comparing blank matrices spiked with CBD vs. acetonitrile spiked at the same concentrations of CBD. Matrix effect was determined by the following equation:

Matrix effect= (recovery in sample/recovery in acetonitrile) *100

Ion enhancement: Matrix effect> 100%

Ion suppression: Matrix effect< 100%

To assess carryover, an injection of blank matrix was performed after injection of ULOQ, the highest calibrator. To be acceptable, carryover could not exceed 20% of the LLOQ.

2.6 *Ex Vivo* Experiments

2.6.1 Preparation of Cyclodextrin Formulations Containing CBD

Solutions were prepared fresh on the day of experimentation. 10% RAMEB and HPβCD in deionized water were used to dissolve CBD. The concentration of cyclodextrin (CD) was chosen based on previous reports showing solubility of cannabinoids in CD [161-163]. Both CDs under study readily dissolved in deionized water with vortexing. An accurately weighed amount of

CBD was added to 10% RAMEB or HPβCD to a final concentration of 0.1% w/v. Preparations were sonicated to facilitate CBD dissolving.

2.6.2. Corneal Penetration Study

The *ex vivo* whole eye corneal penetration experiments were conducted as published previously [164,165]. Freshly enucleated porcine eyes were collected from the local butcher shop (JBS Swift, Louisville KY) and transported on ice to the laboratory. Only intact eyes with no corneal lacerations or opacification were used within 2 hours of enucleation. Eyes were trimmed of excess tissue and placed into six-well cell culture plates. Eyes were held in place from the bottom using modeling clay and lined with cling film to prevent the eyes from rolling. To mimic a donor chamber of a Franz diffusion cell, a corneal sleeve with a diameter of 0.5'' was held in place on top of the eye with cling film [164,165]. 50 µL of CBD formulations were injected into the donor chamber (the corneal sleeve). The entire setup was incubated at 37°C. At pre-determined time points, eyes were removed from the setup and rinsed thoroughly with deionized water. Aqueous humor was removed by paracentesis with a 28G needle and transferred to an Eppendorf tube. Aqueous humor samples were stored at -20°C until analysis by UPLC-MS/MS method described above.

2.7 *In Vivo* Experiments

2.7.1 Animal Maintenance and Housing

Pharmacokinetic assessments were performed with female wild type C57/B6 mice aged 18-20 weeks (Jackson Laboratory, Bar Harbor, ME). IOP measurements were performed with female wild type C57/B6 mice aged 2-5

months (Jackson Laboratory, Bar Harbor, ME). Experiments were conducted at University of Louisville health sciences campus. All mice were handled according to IACUC approved methods (protocol number 21941). Animals were housed in clean, federally regulated and AAALAC-accredited facilities operated by the University of Louisville School of Medicine Department of Animal Care. Animals were housed four per cage with free access to food and water, in humidity and temperature-controlled room with a 12h light/dark cycle. All animals utilized in this project were monitored daily for evidence of discomfort, distress, pain or injury.

2.7.2 Drug Preparation and Administration

CBD in crystalline form was dissolved in ethanol and added to Tween20 solution (3% ethanol, 3% Tween 20, 94% normal saline). CBD was administered (100 µL) intraperitoneally at a dose of 50 mg/kg. Control animals were administered corresponding amounts of the vehicle. The doses used in the current study were selected according to previous literature; the highest dose of CBD used in a clinical setting is 50 mg/kg, mainly for drug resistant epilepsy and secondary epilepsies [166].

2.7.3 Pharmacokinetic Analysis and Tissue Collection

A single dose aqueous humor pharmacokinetic study using C57BL/6J (B6) mice was conducted. CBD preparations were administered intraperitoneally at a dose of 50 mg/kg. Mice were anesthetized and euthanized by intracardiac puncture. Blood was collected by intracardiac puncture at 5 min, 15 min, 30 min, 1h, 2h, 3h, 4h, 6h, 16h, and 24h post- administration and serum was separated.

Eyes were enucleated at these time points and aqueous humor was collected post mortem using a previously published method [167]. Each experimental group for each time points comprised of 5 animals. The total number of mice used for pharmacokinetic assessment was 50 (n= 5 per group). Samples were stored at -20°C until UPLC-MS/MS analysis. All samples were processed and analyzed as described above.

2.7.4 Intraocular Pressure Measurement

IOP was measured by rebound tonometry with an ICARE TONOLAB tonometer (Icare Finland Oy, Vantaa, Finland). Baseline IOP measurements were taken in both eyes without anesthesia since anesthesia can affect IOP [168-170] and anesthesia can be avoided with proper animal conditioning [168]. IOP was measured 6 times per eye, and the average for each eye was used for analysis. Following baseline measurement, a single intraperitoneal injection of 50 mg/kg CBD or vehicle (3% ethanol, 3% Tween20, 94% normal saline) was administered. IOP was then measured in both eyes at 3 time points (0.5, 1.5, and 4 hours post CBD administration). The number of mice used for IOP measurement was 14 (n=7 per group).

2.8 Data Analysis

All statistical analyses were performed with GraphPad Prism 9.1.2 software (San Diego, CA). For corneal penetration studies, differences in CBD concentrations applied with CD formulations at each time point were compared by t-test and significance was set at $p<0.05$. Pharmacokinetic parameters were determined by non-compartmental analysis using GraphPad Prism. Maximal

drug concentration (C_{max}), time of maximal concentration (T_{max}), apparent elimination half-life ($t_{1/2}$), AUC and aqueous humor bioavailability based on $AUC_{(0\text{-}16)}$ aqueous humor/serum and dose ratios were evaluated for serum and aqueous humor following intraperitoneal administration of CBD. Analysis of IOP measurements following drug administration was performed using a *t*-test with a significance threshold fixed at $p<0.05$.

3. Results

3.1. Method Validation

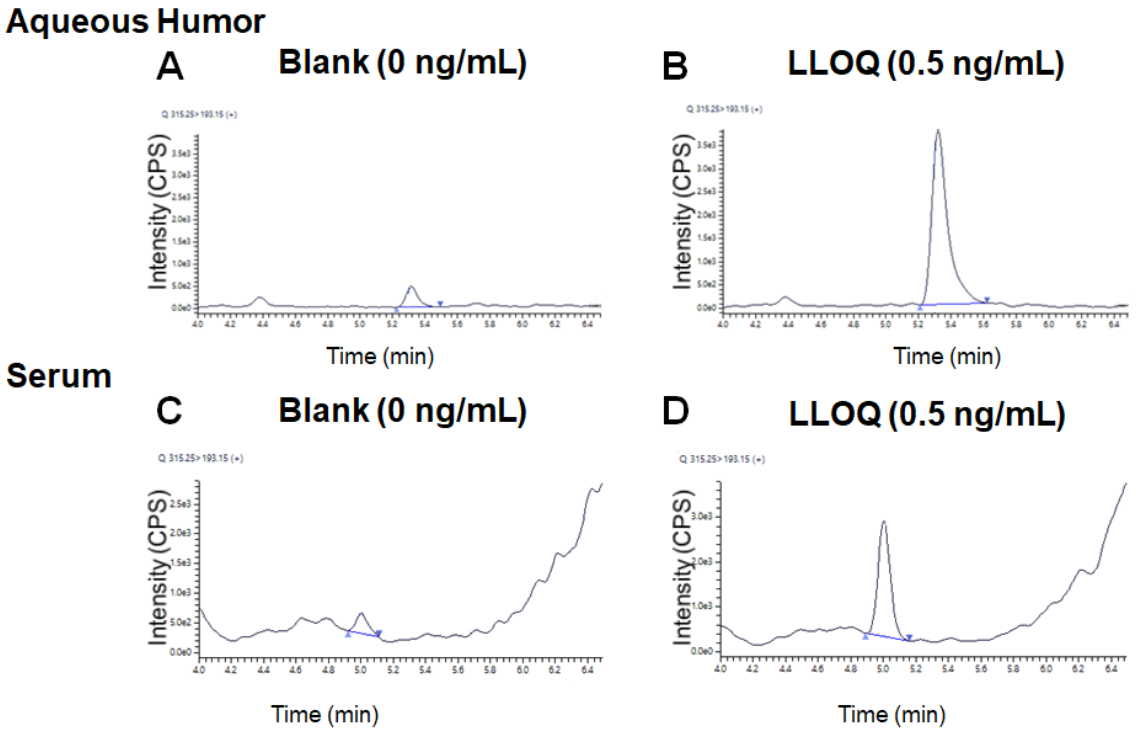
3.1.1. Selectivity and Specificity

Six blank aqueous humor from different eyes and serum samples were checked for the method specificity and selectivity. Representative chromatograms of blank and LLOQ (0.5 ng/mL) aqueous humor and serum are shown in Figure 6. The method is highly selective and specific for detecting CBD at a retention time of 5.33 minutes. Further, a concentration of 0.0012 ng/mL was detected in blank aqueous humor and 0.0083 ng/mL in blank serum (Figure 1A and 1C), which is 0.27% and 1.66% of the LLOQ, respectively. Thus, at the ion transitions used in this method, no endogenous substances interfered with the ionization of analytes in any blank matrix samples at retention times of CBD or CBD-d_3.

3.1.2. Sensitivity

Sensitivity was determined for the method by comparison of blank aqueous humor/serum samples and LLOQ. The method demonstrated acceptable sensitivity; LLOQ for CBD were accurate and precise within ±10 of the nominal concentration for both tissue types. Measured concentrations in blank aqueous humor and serum, if any, were less than 1% of the LLOQ. Thus, the response at LLOQ was greater than ten times the analyte response (S/N>10:1) of the zero calibrator for both analytes under study. LOD was determined to be 0.1 ng/mL for both tissue types.

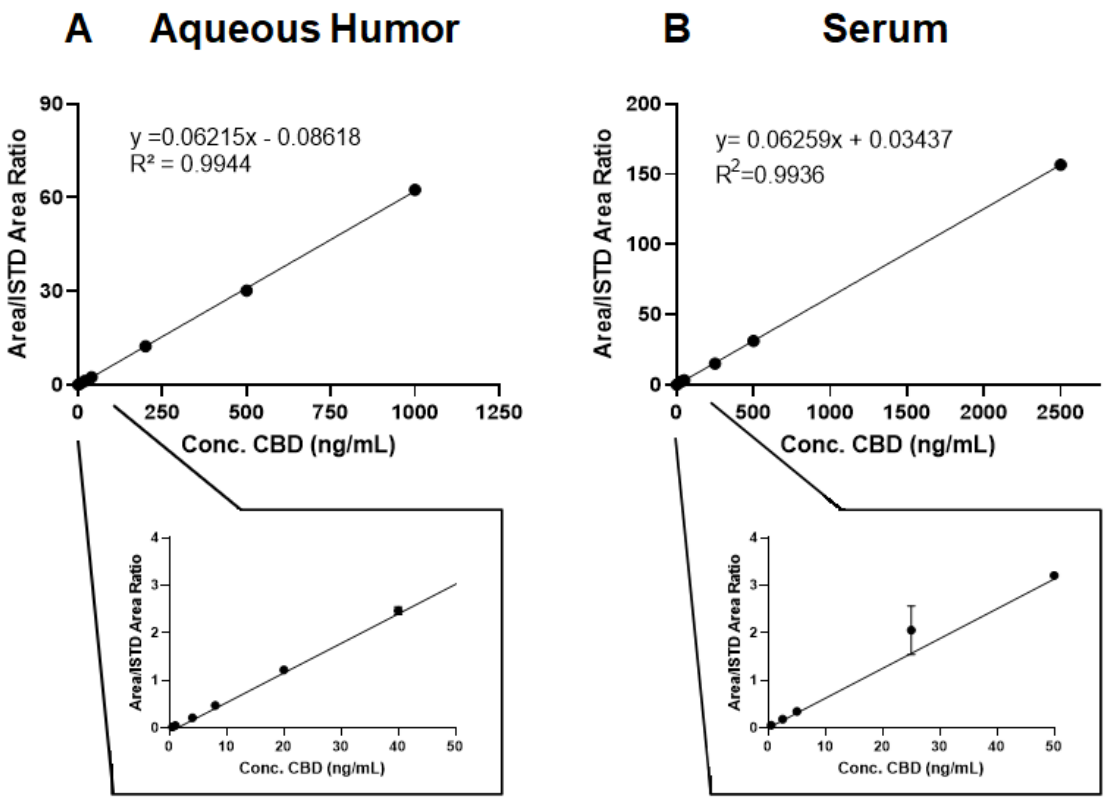
Figure 6. Representative UPLC-MS/MS Chromatograms of CBD Analytes in Serum. (A) aqueous humor blank and (B) aqueous humor spiked at LLOQ (0.5 ng/mL); (C) serum blank and (D) serum spiked at LLOQ (0.5 ng/mL). CBD, cannabidiol; CPS, counts per second.



3.1.3. Calibration Curve and Linearity

A 9- point calibration curve was established for aqueous humor and serum by plotting the ratios of chromatogram peak areas of CBD to internal standard versus CBD nominal concentrations. As shown in Figure 7, calibration curves were linear (R^2> 0.99) within the calibration range for aqueous humor (0.5-1000 ng/mL) and serum (0.5-2500 ng/mL). The weighted linear regression equations for CBD in aqueous humor and serum were y= 0.06215x – 0.08618 and y=0.06259x+0.03437 respectively, where y represents the peak area ratio of CBD to internal standard, and x denotes the concentration of analytes in matrix. The LLOQ of CBD was 0.5 ng/mL in both tissue types based on a S/N ratio of >10:1, and adequate precision and accuracy within ±20% was achieved.

Figure 7. Calibration Curve for CBD in (A) Aqueous Humor and (B) Serum**.** Linearity determined with a weighted (1/x) least squares regression, and equation is displayed. The determination coefficient (R^2) and equation are shown. conc., concentration.



3.1.4. Intra- and Inter- day Precision and Accuracy

The intra- and inter- day accuracy and precision of CBD in porcine aqueous humor and murine serum at 4 concentrations (aqueous humor: LLOQ 0.5 ng/mL, 4 ng/mL, 500 ng/mL, and ULOQ 1000 ng/mL; serum: 2.5 ng/mL, 25 ng/mL, 500 ng/mL, and ULOQ 2500 ng/mL) are shown in Table 5. Precision is reported as coefficients of variation, and accuracy is reported as % bias. Precision and accuracy for all concentrations were less than 10% for CBD. These data demonstrate that the method is acceptable for measurement of CBD in porcine aqueous humor and murine serum per FDA guidance [160].

Table 5. Inter-day and Intra-day Accuracy and Precision of CBD Quantification in Porcine Aqueous Humor and Murine Serum. Samples were prepared on 5 different days (*n*=5) for inter-day assessments, and same day (*n*=5) for intra-day assessments.

Aqueous Humor						
	Inter- day			**Intra-day**		
Conc. (ng/mL)	Mean ± SD	Accuracy (Bias %)	Precision (C.V. %)	Mean ± SD	Accuracy (Bias %)	Precision (C.V. %)
0.5	0.4951±.03770	-0.9880	7.620	0.5059±0.02226	1.170	4.401
8	7.961±0.4024	-0.4930	5.055	8.110±0.2778	1.370	3.426
500	500.9±23.73	0.1735	4.737	499.3±29.11	-0.1476	5.830
1000	1002±27.30	0.2024	2.725	999.1±35.42	-0.08647	3.545
Serum						
	Inter- day			**Intra-day**		
Conc. (ng/mL)	Mean ± SD	Accuracy (Bias %)	Precision (C.V. %)	Mean ± SD	Accuracy (Bias %)	Precision (C.V. %)
2.5	2.492±0.09556	-0.3100	3.834	2.491±0.1570	-0.3700	6.302
25	24.45±0.9201	-2.218	3.764	24.49±1.412	-2.048	5.765
500	498.8±8.987	-0.2460	1.802	493.9±11.57	-1.648	2.342
2500	2504±17.67	0.1420	0.7058	2502±14.42	0.2225	0.5763

3.1.5. Extraction Recovery, Matrix Effects, and Carryover

Extraction recoveries and matrix effects and are reported in Table 6. Extraction recovery of CBD was assessed at 4 concentrations along the calibration curve by comparison of blank matrix (aqueous humor or serum) spiked with CBD pre- and post- protein precipitation. The absolute CBD recoveries in aqueous humor and serum were 66.06±5.146% and 78.32±5.017% respectively, demonstrating sufficient recovery of CBD in both tissue types.

Matrix effects were determined by comparing blank matrix (aqueous humor or serum) spiked with CBD and acetonitrile spiked at the same concentrations at four concentrations along the calibration curve. CBD had insignificant ion suppression of 7.318±8.197% in aqueous humor and ion enhancement of 28.20±8.681% in serum; matrix effects were consistent across each concentration measured. The matrix effects were controlled for by using deuterated internal standard.

Table 6. Recovery and Matrix Effects of CBD in Porcine Aqueous Humor and Murine Serum. CBD recovery and matrix effects at four concentrations along the calibration curve (*n*=3 at each level).

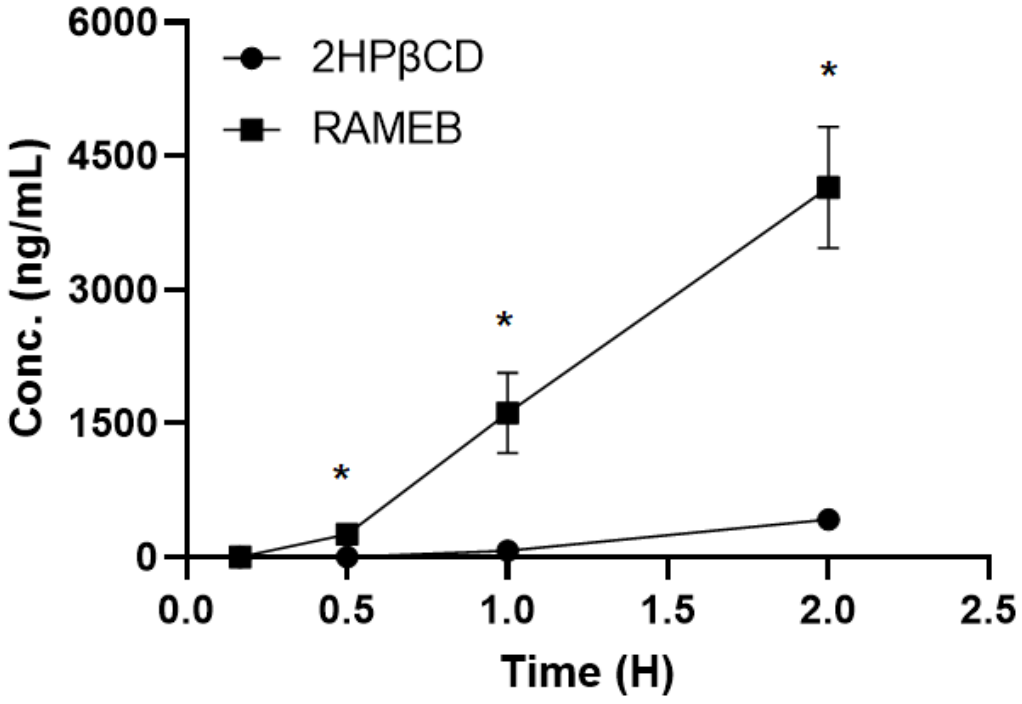
Aqueous humor			**Serum**		
Conc. (ng/mL)	Recovery (%)	Matrix Effect (%)	Conc. (ng/mL)	Recovery (%)	Matrix Effect (%)
0.5	71.43	83.62	2.5	71.57	138.8
8	69.27	99.49	25	82.52	125.3
500	60.47	99.75	500	81.72	118.2
1000	63.05	87.87	2500	77.46	130.5

Injection carryover was assessed by injecting blank samples after injection of the highest calibrator. Insignificant carryover was set at less than 20% of the LLOQ. Carryover of CBD in blank aqueous humor sample was low following the ULOQ (7.093±10.13%). Carryover was higher in serum blank samples however following the ULOQ (21.50±29.69) A second blank injection was performed following the first and shown to have no carryover (0.00±0.00%). Thus, to control for high carryover, a blank injection was performed following the highest calibrator.

3.2. Corneal Penetration Study

The validated LC-MS/MS method was used to analyze aqueous humor samples collected during the *ex vivo* corneal permeation experiment to assess the penetration of CBD through the porcine cornea. Porcine aqueous humor samples were diluted five-fold during sample preparation for UPLC-MS/MS, so that measured concentrations were within the established standard curve. Aqueous humor concentrations of CBD increased in a time dependent manner for both CDs under study (Figure 8). The concentration of CBD in aqueous humor was significantly different between the two CD formulations at all the time points except 10 minute (*t*-test, $p<0.05$), with RAMEB formulation measured at roughly 10 times higher aqueous humor CBD concentration than the 2HPβCD formulation (4148±1521 ng/mL, and 424.8±213.8 ng/mL, respectively).

Figure 8. Corneal Penetration of CBD From Cyclodextrin Formulations. The amount of cannabidiol recovered (ng/mL) in aqueous humor after application of a single 50 µL 1 mg/mL dose of cyclodextrin (either randomly methylated β CD or 2-hydroxypropyl-β-CD) formulations plotted against time (*n*=5; mean±SEM). The significance between formulations as determined by *t*-test is noted with *, *p*<0.05.



3.3. Pharmacokinetic Study

The validated method was used to measure unknown aqueous humor samples collected at 8 time points. Five mice were used per time point (5 min, 15 min, 30 min, 1h, 2h, 3h, 4h, 6h, 16h, and 24h). All concentration values measured were within the range of the validated method. CBD was not detectable in aqueous humor at the 16 and 24h time points. The mean±SEM concentration-time curves are shown in Figure 9. The main pharmacokinetic parameters determined with GraphPad Prism are shown in Table 7. In our study, T_{max} was 2 hours in aqueous humor and 15 minutes in serum, C_{max} was 71.55 ng/mL in aqueous humor and 4826 ng/mL in serum (Figure 9, Table 7). Elimination of CBD from the aqueous humor was rapid with a $t_{1/2}$ of 1.046 hours, and prolonged in serum ($t_{1/2}$ 3.854 hours).

Figure 9. Concentration Versus Time Plots of CBD in Aqueous Humor and Serum. Mean±SEM (A) aqueous humor and (B) serum concentration-time profiles following a single intraperitoneal administration of 50 mg/kg CBD (*n*=4 at each time point).

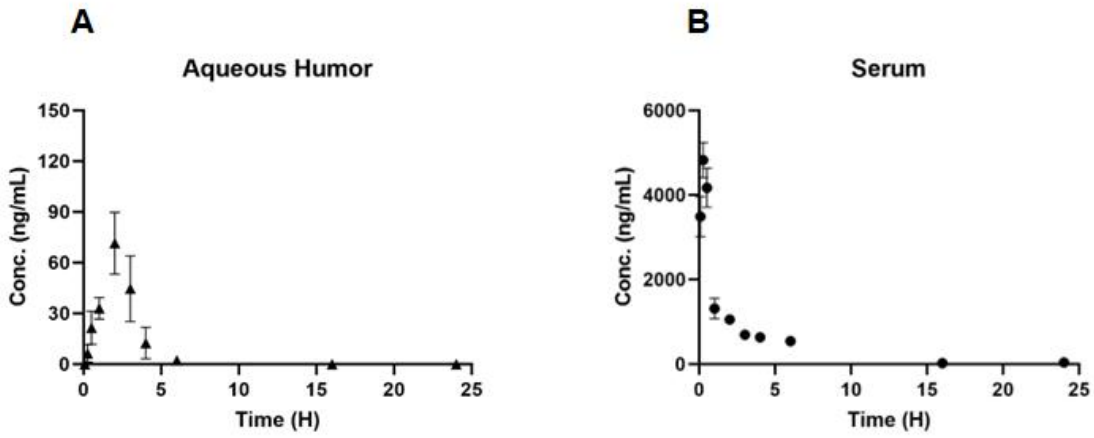


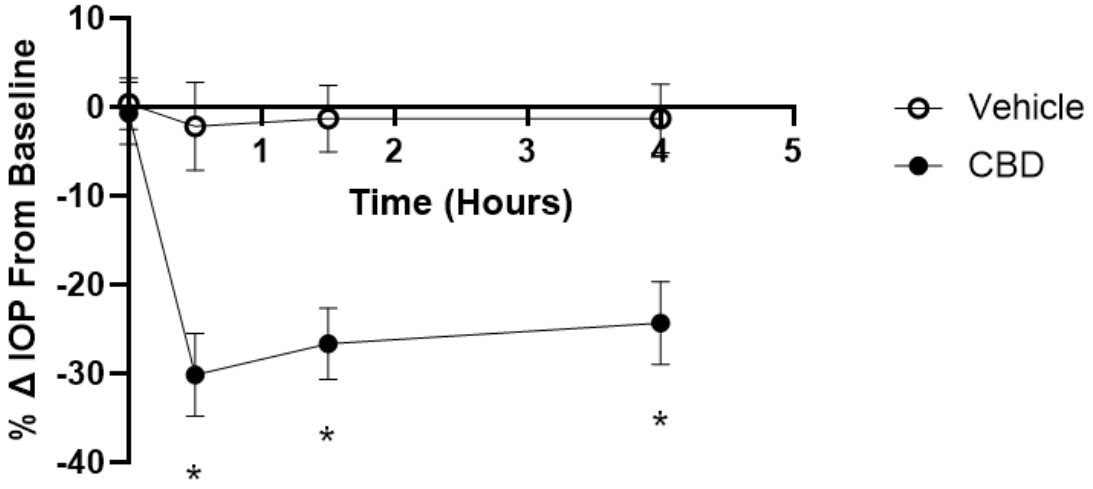
Table 7. Pharmacokinetic Parameters for CBD in Mouse Aqueous Humor and Serum Following Single Intraperitoneal (50 mg/kg) Administration (*n*=4).

PK parameter	Aqueous Humor	Serum
C_{max} (ng/mL)	71.55	4826
T_{max} (h)	2	0.25
Apparent $t_{1/2}$ (h)	1.046	3.854
$AUC_{0\text{-}16h}$ (ng/mL*h)	183.4	10002
F%	1.835	

3.4 Intraocular Pressure Changes Induced by CBD

The effect of a single intraperitoneal injection of 50 mg/kg CBD on IOP in WT C57BL/6J mice was measured. IOP was measured at 3 time points following injection of vehicle only or vehicle containing CBD. Changes in IOP following control or CBD treatment are shown in Figure 10. Mice given vehicle only were devoid of any significant change in IOP from baseline. Conversely, CBD produced a significant (31.01 ± 16.08%) decrease from baseline at 30 minutes post-administration. Compared to the vehicle treated group, CBD significantly decreased IOP at each time point post-administration up to the 4-hour observation period.

Figure 10. Fall in Murine IOP After Single Intraperitoneal Injection of CBD. Values displayed are the mean ± SEM for both eyes from 7 mice per treatment. A single intraperitoneal injection of CBD (50 mg/kg) lowers IOP relative to control and baseline IOP measurements. Statistical significances between CBD treated versus control treated mice were analyzed by *t*-test, **p*<0.05.



4. Discussion

The overall goal of the current work was to measure CBD in aqueous humor, and correlate the concentration with IOP effect following intraperitoneal administration. In order to achieve this goal, an UPLC-MS/MS method was developed. To our knowledge, the current paper reports the first validated method for measurement of CBD in aqueous humor via UPLC-MS/MS. The novel method described herein is specific, accurate and precise. CBD peaks were well resolved with a retention time of 5.33 minutes. The concentration of CBD varied linearly with the peak areas with a determination coefficient >0.99; recovery was >60% and no significant matrix effects were present. Accuracy and precision were within 10% for within and between day measurements.

To measure physiologically relevant concentrations of CBD in small volumes of aqueous humor, a method with a low LLOQ and small sample requirements is necessary. Our LC-MS/MS method has a LLOQ of 0.5 ng/mL, sample volume requirement of 5 µL, and injection volume of 5 µL. This LLOQ in aqueous humor is comparable to LLOQ of CBD in other biological tissues measured by LC-MS/MS [153,154].

Aqueous humor outflow is the largest contributing factor to IOP [109]. A hypotensive agent has to be able to penetrate the cornea, and reach the aqueous humor to regulate outflow through the TM. In this study, we measured CBD concentration in aqueous humor following topical application *ex vivo*. CBD (1 mg/mL) was applied in 10% RAMEB and 2HPβCD since cyclodextrins can increase solubility of cannabinoids in aqueous solution [163,171]. Both RAMEB

and 2HPβCD facilitated corneal penetration through the cornea, resulting in a time dependent increase in CBD aqueous humor concentration. Maximum CBD aqueous humor concentrations of 4148±1521 ng/mL and 424.8±213.8 ng/mL were achieved at 2 hours following application in RAMEB and 2HPβCD, respectively.

The UPLC-MS/MS method was utilized to conduct pharmacokinetic assessment of CBD in aqueous humor and serum samples following a single intraperitoneal administration of 50 mg/kg CBD. Pharmacokinetics of CBD has been extensively studied in humans [172], however study of CBD ocular pharmacokinetics is not widely studied. One group [159] was unable to measure CBD in ocular tissue following topical application. Administration of CBD intraperitoneally avoids gastric first pass metabolism of CBD which hinders bioavailability of the cannabinoid [173]. The intraperitoneal route was also chosen to determine if CBD administered systemically can penetrate the blood-retinal barrier and reach aqueous humor. In the present study, the use of vehicle containing ethanol and surfactant Tween20 enabled a C_{max} of 4.8 µg/mL in serum. This finding is on par with previous work showing high concentrations of cannabinoids following i.p, administration in a lipid soluble vehicle in other tissues [174,175]. Interestingly, a C_{max} of 71.55 ng/mL was observed in aqueous humor.

Next, IOP was measured following a single 50 mg/kg intraperitoneal injection of CBD. The delivered dose of CBD decreased IOP beginning at 30 minutes and lasting the duration of observation (4 hours). These results also show that CBD applied systemically in the Tween-based solvent can penetrate

the blood-retinal barrier and lower IOP. Putting together pharmacokinetics and tissue concentrations, T_{max} in aqueous humor (2 hours) did not correspond with maximal reduction in IOP (30 min). Though the $t_{1/2}$ was 1.046 hours, CBD reduced IOP for the duration of measurement (4 hours), which suggests that low concentrations of CBD can also mediate changes to IOP. In Chapter II it was observed that 1 µM (314 ng/mL) increased aqueous humor outflow, indicative of possible IOP regulation. Our work shows that CBD at low micromolar (0.23-1 µM) can alter IOP and AH outflow.

5. Conclusions

In summary, in this study we have developed and validated a sensitive, specific, accurate, and precise UPLC-MS/MS method for measurement of CBD in aqueous humor and serum. With the low limit of quantitation (0.5 ng/mL) and the small sample volume requirement (5 µL), the method was applied to measurement of CBD in aqueous humor for pharmacokinetic assessment. Further, we were able to correlate the concentrations of CBD in aqueous humor with its IOP lowering effects following intraperitoneal administration in mice. We have also used the method to successfully study the transcorneal penetration of CBD dissolved in RAMEB and HPβCD aqueous vehicles *ex vivo*. Therefore, the development and validation of this UPLC-MS/MS method is an important step toward assessing the aqueous humor concentrations of CBD and correlating the concentrations of this phytocannabinoid with its pharmacological effects (such as IOP regulation) in the eye.

CHAPTER IV

OCULAR APPLICATION OF CBD: EFFECT ON INTRAOCULAR PRESSURE AND CORNEAL PAIN

1. Introduction

Cannabidiol (CBD) is a phytocannabinoid derived from *Cannabis sativa*. While non-psychotropic, CBD is psychoactive mediating therapeutic benefits in the central nervous system. CBD was FDA approved for treatment of pediatric epilepsies in 2018 [23]. In addition, CBD is well documented to have immune suppressive [176], anti-inflammatory [27], anti-oxidant [27], and neuroprotective effects [31]. These properties have been demonstrated in the eye as well [123]. Glaucoma is a progressive neurodegenerative disease of the eye [109,111,177]. Glaucoma pathology is marked by elevation of intraocular pressure (IOP), optic nerve damage, loss of retinal ganglion cells, and eventual blindness if left untreated [109,111,177]. Over the years, several studies have shown CBD lowers IOP, the leading risk factor for glaucoma [74-76,108].

However, there is a lot of controversy in the literature regarding the effects CBD applied topically to the eye on IOP [63,75,76,108]. This controversy could be due to poor delivery. Contributing to poor delivery, CBD is highly hydrophobic with an aqueous solubility of 0.06 µg/mL, making ocular delivery difficult [178].

Previously, for topical delivery, CBD had to be dissolved in oily carriers such as mineral oil or polyethylene glycol (PEG) [34,105]. However, both mineral oil and PEG are known to be irritating and toxic to the eye [34,105]. In addition, IOP-lowering effects of CBD appeared to have a short duration of action. In a previous study, application via osmotic minipump was required to maintain IOP reduction in cats [76]. To these ends, application of CBD in a vehicle which improves solubility, corneal penetration and allows extended release should increase CBD ocular bioavailability and enable prolonged reduction in IOP.

The cornea is one of the most densely innervated tissues in the body. Damage to corneal neurons resulting from surgery, trauma, disease or infection can result in chronic pain and inflammation [38]. There are a couple reports showing that CBD has promise as treatment for ocular inflammation and pain [39,108]. Following induction of hyperalgesia, topical application of CBD reduced pain score and neutrophil infiltration [39]. The antinociceptive and anti-inflammatory action of CBD was not blocked by CB1 antagonist, but was blocked by 5HT1A antagonist WAY100635 [39]. In an LPS-induced keratitis inflammation model, topical application of CBD nanoemulsions reduced inflammatory cytokines KC, IL-6, MIP-2 and GCSF dose-dependently [108]. For treatment of cornea pain or inflammation, an ideal vehicle should allow the delivery of CBD to the cornea but not inside the eye.

Cyclodextrins (CDs) are a class of amphiphilic compounds that can improve solubility and bioavailability of hydrophobic drugs. CDs are cyclic oligosaccharides ranging from 1000-1500 g/mol, easily soluble in aqueous tear film due to the outer

hydrophilic region of the CD molecules but too large to penetrate the cornea [179]. Complexation of drugs with CDs through an inclusion complex which forms no covalent bonds enhances penetration of hydrophobic drugs through increased drug-epithelial surface interaction and availability at the corneal barrier, increases solubility and stability, limits degradation, and masks irritation from the drug itself [179,180]. Currently, several cyclodextrin-containing eye drops, including those containing either methyl-β-cyclodextrin are on the market for the topical delivery of hydrophobic drugs to the eye [179,180].

CDs have been shown to improve the solubility of various classes of cannabinoids in aqueous solution including endocannabinoids [181], phytocannabinoids [161-163], and synthetic cannabinoids [182,183]. CDs also increase transcorneal permeability of cannabinoids [163,171]. Additionally, CDs have been shown to increase aqueous solubility of CBD [178]. However, so far CDs have not been reported as a vehicle for ocular delivery of CBD.

A sol-to-gel system is one which changes from a liquid to a semi-solid gel in response to stimuli such as pH, electrolytes, temperature and light exposure [184,185] [186]. *In situ* sol-to-gel forming systems are extensively used for ocular delivery. Once the gel is formed, it adheres to the cornea and drugs suspended in the hydrogel are protected from drainage from the precorneal area which subsequently increases precorneal residence time and ocular bioavailability [184,185]. For ocular application, there are 2 FDA approved drugs for glaucoma containing hydrogels: Pilopine HS and Timoptic XE.

Hydrogels alone are not sufficient to deliver hydrophobic drugs due to the inherent aqueous environment, so a frequently used strategy is to encapsulate the hydrophobic drug in a carrier suspended in the hydrogel [187]. Previous reports have shown that poorly soluble drugs are able to be complexed with CDs and loaded into gellan gum (GG) hydrogels without disrupting gel formation. Further, combination of CD and GG increases permeability of poorly soluble drugs [187]. CD- hydrogel systems have been employed for topical delivery of a variety of hydrophobic drugs [187-191]. However, hydrogels have not been reported in ocular delivery of CBD.

Semifluorinated alkanes (SFAs) are a novel class of compounds capable of delivering hydrophobic drugs [165,192,193]. SFAs are amphiphilic molecules, have low viscosity and surface tension, possess a similar refractive index to water (thus no blurry vision), and do not require preservatives since the drops are water free and microbial growth is not possible [165,192,193]. Topical use of SFAs avoids spillover and loss of drug which increases drug bioavailability. SFAs are physically, chemically and physiologically inert, metabolized by the human body, and are well tolerated [165,192,193]. Novaliq, a German company, produces ocular pharmaceuticals containing SFAs. NovaTears™ is approved for over-the-counter use in Europe for the treatment of dry eye disease. Novaliq also has drugs in various stages of clinical trials for dry eye disease (CyclASol and NovaTears Rx) and glaucoma (BTQ 1901/ BTQ 190, timolol and nebivolol respectively) [194]. As yet, CBD has not been applied in an SFA, and the corneal penetrative capacity of CBD with this carrier is unknown.

In the currently study, CBD was applied topically to the eye with vehicles intended to improve its ocular delivery through improved solubility, corneal permeability and extended release. These vehicles include: randomly methylated β cyclodextrin (RAMEB), RAMEB in combination with Gelrite® GG (RAMEB+GG), or the SFA 1-(perfluorobutyl)pentane (PFBP). Using an *ex vivo* porcine eye model, aqueous humor and cornea concentrations of CBD were measured following topical application. Furthermore, using mice, changes in IOP and corneal pain following topical administration of CBD in different formulations were assessed.

2. Materials and Methods:

2.1 Chemicals and Reagents

CBD standard solution for chromatographic and spectrophotometric analysis was purchased from Cerilliant (Round Rock, TX). For chromatographic and spectrophotometric analysis, HPLC grade methanol and acetonitrile were purchased from Fischer Scientific (Waltham, MA). CBD for hydrogel release, corneal penetration, and IOP assessments was purchased from Extract Labs (Lafayette, CO). For all experiments, RAMEB was purchased from Sigma (St. Louis, MO). Gelrite™ GG was purchased from Research Products International (Mount Prospect, IL), and PFBP was purchased from Fluoryx Labs (Carson City, NV).

2.2 Preparation of Formulations Containing CBD

2.2.1 RAMEB

RAMEB was accurately weighed and dissolved in deionized water with vortexing and sonication. The percentage of 10% RAMEB was selected because it was previously shown to improve aqueous solubility of CBD [178]. After RAMEB fully dissolved, an accurate weight of CBD was added to a final concentration of 0.1 mg/mL. CBD readily dissolved in the 10% RAMEB solution with sonication. Solutions were stored overnight at 4°C to ensure complete drug-inclusion complex formation.

2.2.2 RAMEB+GG Formulations

First, the 10% RAMEB containing CBD solution as prepared as described in section 2.2.1. Subsequently on the following day, GG was added to a final concentration of 0.4%. A percentage of 0.4% was selected due to the immediate and persistent gelation in preliminary experiments. Gel-containing formulations were heated at 85°C with vortexing for 5 minutes. The solution was then allowed to cool and hydrate at room temperature for 10 minutes. Solutions were prepared on experimental days for *in vitro* release, corneal penetration, and IOP experiments. Formulations were tested for gel formation by addition to PBS prior to experimentation.

2.2.3 PFBP

CBD was accurately weighed and PFBP was added to a final concentration of 1 mg/mL. CBD readily dissolved in PFBP with sonication. Solutions were prepared fresh for corneal penetration and eye wipe experiments.

2.3 *Ex Vivo* Corneal Penetration Study

Ex vivo corneal penetration experiments were conducted as published previously [195]. Briefly, freshly enucleated porcine eyes were placed in six-well culture plates containing modeling clay covered with cling film, and corneal sleeve was held to the cornea. 50 µL of RAMEB, RAMEB+GG, or PFBP formulations containing 0.1 mg/mL (RAMEB, RAMEB+GG) or 1 mg/mL (PFBP) CBD were applied to the corneal sleeve. Eyes were incubated with different formulations of CBD at 37°C for 1,2, and 4 hours. After incubation, corneal sleeves were removed and eyes were thoroughly rinsed with deionized water before collection of tissues. Aqueous humor was collected by paracentesis, and

corneas were collected through excision. Five replicates were used per time point (*n*=5) for each formulation. Samples were stored at -20°C until analysis by High Performance Lipid Chromatography-Ultraviolet Visible spectroscopy (HPLC-UV/VIS) described below.

2.4 *In Vitro* Drug Release Study

In vitro CBD release from RAMEB+GG formulations was conducted similarly to previously published methods [188,196,197]. Briefly, 0.5 mL formulation was added to 200 mL phosphate buffered saline (PBS: 0.137M sodium chloride, 0.0027 M potassium chloride, 0.01 M sodium phosphate dibasic, and 0.0018 M potassium phosphate monobasic, pH 7.2) with care to ensure no air bubbles were present inside the hydrogel solutions. Upon interaction with PBS, the ion-sensitive GG formed a hydrogel. Containers with PBS and RAMEB+GG (or RAMEB as a control) were maintained at 37°C at 60 revolutions per minute using an orbital shaker (Gyromax™ 767, Amerex, Concord, CA). Aliquots (1 mL) were collected directly into HPLC vials at each sampling time, and replaced with an equal volume of PBS. The amount of CBD in collected PBS samples was analyzed by HPLC-UV/VIS. The experiment was performed in triplicate.

2.5 Sample Preparation for HPLC-UV Analysis

PBS samples were not modified prior to analysis. Aqueous humor samples were first protein precipitated and diluted prior to analysis: 50 µL aqueous humor samples collected from the *ex vivo* corneal penetration study were diluted with 100 µL ice cold acetonitrile to precipitate protein. Samples were

then vortexed for 15 seconds, centrifuged at 1600g for 10 minutes at room temp, and 100 µL of supernatant was transferred to a 250 µL glass insert in an HPLC vial for analysis as described below.

Corneal samples were rinsed with deionized water after removal, weighed, minced, 1 mL acetonitrile added and homogenized using a Polytron tissue homogenizer (Kinematica, Bohemia, NY). After homogenization, an additional 1 mL acetonitrile was added (total volume 2 mL), and samples vortex mixed. To fully extract CBD from corneal tissue, samples incubated for 30 minutes at room temperature. Following extraction, samples were centrifuged for 10 minutes at a speed of 4000 revolutions per minute (3270 x *g*) at room temperature. After centrifugation, 1 mL of supernatant was collected for HPLC-UV/VIS analysis.

2.6 Quantification of CBD in PBS, Aqueous Humor, and Cornea by HPLC

An HPLC-UV/VIS method was used to quantify CBD in matrices under study (PBS, aqueous humor, and corneal tissue). Standards and samples were run on a Flexar FX-15 (Perkin Elmer, Waltham MA) system connected to a UV-VIS spectrophotometer. The analytical column was Raptor ARC-18 column 150x 2.1mm, 1.8 µm analytical column (Restek, Bellefonte, PA). The mobile phase was a mixture of methanol and water (80:20 v/v) and degassed in an ultrasonic bath before use. An isocratic analysis at a flow rate of 0.3 mL/min was performed with column oven set to 55°C. The injection volume was 5 µL and the detection wavelength was set at 207 nm. Samples were analyzed at 207 nm, since it was demonstrated to yield maximum absorption in preliminary experiments, and 205-210 nm was previously shown to be the maximum absorption for CBD [198,199].

The quantification of CBD was performed by measuring the peak area count compared to the analytical reference standard solution of CBD in PBS, aqueous humor or cornea extracted acetonitrile at calibrator concentrations. Calibration solutions were stored at -20°C until time of analysis. Calibration standards containing known amounts of CBD were prepared by spiking PBS, aqueous humor, or corneal extracted acetonitrile with CBD standard (Cerilliant, Round Rock, TX).

A calibration curve was run for PBS was run (100-5000 ng/mL) on the same day as sample analysis. The method was further validated for biological samples (aqueous humor and cornea extracted acetonitrile) with linearity, limit of quantitation, limit of detection, within day precision and accuracy. Linearity was determined using freshly made calibration curves (aqueous humor: 100-5000 ng/mL and cornea: 100-25000 ng/mL) prepared from CBD stock solutions (n=3). The limit of detection (LOD) and limit of quantitation (LOQ) were defined as signal-to-noise ratios of 3:1 and 10:1, respectively. Intra-day precision of the method was evaluated variability, and intra-day accuracy of the method was evaluated with bias from the nominal value. For both precision and accuracy, 3 replicates of each concentration along the calibration curve were run on the same day. the mean concentration values, coefficient of variance (C.V.) and accuracy (% bias) were calculated.

2.7 Animals and Animal Housing

IOP assessment was performed with female wild type C57/B6 mice aged 11-19 weeks, and eye wipe assessments were performed with male wild type

C57/B6 mice aged 11-18 weeks (Jackson Labs, Bar Harbor, ME). The number of mice used for the IOP test were 28 (*n*=7 per group), and eye wipe test were 21 (*n*=7 per group). All experiments were conducted with University of Louisville IACUC approved methods (protocol number 21941). Animals were housed in clean, federally regulated and AAALAC-accredited facilities operated by the University of Louisville School of Medicine Department of Animal Care. Animals were housed four per cage with free access to food and water, in humidity and temperature-controlled room with a 12h light/dark cycle. All animals utilized in this project were monitored daily for evidence of discomfort, distress, pain or injury.

2.8 IOP Experimental Design

To test the effects of topically applied CBD, IOP was measured by rebound tonometry with an ICARE TONOLAB tonometer (Icare Finland Oy, Vantaa, Finland). IOP measurements were conducted without anesthesia since anesthesia can affect IOP [168-170] and anesthesia can be avoided with proper animal conditioning [168]. Baseline IOP measurements were taken in both eyes. Subsequently, one eye was treated with 5 µL of vehicle (RAMEB or RAMEB+GG) containing CBD, and the contralateral eye vehicle only. A dose of 0.1 mg/mL CBD was applied. IOP was measured in both eyes after application periodically for 22 hours. Animals were returned to their home cage between measurements.

2.9 Corneal Pain Eye Wipe Experimental Design

The eye wipe test was used to evaluate the ability of CBD to relieve corneal pain following noxious stimuli as published previously [200-203]. The eye wipe test was used to evaluate the ability of CBD to relieve corneal pain following noxious stimuli as published previously [200-203]. Mice were habituated to the testing environment (30x30 cm plexiglass lidless chamber) for 10 minutes prior to baseline measurement. To apply test solutions, mice were gently grasped and 5 µL of saline (negative control), PFBP only (to determine if the formulation alone alters corneal pain), or PFBP containing 1 mg/mL CBD was administered to the center of the corneal surface. After application of test solutions, mice were returned to their home cage for 10 minutes. To induce irritation, 5 µL hypertonic saline (5M NaCl in deionized water) was applied to the same eye that received the test formulation. Ten seconds after application of hypertonic saline, mice were placed in the Plexiglas chamber. Animals immediately began to wipe the eye with the ipsilateral forepaw which is noticeably different from grooming activity; the number of eye wipes were counted for 30 seconds. Differences in the count of eye wipes was used to determine if PFBP or PFBP containing CBD produced hypoalgesia. After measurement, entire ocular surface was irrigated with 10 mL normal saline and mice were returned to their home cage.

2.10 Statistical Analysis

All statistical analyses were performed with GraphPad Prism 9.1.2 software (San Diego, CA). For corneal penetration studies, differences in CBD concentrations in aqueous humor applied with RAMEB and RAMEB+GG were compared by 2-way ANOVA with Bonferroni's post-hoc test and significance was

set at $p<0.05$. IOP measurements following drug administration and eye wipe counts following induction of corneal pain were analyzed by *t*-test with significance set at $p<0.05$. IOP measurements compared differences between RAMEB versus RAMEB containing CBD and RAMEB+GG versus RAMEB+GG containing CBD at each time point using a *t*-test with significance set at $p<0.05$. A *t*-test was selected over an ANOVA due to the fact that IOP follows a diurnal pattern [204]. Eye wipe counts compared vehicle and CBD treated eyes to saline treated eyes using a *t*-test; significance was set at $p<0.05$.

3. Results

3.1 Analytical Method

A sharp peak was separated from the solvent front in each sample type. The results of method validation are displayed in Table 8. In biological sample types, the assay was linear from 100-5000 ng/mL in aqueous humor, and 100-25,000 ng/mL in cornea extracted acetonitrile (determination coefficients of 0.994, and 0.998 respectively). The data was best fitted with a simple linear regression model. The LOD and LOQ were determined to be 20 ng/mL and 100 ng/mL in aqueous humor, and 10 ng/mL and 30 ng/mL in cornea extracted acetonitrile. Regarding precision, the CV values obtained for intraday variability were within 10% for both sample types. Regarding accuracy, the % bias values obtained for intraday accuracy were within 11% for aqueous humor and cornea extracted acetonitrile samples.

Table 8. HPLC-UV/VIS Validation Data

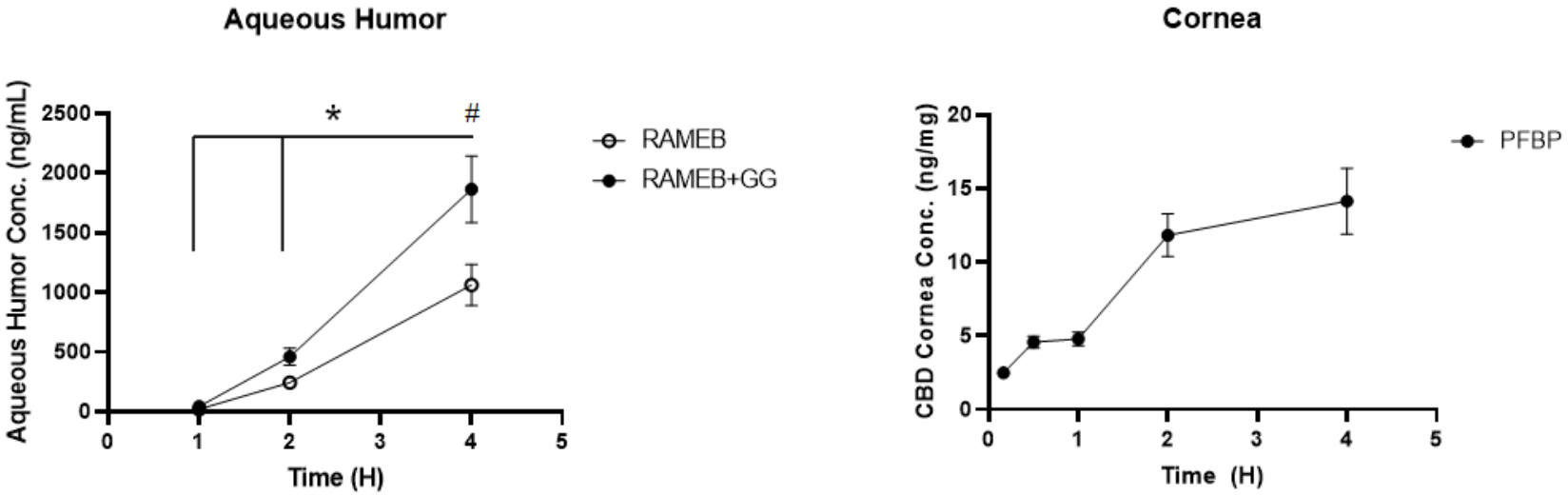
Aqueous Humor				Cornea			
Conc. (ng/mL)	average conc.	% Bias	CV%	Conc. (ng/mL)	average conc.	% Bias	CV%
100	106.0±6.67	6.02	6.30	100	99.09±9.500	-0.91	9.58
200	202.6±11.65	1.30	5.75	1000	1105±65.67	10.47	4.50
500	453.7±11.65	-9.24	2.73	5000	4926±251.8	-1.47	5.11
1000	920.1±29.66	-7.99	3.22	10000	10182±526.2	1.82	5.17
5000	5123±87.42	2.46	1.71	25000	26249±714.7	4.99	2.72
Linearity (R^2)	0.9942				0.9982		
LOD	20 ng/mL				10 ng/mL		
LOQ	100 ng/mL				30 ng/mL		

3.2 Corneal Penetration of CBD

Amount of CBD present in aqueous humor following topical application (0.1 mg/mL) in RAMEB or RAMEB+GG formulations to enucleated porcine eyes is shown in Figure 11A. RAMEB formulation containing CBD was used as comparison to determine if addition of hydrogel affected transcorneal permeability. CBD was not detectable or below the LOQ at 30 minutes; from 1 hour on, concentrations increased time dependently. A two-way ANOVA was performed to analyze the effect of time and vehicle (RAMEB or RAMEB+GG) on aqueous humor CBD concentrations. A two-way ANOVA revealed that there was a statistically significant interaction between the effects of time and vehicle ($F(2,23) = 2.474$, $p = 0.013$). Further, simple main effects analyses showed that both time and vehicle used had statistically significant effects on aqueous humor concentration of CBD ($p<0.0001$ and $p<0.0022$). The maximal concentration reached at 4 hours post-application was 1061±389.3 ng/mL with RAMEB application, and 1864±559.0 ng/mL with RAMEB+GG application. These results show that concentration of CBD increased time dependently, and RAMEB+GG enabled a significantly greater penetration of CBD than RAMEB only in this model.

CBD was not detected in aqueous humor following topical application in PFBP. In contrast, as shown in Figure 11B, CBD concentration time-dependently increased in cornea post-topical application. At 4 hours post-topical application, CBD concentrations as high as 14±5 ng/mg were detected in cornea.

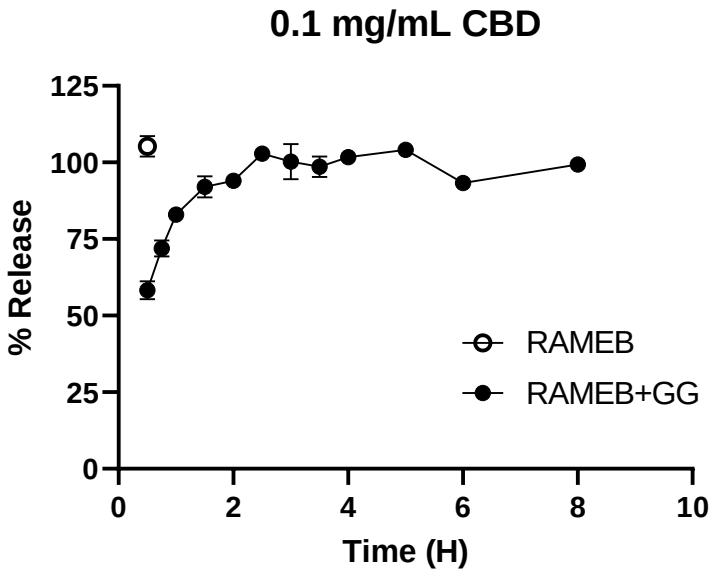
Figure 11. Corneal Penetration of CBD from Cyclodextrin, Cyclodextrin-hydrogel, and Semifluorinated Alkane Formulations. The amount of cannabidiol recovered (ng/mL) in (A) aqueous humor or (B) cornea after application of a single dose applied in RAMEB, RAMEB+GG, or PFBP formulations plotted against time (n=5; mean±SEM). RAMEB and RAMEB+GG formulations contained 0.1 mg/mL CBD, and PFBP contained 1 mg/mL CBD. * Represents statistically significant differences between the 4 hour time point and 1 and 2 hour time points (p<0.05). # Represents significant differences between RAMEB and RAMEB+GG treatments at 4 hours (p<0.05).



3.3 *In Vitro* Release of CBD From RAMEB+GG

Cumulative amount of CBD (0.1 mg/mL) released from RAMEB+GG is shown in Figure 12. RAMEB formulation was used as control; CBD concentrations reached maximum immediately when RAMEB is used. RAMEB+GG rapid gelation upon immersion in PBS; the resulting gel was clear and gels persisted for entirety of measurement (8 hours). Compared with RAMEB, RAMEB+GG prolonged CBD release for approximately 2.5 hours.

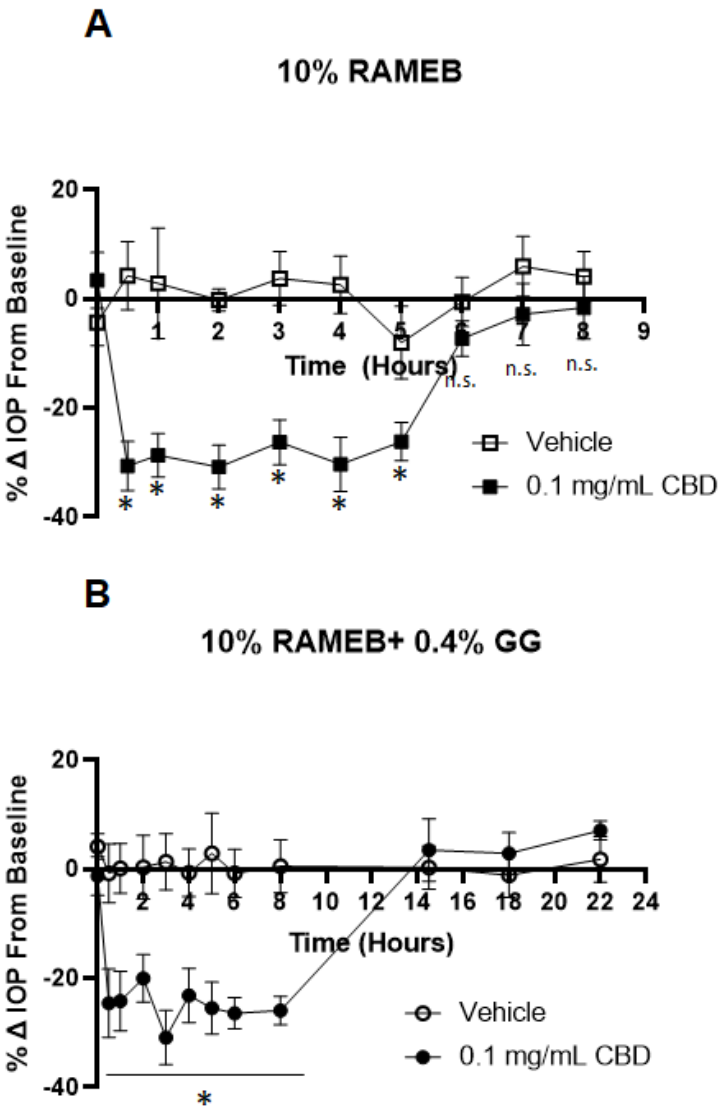
Figure 12. *In Vitro* Release Profile of CBD from CD Loaded Hydrogel. The amount of CBD (ng/mL) released in PBS from RAMEB and RAMEB+GG loaded with 0.1 mg/mL CBD plotted against time (mean±SEM, *n*=3).



3.4 CBD Effect on IOP

Next the effect of CBD (0.1 mg/mL) delivered in RAMEB and RAMEB+GG on IOP of normotensive mice was evaluated. IOP was measured at baseline first, followed by topical applications of vehicles (RAMEB or RAMEB+GG) or vehicles containing CBD and period measurement of IOP for 22 hours. IOP versus time of measurements for each formulation tested are presented in Figure 13. Eyes treated with either vehicle had no statistically significant differences between baseline and different time points evaluated. It is only when CBD was present in the formulations that IOP was lowered. Indeed, the 0.1 mg/mL CBD RAMEB formulation decreased IOP compared to RAMEB without CBD after 30 minutes (Figure 13A). A statistically significant difference between vehicle (RAMEB) and CBD was present at each time point lasting for 5 hours. IOP returned to baseline by 6 hours. Interestingly, with RAMEB+GG as vehicle, a statistically significant decrease in IOP was present from 30 minutes and each time point lasting up to 8 hours (Figure 13B). IOP had returned to baseline by 14.5 hours.

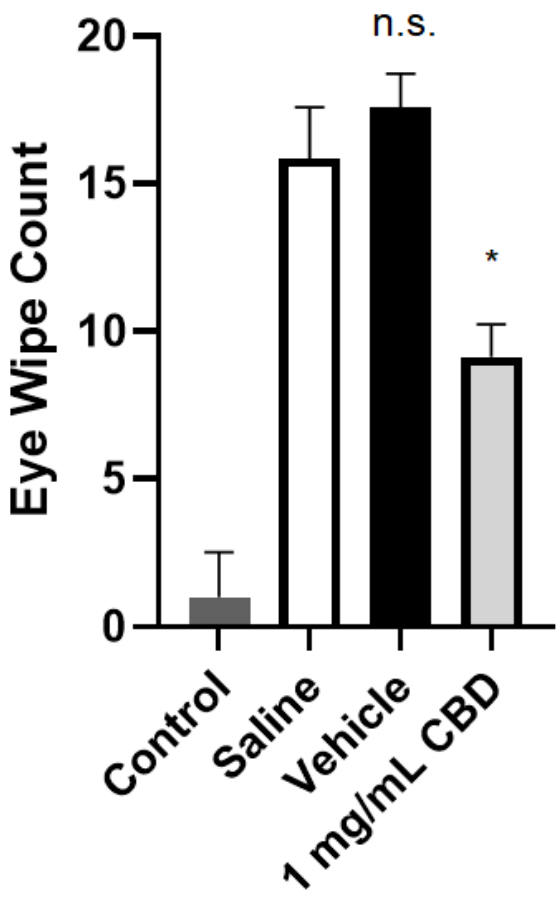
Figure 13. CBD Dependent Regulation of IOP Following Topical Application in Normotensive Mice A comparison of the IOP values obtained following topical application of control (0 mg/mL) or 0.1 mg/mL CBD in either (A) RAMEB or (B) RAMEB+GG formulations. IOP data shown are the mean±SEM (*n*=7). Statistical significances between RAMEB and RAMEB+GG treatments at each time point were derived from *t*-test (*p<0.05).



3.5 CBD Effect on Corneal Pain

In the final set of experiments, we evaluated the ability of the CBD in PFBP to inhibit corneal pain induced by application of hypertonic saline solution. Animals were pretreated with PFBP containing concentrations of CBD (0, 1mg/mL). A saline only group was included as a control to demonstrate normal eye wipe counts in absence of corneal pain. Number of eye wipes from each group are presented in Figure 14. Eye wipe counts were higher following 5M NaCl compared with saline control, indicating corneal pain was induced. Following corneal pain induction, PFBP alone did not alter eye wipe counts. However, 1 mg/mL CBD significantly lowered eye wipe counts compared with saline control, supporting the analgesic effect of CBD on corneal pain.

Figure 14. Corneal Analgesic Effect of CBD. Eyes were pre-treated with topical CBD (0 and 1 mg/mL) in 1-(perfluorobutyl)pentane. Eye wipes were counted following topical application of 5M saline. Results are expressed as mean ± SEM (*n*=7). Treatment groups are compared to normal saline control with *t*-test, *p*< 0.05.



4. Discussion

Hydrophobicity of CBD is the major challenge to ocular application. Previous studies have employed numerous lipophilic vehicles such as sesame oil and mineral oil to apply cannabinoids topically [39,75]. However, such vehicles can blur vision and cause irritation [34,105]. In the present work a cyclodextrin, a sol-to-gel hydrogel, and a semifluorinated alkane were used to enhance CBD solubility and thus ocular delivery.

Cyclodextrins are large amphiphilic compounds capable of increasing aqueous solubility of CBD [178] and transcorneal permeability of other cannabinoids [161-163,181-183] (Chapter III). In the present study 10% RAMEB was used to administer 0.1 mg/mL CBD and determine aqueous humor concentration. RAMEB afforded a time dependent increase in aqueous humor concentration, reaching an aqueous humor concentration of 1061 ng/mL.

Combining CDs and hydrogel systems is a strategy which has been employed for numerous hydrophobic ophthalmic drugs [187-191]. Sol-to-gel forming hydrogel systems increase ocular delivery through mucoadhesion to the cornea. GG forms a clear gel in the presence of mono- or divalent cations, such that gelation occurs upon interaction with tear fluid. The embedded drug is thus resistant to drainage, residence time is prolonged, and bioavailability is increased [186]. In our study RAMEB was combined with GG to apply CBD topically. Our results show a time- dependent and vehicle- dependent increase in aqueous humor concentrations when RAMEB+GG was used compared to RAMEB only.

RAMEB+GG enabled aqueous humor concentrations of 1864 ng/mL after 4 hours. Further, RAMEB+GG extended release of CBD *in vitro*. Our finding is consistent with previous reports that determined hydrogel increased permeability of other hydrophobic drugs [187-191].

When applied in RAMEB+GG formulation, CBD was hypotensive for 8 hours. Sol-to-gel forming hydrogel systems increase ocular delivery through mucoadhesion to the cornea. GG forms a clear gel in the presence of mono- or divalent cations, such that gelation occurs upon interaction with tear fluid. The embedded drug is thus resistant to drainage, residence time is prolonged, and bioavailability is increased [186]. It is likely through prolonged residence time with the cornea that hydrogels afforded a longer duration of IOP reduction than CD only formulation.

Regarding CBD effect on IOP, only four IOP reports applied CBD topically [63,75,76,108]. One showed decrease in IOP at CBD concentrations ranging from 0.0001- 1.0% (1 µg/mL to 10 mg/mL) applied in mineral oil [75]; the second report observed an IOP decrease in CBD doses ranging 250-1000 µg (roughly 60-250 µg/kg) in cats; the effect was more pronounced at the lower dose [76]. the third report showed an IOP increase at 5 mM (1.6 mg/mL) CBD applied in Tocrisolve® [63]. Interestingly, Rebibo et al. [108] reported that concentrations of 0.4% (4 mg/mL) and 1.6% (16 mg/mL) CBD applied in a nanoemulsion lowered IOP whereas 0.8% (8 mg/mL) did not affect IOP.

Unfortunately, none of these studies reported on aqueous humor concentrations of CBD following topical application, so it is impossible to know

what concentration penetrates through the cornea to exhibit an effect. In this study therefore, we sought to apply a lower dose of CBD and determine the effect on IOP. Following a 0.1 mg/mL application of CBD *in vivo* with the RAMEB vehicle, IOP was lowered by 25-30% lasting for 5 hours. In contrast, RAMEB+GG induced a prolonged hypotensive effect lasting at least 8 hours. Based on this hypotensive effect and an aqueous humor concentration range of 1061-1864 ng/mL (3.37-5.93 µM), we estimate that CBD is effective at lowering IOP at low micromolar tissue concentrations.

The findings of this chapter are consistent with conclusions from Chapter II which demonstrated that 1 µM CBD increases aqueous humor outflow, and Chapter III which demonstrated that a 50 mg/kg dose of CBD decreases IOP that correlated with an aqueous humor concentration maximum of 71.55 ng/mL. However, a correlation cannot be drawn as a result of this study between the concentration of CBD in aqueous humor and effect on IOP. In this study, aqueous humor concentrations were determined *ex vivo* in a porcine model, while hypotensive effects were measured *in vivo* in a murine model. In the future, a correlation study should be conducted *in vivo* in one species.

Finally, SFAs are amphiphilic compounds with low viscosity and surface tension which can deliver hydrophobic drugs [165,192,193]. THC has been applied using an SFA, PFBP, in a dry eye disease mouse model of desiccating stress [205]. As yet, CBD has not been applied topically in the eye with an SFA and effect of corneal pain has not been studied. In the present work CBD was applied in PFBP, and aqueous humor and corneal concentrations were measured *ex vivo*.

CBD was not present in aqueous humor following topical application, but was localized to the cornea. Delivery of CBD in PFBP therefore can be used for delivery of CBD to cornea without penetrating into the anterior chamber and possibly causing side effects within the eye.

The cornea is frequently used for nociception studies of trigeminal nerve pain since the cornea is heavily innervated by the ophthalmic branch of the trigeminal nerve [206]. The eye wiping test used herein has previously been validated as a method in trigeminal pain studies, and compared with hot plate pain tests [200]. In the corneal pain eye wipe model used, CBD demonstrated analgesic effect at applied 1 mg/mL dose mg/mL (3 mM). Our results are consistent with Thapa et al. [39] which demonstrated 5% (50 mg/mL, 158 mM) CBD reduced corneal pain following cauterization and capsaicin challenge, though we used a smaller dose. A smaller dose requirement to achieve analgesia may be attributed to effective delivery of PFBP.

CBD at doses ranging from 0.4-1.6% (4-16 mg/mL) has also been shown to reduce inflammatory markers in an LPS-induced keratitis inflammation model [108]. Though corneal inflammation was not studied in the present study, future study of lower doses of CBD administered in PFBP for modulation of corneal inflammation is justified.

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5. Conclusions

In this study topical drug delivery systems were used to deliver CBD and assess tissue concentrations in the anterior segment of the eye. Based on the tissue concentrations, pharmacologic assessment (either IOP or corneal pain) was assessed. RAMEB and RAMEB+GG formulations enabled penetration through the cornea. *In vivo*, CBD (0.1 mg/mL) delivered in RAMEB only vehicle induced a decrease in IOP, and the effect was prolonged in the RAMEB+GG vehicle. PFBP did not enable penetration to aqueous humor but instead localized CBD to the cornea. *In vivo*, CBD (1 mg/mL) lowered eye wipe counts indicative of analgesic effect in a model of corneal pain. The results highlight not only the importance of vehicle for the pharmacologic effect, but also tissue localization of CBD. CBD may have benefits for different ocular diseases such as corneal pain and inflammation or glaucoma depending on which vehicle is used to apply the phytocannabinoid.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

1. Restatement of Goals

The overall goal of this dissertation was to correlate the concentration of CBD in ocular tissues with pharmacologic effects. Previous studies of CBD involvement in IOP are inconsistent, perhaps due to difficulties with delivery and poor bioavailability of hydrophobic drugs. The experiments in Chapter II were performed to determine if CBD regulates aqueous humor (AH) outflow and assess the effect of CBD on a trabecular meshwork (TM) cell signaling pathway that is involved in regulating outflow. The results of Chapter II lead to Chapters III and IV, studying effect of CBD on IOP from different routes of administration. In Chapter III, an LC/MS-MS method was developed and validated for measurement of CBD in AH and serum and used to conduct pharmacokinetics and correlate the tissue concentrations with changes to IOP following intraperitoneal administration. Finally, Chapter IV describes quantification of CBD in AH and cornea following topical application using formulations which improve bioavailability; aqueous solutions containing randomly methylated β cyclodextrin (RAMEB) or RAMEB with gellan gum (RAMEB+GG), and a semifluorinated

alkane 1(perfluorobutyl)pentane (PFBP) were tested. Following determination of AH and corneal concentrations, CBD was applied to either IOP measurement or corneal pain assessment. Taken together, these studies provide insight into routes, doses, and AH concentrations that can regulate IOP.

2. Major Findings

2.1 CBD Increases AH Outflow and Modulates Rho/ROCK Signaling in TM Cells

In anterior segment perfusion experiments, AH outflow was increased significantly within 1 hour after adding 1 µM CBD and the effect was sustained over the 5 hours of measurement. Treatment of TM cells with 1 µM CBD significantly decreased TM cell-mediated collagen contraction, inhibited phosphorylation of MLC and MYPT1, and reduced RhoA activation. These results demonstrate CBD can enhance AH outflow and modify TM cell signaling, warranting further study of CBD effect on IOP.

2.2 Following Intraperitoneal Administration, CBD is Detectable in AH and Lowers IOP.

In corneal permeation experiments *ex vivo*, 10% formulations of RAMEB and 2-hydroxyproypl-β-cyclodextrin (2HPβCD) facilitated penetration through the cornea, resulting in a time-dependent increase in CBD AH concentration. RAMEB yielded a higher concentration of CBD in AH compared to 2HPβCD. The pharmacokinetic study demonstrated that CBD delivered through intraperitoneal administration reaches C_{max} of 71.55 ng/mL and AUC was 183.4 ng*h/ml in ocular fluid. The time to C_{max} was 2h, with a half-life of 1.046 min in AH. Further, IOP measurements conducted after intraperitoneal administration of CBD showed a decrease lasting up to 4 hours post-administration. Altogether,

concentrations of CBD were used to calculate pharmacokinetic parameters and correlated with ocular hypotensive effect on IOP.

2.3 CD Formulations Deliver CBD to AH and Lower IOP; SFA Delivers CBD to Cornea and Reduce Corneal Pain

Firstly, both RAMEB and RAMEB+GG formulations enabled corneal, reaching AH concentrations of 1061 ng/mL and 1864 ng/mL respectively. Further, addition of GG to the RAMEB vehicle significantly increased the concentration of CBD over time compared with the RAMEB only vehicle. On the other hand, CBD was not present in AH following 1 mg/mL topical instillation in PFBP but was measured in the cornea. Secondly, in an *in vitro* release experiment, 100% of CBD was released from RAMEB+GG within 2.5 hours of instillation. Ergo, RAMEB and RAMEB+GG formulations were used to apply CBD for IOP measurement, and PFBP was applied in an eye wipe corneal pain model. *In vivo*, 0.1 mg/mL CBD decreased IOP for 5 hours post topical application when delivered in 10% RAMEB. When gellan gum was incorporated however, the 0.1 mg/mL CBD dose produced a significant decrease in IOP for 8 hours and returned to baseline overnight. When CBD was applied at 1 mg/mL and 10 mg/mL in PFBP, eye wipe counts were reduced indicative of a corneal analgesic effect. Our results highlight not only the importance of dose and vehicle, but also tissue concentrations for pharmacologic effects. Our results also point to the potential therapeutic of CBD for glaucoma and also superficial corneal conditions depending on vehicle used to apply the cannabinoid.

3. Significance and Discussion

3.1 CBD Regulation of IOP

The literature suggests that different doses of CBD can mediate different effects on IOP. A previous study found that CBD was effective at lowering IOP even at high concentrations. When applied topically in mineral oil, concentrations ranging between 3-31 µM lowered IOP [75]. It is possible that this occurred because much of the applied dose in mineral oil is not retained on the cornea and can easily be washed out by nasolacrimal drainage, so only a small percentage of CBD enters the eye. Interestingly, Colasanti et al. [76] found that the hypotensive effectiveness of CBD varied with concentration: following topical application in polyethylene glycol, a dose of 250 µg (roughly 60 µg/kg) had a more pronounced decrease in IOP than the 1000 µg (roughly 250 µg/kg) dose. Tomida et al. [64] also showed a dose dependence on CBD-induced IOP effects. At a 20 mg sublingual dose no effect was observed, but at 40 mg a transient IOP increase at 4 hours was observed. Miller et al. [63] also observed an IOP increase following topical application of 5 mM (1.6 mg/mL) in Tocrisolve® based solvent. Finally, Rebibo et al. [108] show that when applied in a nanoemulsion 0.4% (4 mg/mL) and 1.6% (16 mg/mL) CBD solutions reduce IOP, but 0.8% (8 mg/mL) is not effective at IOP reduction. This study did not report tissue concentrations, so it is not clear what mechanism is involved in regulation of IOP. The researchers also reported CBD regulation of ocular surface inflammation [108].

It is impossible to draw a conclusion based on the previous literature because of the variability of dose, vehicle, route of administration, and species. Measuring CBD in AH directly bypasses the issue of bioavailability and allows us to assess what AH concentrations of CBD modulate changes to IOP. In Chapter II we see 1 µM CBD increases AH outflow. In Chapter III, 71.55 ng/mL (0.227 µM) concentration was observed in AH following intraperitoneal injection, and IOP was lowered at this dose. In Chapter IV we find that topical 0.1 mg/mL, resulting in AH concentrations of (1061-1864 ng/mL (3.37-5.92) µM lower IOP. Additionally, we observed AH concentrations of roughly 4148 ng/mL (13 µM) following 1 mg/mL topical application at 2 hours (Chapter III), a dose in the same order of magnitude which showed elevation of IOP (1.5 mg/mL, 5 mM) [63]. Altogether, we observed IOP lowering effects between 0.23-5.36 µM CBD. We suggest that the therapeutic dose for CBD lowering IOP is in the high nanomolar to low micromolar range, and AH concentrations above 13 µM will elevate IOP. However, provided AH turnover and drug washout, 13 µM is probably an overestimate and a dose lower than 13 µM elevates IOP.

The detection of CBD in AH shows what concentration of CBD modulates changes to IOP. The methods developed herein can be used to elucidate the effect of CBD when applied in different doses, routes of administration, vehicles, and species. In this dissertation, only a few concentrations were measured (1 µM in vitro, 1061, 1864, and 4148 ng/mL ex vivo, and 71.55 ng/mL in vivo). The immediate future direction of this research should be aimed at measuring CBD

aqueous humor from different administered doses, routes, vehicles, and species to resolve the controversy of CBD effect on IOP.

3.2 Choice of Vehicle Impacts Tissue Availability

In this dissertation we report that CBD delivered in an SFA PFBP localized delivery of CBD to the cornea, whereas RAMEB enabled delivery to AH. A previous study showed that CBD applied in soybean oil reduced corneal pain and inflammation following topical application [39]. However, tissue concentrations were not reported, and the lipophilic vehicle likely allows CBD to penetrate through the cornea. Further, the high dose used (50 mg/mL) may elevate IOP which was also not reported. Utilization of an SFA vehicle enables delivery of CBD at high concentrations without risk of toxicity to other areas of the eye (e.g., elevated IOP).

3.3 CBD Can be Applied Selectively for Corneal Conditions Such as Corneal Pain and Inflammation

We found CBD reduces corneal pain through an eye wipe test, in agreement with previous literature that CBD is analgesic in a cauterization-capsaicin challenge model of corneal pain [39]. CBD is widely supported as anti-inflammatory in numerous tissues. Anti-inflammatory actions of CBD in the cornea are also reported [39,108], at least partly through 5HT1A [39].

Another receptor which CBD may regulate corneal inflammation is TRPV1; CBD is a TRPV1 agonist [207,208]. TRPV1 is expressed in the cornea and have a prominent role in regulation of corneal inflammation and immune response [209]. CB1 inhibits TRPV1 sensitization following neuropeptide induced

sensitization in afferent neurons [210]. CB1/ CB2 agonist WIN55,212-2 reduced corneal neuronal activity in a corneal pain model [211], as well as inflammation and scarring following corneal injury [212].

4. Future Directions

The last section indicated an immediate future direction of the project studying effect of CBD from application in different doses, vehicles, routes, and species. In the next section, further reaching next steps for the project are proposed.

4.1 Molecular Mechanism of CBD Regulation of IOP

Since this dissertation showed that low micromolar concentrations of CBD can lower IOP, yet high CBD concentrations can elevate IOP [63] suggests complex pharmacology involving multiple possibly conflicting mechanisms of action. To identify the pharmacologic effects of CBD in the eye, the molecular targets should be evaluated. CBD is a promiscuous ligand with 65 known targets [18]. Several of these targets which are expressed in the eye may intermediate CBD's decrease on IOP. A summary of CBD target, affinity, and receptor's role on IOP are shown in Table 9.

CB1: CB1 receptor involvement in regulation by numerous ligands is well documented and under continuous study. Many cannabinoid ligands including THC [122], Δ^8-tetrahydrocannabinol [213], CP55,940 [214], WIN55,212-2 [215,216], and endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [217-219] activate CB1 and decrease IOP. Recently it has also been shown that NAMS of CB1 can lower intraocular pressure. Further, CB1 regulates IOP differently depending on time of day and sex [220].

At high concentrations, CBD is an antagonist of CB1 (K_i 3 μM) [221], and a negative allosteric modulator (NAM) at lower concentrations (0.5-1 μM) [82]. Miller et al. [63] demonstrated at a concentration able to antagonize CB1 (5 mM), CBD elevated IOP through a CB1 dependent mechanism. Several others however have demonstrated that CBD lowers IOP (Chapters III and IV) [74-76,108], indicating complex pharmacology likely involving multiple receptors.

Table 9. Target, Receptor-IOP Effect, Ocular Tissue Expression, CBD-IOP Effect

Target	Receptor Effect IOP [ref]	Tissue Expression	CBD Activity/ Affinity
CB1	Activation ↓ IOP: Δ^9-THC [122], Δ^8-THC [213], CP55,940 [214], WIN55,212-2 [215,216], AEA [217-219], 2-AG [217-219]	Cornea [83,222]	Antagonist: IC_{50} 30 μM [223] Antagonist: Ki 4.9 μM [224] Antagonist: Ki 3.2 μM [221] NAM: EC_{50} 0.5-1 μM [82]
		TM [83,134]	
		Ciliary body/epithelium [83,84]	
		Schlemm's Canal [83]	
GPR18	Activation ↓ IOP: Abn-CBD [88], NAGly [88]	Cornea [88]	Partial agonist/antagonist: 51.1 μM [225] Biased agonist: 0.3 μM [87]
		TM [88]	
		Ciliary body/epithelium [88]	
GPR55	Activation ↓ IOP: PEA [226]	TM [226]	Antagonist: IC_{50} 0.45 μM [223]
		Retina [227]	
GPR119	Activation ↓ IOP: 2-OG [228], PEA [226]	Cornea [228]	N/A
		TM [228]	
		Iris [228]	
TRPV1	N/A	Cornea [229]	Agonist: EC_{50} 3.2-3.5 μM [207] Agonist: EC50 1 μM [208] Agonist: EC_{50} 1.9 μM [221]
		TM [230]	
		Ciliary body [229]	
5HT1A	Activation ↓ IOP: 8-OH-DPAT [231], Flesinoxan [232], 5-methylurpuradil [233]	Cornea [234]	Agonist: EC_{50} 7 nM [235] Agonist: EC_{50} 16 μM [78]
		Ciliary body [231,236]	
		Retina [237]	
FAAH	Inhibition ↓ IOP: URB597 [220,238]	TM [136]	Antagonist: IC_{50} 15 μM [208] Antagonist: IC_{50} 19.8 μM [221]
		Retina [239]	
FABP/ cannabinoid transporters	Inhibition ↓ IOP: SBF1-26 [220], WOBE437 [220]	Retina [240]	Antagonist: Ki 1-2 μM [241]

CB1: cannabinoid receptor type 1; CB2: cannabinoid receptor type 2; GPR18: G coupled protein receptor 18; GPR55: G coupled protein receptor 55; GPR119: G coupled protein receptor 119; TRPV1: transient receptor potential vallinoid type 1; 5HT1A: Serotonin type 1A receptor; FAAH: fatty acid amide hydrolase; FABP: fatty acid binding proteins; Δ^9-THC: Δ^9-tetrahydrocannabinol; Δ^8-THC: Δ^8-tetrahydrocannabinol; AEA: anandamide; 2-AG: 2-arachidonoylglycerol; Abn-CBD: abnormal cannabidiol; NAGly: N-arachidonoyl glycine; PEA: palmitoylethanolamide; 2-OG: 2-oleoylglycerol; 8-OH-DPAT: 8-Hydroxy-2-(di-n-propylamino)tetralin; NAM: negative allosteric modulator

TRPV1: Transient receptor potential vanilloid type 1 (TRPV1) receptor is a temperature sensitive ion channel expressed in several anterior ocular tissues including trabecular meshwork cells [230], cornea and ciliary body [229]. While TRPV1 activation has not be implicated in regulation of IOP, it is activated by many ligands shown to lower IOP such as AEA, 2AG, and CBD [219]. CBD EC_{50} for TRPV1 ranges between 1-3.5 μM [208,221]. At concentrations used in this dissertation therefore, CBD may be modulating IOP partially through activation of TRPV1.

5HT1A: A receptor which has a high affinity for CBD that could regulate IOP is the serotonin 5HT1A receptor. CBD has a high affinity as a 5HT1A agonist (EC_{50} 7 nM) [235]. A number of reports indicate that 5HT1A agonism lowers IOP [231-233]. Based on CBD affinity and agonism, and ocular hypotensive effect of 5HT1A agonism with other agents, this target seems the most likely to regulate CBD-mediated decrease in IOP. It has yet to be demonstrated if the hypotensive effect of CBD will be present when the serotonin receptor is knocked down or antagonized.

Regulation of Endocannabinoid Tone: GPR18 and GPR119 are putative cannabinoid G protein coupled receptors, and activation is involved in IOP regulation. Agonist abnormal cannabidiol (Abn-CBD) and n-arachidonoyl glycine (NAGly) decrease IOP via GPR18 independently of CB1, CB2, and GPR55 [88], and agonist 2-oleoylglycerol (2-OG) decreased IOP via GPR119 [228]. GPR119 was also indirectly implicated in palmitoylethanolamide (PEA) changes in AH outflow [226], and later it was shown that PEA is a GPR119 agonist [242]. There

is limited data supporting CBD activity at GPR18 [87,225], and currently no support for CBD agonism/antagonism of GPR119. As such, the future may show that CBD indirectly activates these and other receptors through modulation of endocannabinoid tone.

The endocannabinoid system is currently comprised of the known cannabinoid receptors CB1 and CB2, and endocannabinoids AEA and 2-AG, all of which have a well-documented role in regulation of IOP. Endocannabinoid tone refers to regulation of synthesis and degradation of endocannabinoids. The two endocannabinoids are AEA and 2-AG, both of which have a well-documented role in regulation of IOP. AEA is primarily metabolized by fatty acid amide hydrolase (FAAH) [243], and 2-AG by monacylglycerol lipase [244].

CBD regulates both reuptake and degradation of endocannabinoids. CBD inhibits FAAH at micromolar concentrations (IC_{50} 15-20 µM) [208,221]. FAAH expression is confirmed in the retina [239] and TM [136], and inhibition of FAAH with inhibitor URB597 lowers IOP [220,238]. CBD also inhibits fatty acid binding proteins that facilitate reuptake of AEA and 2-AG, which subsequently increases cellular concentrations of AEA [221,241]. Given the multitude of CBD receptor targets and involvement in endocannabinoid tone, CBD may regulate IOP additively through multiple targets. Further study of CBD action will not only elucidate the molecular pathway through known targets but could also shed light on the intricacy of endocannabinoid regulation on IOP.

4.2 CBD Application in a Glaucomatous Model

Elevation in IOP, while the major hallmark for glaucoma, is not solely causative or the only physiological marker of the disease. Glaucoma pathogenesis involves damage to the trabecular meshwork with subsequent changes to AH outflow and IOP, damage to the optic nerve head which further damages the optic nerve axons, and damage and loss of retinal ganglion cells [177].

Excessive extracellular glutamate leading to excitotoxicity in the retina is a consequence of glaucoma [245]. Cannabinoid and cannabinoid- like compounds have shown neuroprotective and pro-survival effects following NMDA induced excitotoxicity [245,246]. The protective effects of CBD on retinal excitotoxicity and neurotoxicity are also established. CBD protects against excitotoxicity in an NMDA induced retinal toxicity model through reduction of peroxynitrite production and apoptosis [51]. CBD also reduces endotoxin induced retinal inflammation *in vitro* in retinal microglial cells [44]. In diabetic retinopathy rodent model, CBD reduces inflammation, vascular permeability, and neurotoxicity [58].

This dissertation only assessed IOP in normotensive mice. To determine if CBD is truly therapeutic for glaucoma, several pathologic markers (IOP, AH outflow dynamics, retinal ganglion cell loss, inflammation, ROS production) should be assessed in a glaucomatous model. Optic nerve health and visual function should also be assessed. Rodents are a cost-effective species for evaluation of glaucoma, and rodent eyes share similarities to human eyes. Several rodent models for glaucoma exist including inducible models and genetic models, as well as pressure-dependent and independent models [177]. Given the

CBD anti-inflammatory effects, increase in AH outflow, decrease in IOP, further study is warranted.

4.3 Effect of CBD on Dry Eye Disease

Dry eye disease (DED) is a chronic condition marked by lack of adequate tear production. DED can be the evaporative subtype, or reduced tear production subtype [247]. Pathology of DED includes tear film instability and evaporation, dysfunction of meibomian glands, inflammation, and subsequently corneal epithelial cell damage and changes to corneal nerve function [248].

There is recent data on the involvement of the endocannabinoid system in DED. Interestingly, a recent abstract showed that THC applied in PFBP relieved desiccating stress in a DED mouse model [205]. Additionally, Novaliq is collaborating with the University of Cologne in development of a cannabinoid drug for dry eye treatment [249]. Cannabinoid receptors are expressed in the cornea, and are upregulated under desiccating stress following induction of DED [248]. CB1 and CB2 expression were subsequently lowered with THC and CB1 and CB2 antagonist treatment (SR141716A and SR144528 respectively). Cannabinoid treatment also modulated tear production, maintained corneal sensitivity, lowered IL-1β expression and $CD4^+$ T-cell infiltration, and protects corneal morphology in DED induced mice [248]. Further indicative of cannabinoid involvement in DED, a frequently reported symptom of cannabis use is dry, irritated eyes [250]. In 2020, it was revealed that CB1 expressed in the lacrimal gland and regulation by agonists THC and CP55,940 affects tear production sex independently [250].

The role of CBD in dry eye disease has not yet been investigated. CBD regulates inflammation and interacts with TRPV1 which may indicate that CBD can produce an effect on DED. CBD is a TRPV1 agonist with an EC_{50} of 3.2-3.5 μM [207], and TRPV1 expression has been demonstrated in trigeminal nerve endings located in cornea tissues [209,251]. Injury to epithelial cells induces proinflammatory cytokines and chemoattractants which activate TRPV1 [252]. CB1/CB2 agonism results in blockage of inflammatory pathways activated by TNFα in conjunctival epithelium [253]. CB1 activation also induces a protein-protein interaction between CB1 and TRPV1, which in turn downregulates the pro-inflammatory signaling induced by TRPV1 activation [252]. Given CBD anti-inflammatory effect in ocular tissues including the retina [44,254] and cornea [39], as well as CBD indirect regulation of CB1 through anandamide, and direct activation and desensitization of TRPV1 [207], there is justification to study the effect of CBD in DED.

Another receptor which CBD is an agonist upon that may modulate DED effects is 5HT1A. CBD is a 5HT1A agonist with an EC_{50} of 7 nM [235]. 5HT1A are expressed in the cornea, and activation alleviates corneal lesions in dry eye following through regulation of ROS and autophagy [234]. Further, Thapa et al. [39] showed that CBD was anti-inflammatory in the cornea partially through a 5HT1A activation.

5. Concluding Remarks

This dissertation tested the hypothesis that the effect of CBD on IOP depends on tissue concentration. Herein we demonstrate that measuring tissue concentrations can be correlated with pharmacologic effects, providing insight regardless of which dose, vehicle, or route of administration is used. We have also corroborated previous reports indicating CBD-mediated reduction in IOP, and analgesic effect in the cornea. The methods described in this dissertation enables further characterization of CBD for ocular pharmacologic and pharmacokinetic assessments. Further study is required to determine which mechanisms are involved in pharmacologic effects, and if CBD truly has therapeutic benefit for glaucoma, corneal pain/inflammation, or dry eye disease.

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CURRICULUM VITAE

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Education

2018-2022	Ph.D. in Pharmacology and Toxicology, University of Louisville, Louisville KY
2015- 2018	M.S. in Pharmacology and Toxicology, University of Louisville, Louisville KY
2008-2014	B.S. Biology and Psychology, Chemistry minor, University of Louisville, Louisville KY

Professional Experience

2015- present	Graduate Research Assistant, University of Louisville, Department of Pharmacology and Toxicology
2019-2021	Senior Scientist, Pearl Medical Practice
2019-2020	Lab Technician, Delta-9 Labs
2014, 2015	NIH R25 Cancer Education Program Trainee, University of Louisville
2014-2015	Undergraduate Research Assistant, University of Louisville, Department of Pharmacology and Toxicology

Honors and Awards

2021	*Acta Pharmacologica Sinica* Outstanding Author Contribution Award
2018	NIEHS NRSA T32 Pre-doctoral Fellowship
2017	School of Interdisciplinary and Graduate Studies Travel Award
2017	International Cannabinoid Research Symposium Travel Award
2015-2017	Graduate Research Fellowship, Integrated Programs in Biomedical Sciences, University of Louisville
2015	School of Interdisciplinary and Graduate Studies Travel Award
2015	International Cannabinoid Research Symposium Travel Award

Publications

[1]Aloway, A., Kumar, A., Laun, A. S. & Song, Z. H. in *Handbook of Cannabis and Related Pathologies* (ed V. Preedy) Ch. 77, (Elsevier, 2017).
[2]Song, Z. H., Laun, A. S. & Cai, J. Mass Spectrometry Analysis of Human CB2 Cannabinoid Receptor and Its Associated Proteins. *Methods Enzymol* **593**, 371-386, doi:10.1016/bs.mie.2017.06.019 (2017).
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[9] Aebersold, A., Song, Z.H., The Effects of Cannabidiol on Aqueous Humor Outflow and Trabecular Meshwork Signaling (In preparation).
[10] Aebersold, A., Song, Z.H., Ocular Application of CBD: Effect on Intraocular Pressure and Corneal Pain (In preparation).

Presentations

Oral Presentations

[1] Cannabigerol Modulates the Efficacy of Anandamide on the CB2 Cannabinoid Receptor, International Cannabinoid Research Society, Wolfville Nova Scotia, CA, July 2, 2015
[2] The Role of the Oprhan GPR3 Receptor in Alzheimer's Disease, University of Louisville, Department of Pharmacology and Toxicology Seminar, April 14, 2016
[3] Cannabidiol, the First Biased Inverse Agonist for GPR3, University of Louisville, Department of Pharmacology and Toxicology Seminar, June 22, 2017
[4] Cannabidiol, a Novel Biased Inverse Agonist for GPR3, International Cannabinoid Research Society, Montreal, CA, June 26, 2017.
[5] A Study of Cannabidiol Biased Inverse Agonism At GPR3 and GPR6, University of Louisville, Department of Pharmacology and Toxicology Seminar, April 20, 2018
[6] Detection of CBD and its Potential Therapeutic Effect in the Eye, University of Louisville, Department of Pharmacology and Toxicology Seminar, November 19, 2020
[7] Keeping An Eye On Cannabidiol Pharmacokinetics, University of Louisville, Department of Pharmacology and Toxicology Seminar, December 7, 2021

Poster Presentations

[1] A.S. Laun, P. Kumar, Z.H. Song, Cannabigerol Modulates the Efficacy of Cannabinoids on CB2 Receptor, UofL NCI R25 Cancer Education Program, Louisville, KY, USA, 2014.
[2] A.S. Laun, P. Kumar, Z.H. Song, Cannabigerol Modulates the Efficacy of Anandamide on the CB2 Cannabinoid Receptor, 25th Annual Symposium of the International Cannabinoid Research SocietyWolfville, Nova Scotia, 2015, pp. 60.
[3] A.S. Laun, Z.H. Song, The Modulatory Effects of Pregnenolone On CB1 and CB2 Cannabinoid Receptors, Research! Louisville, Louisville, KY, USA, 2016.
[4] A.S. Laun, Z.H. Song, GPR3 and GPR6, Novel Molecular Targets for Cannabidiol, Research! Louisville, Louisville, KY, USA, 2017.
[5] K.J. Brown, A.S. Laun, Z.H. Song, The Effect of Various Classes of Cannabinoids on GPR12, UofL NCI R25 Cancer Education Program, University of Louisville, 2017.
[6] Z.H. Song, A.S. Laun, GPR3 AND GPR6, NOVEL MOLECULAR TARGETS FOR CANNABIDIOL, 27th Annual Symposium on the Cannabinoids, International Cannabinoid Research Society, Montreal, Quebec, Canada, 2017, pp. P3-9.
[7] J. Mnpotra, A.S. Laun, Z.H. Song, A. Griffith, H. Seltzman, D.P. Hurst, P.H. Reggio, Can a Ligand Switch CB1 Signaling From Inhibitory (Gi) to Stimulatory (Gs) G Protein?, 27th Annual Symposium of the International Cannabinoid Research SocietyMontreal, QC, Canada, 2017, pp. P1-28.

[8] I. Isawi, P. Morales, A.S. Laun, D.P. Hurst, Z.H. Song, P.H. Reggio, Structural Relationship of the Class A Orphan GPCR, GPR6 With the Cannabinoid CB1 and CB2 Receptors, 27th Annual Symposium of the International Cannabinoid Research SocietyMontreal, QC, Canada, 2017, pp. P1-29.
[9] P. Morales, A.S. Laun, D.P. Hurst, Z.H. Song, P.H. Reggio, Functional Selectivity of CBD in the Orphan Receptor GPR3: A Structural Focus, 27th Annual Symposium of the International Cannabinoid Research SocietyMontreal, QC, Canada, 2017, pp. P3-12.
[10] S.H. Shrader, A.S. Laun, H. Seltzman, F. Navas, P.H. Reggio, Z.H. Song, THE EFFECTS OF SR144528 ANALOGUES ON GPR3 AND GPR6, 28th Annual Symposium of the International Cannabinoid Research SocietyLeiden, Netherlands, 2018, pp. P1-35.
[11] A.S. Laun, P.H. Reggio, Z.H. Song, CANNABINOIDS AS INVERSE AGONISTS FOR GPR3 AND GPR6, 28th Annual Symposium of the International Cannabinoid Research SocietyLeiden, Netherlands, 2018, pp. P2-14.

Skills

Technical Skills

Pharmacokinetics, immunoblotting, cell culture, centrifugation, PCR, RT-PCR

COVID-19, spectrophotometry, LC-MS/MS, HPLC, method development,

maintenance, generation of SOPs, urine toxicology, radioligand binding,

microscopy, siRNA knockdown, cellular transfection, rat and mouse animal

handling, etc.

Computer Skills

Microsoft Office, proficient typist, ImageJ, EndNote bibliography software,

GraphPad/Prism, LIMS (Labdaq).