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CBD Levels and Localization in the Body

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Abstract

Cannabidiol (CBD) is a cannabinoid derived from the *Cannabis Sativa* plant. Although CBD has the same chemical formula ($C_{21}H_{30}O_2$) and is a nearly structurally identical isomer to Δ^9-Tetrahydrocannabinol (THC), it has vastly different effects on the brain and body. Cannabidiol is non-intoxicating and non-psychedelic. It has anti-inflammatory, anti-convulsant, anti-anxiolytic and anti-psychotic effects. Recent research has shown that this small molecule has unique properties that reduce neuroinflammation and act as an antioxidant; thus, supporting its potential role as an effective adjunctive treatment for neurodegenerative and psychological disorders. CBD is widely used by the public for numerous applications including pain relief, nausea, and anxiety. Growing clinical research supports CBD in treatment of traumatic brain injury (TBI) recovery, epilepsy, and schizophrenia. There is still little known about the long-term effects of CBD on adults and adolescents. Therapeutic effects can range widely depending on dose and route of administration. CBD can readily cross the blood brain barrier (BBB) and interact with CB1 and CB2 receptors, though exactly how it interacts with these receptors is not fully understood. Current research has sparked interest in the administration of CBD in reducing drug-seeking behavior in patients with substance use disorders (SUDs). This pilot study aims to analyze how CBD alters addiction behavior as well as how it can be detected in the blood following intraperitoneal (IP) injection using an animal model. Our pilot suggests that rats receiving CBD injection show increased rates of active responding for sucrose compared to vehicle group. Further, following 20 mg/kg body weight IP injection of CBD, we can detect CBD in plasma via liquid chromatography-quadrupole time of flight mass spec (LC-QTOF). The ability to detect CBD levels in blood will allow for better analysis of ideal dosing for treatment of various symptoms as well as analyzing how CBD uptake varies by sex and its potential applications in treating SUDs.

Introduction

Cannabidiol use has recently increased tremendously and as now used globally as a therapeutic substance[1]. CBD is a naturally occurring cannabinoid found in the *cannabis sativa* plant, though it can be produced synthetically. CBD is a 21-carbon terpenophenolic compound that is nearly structurally identical to Δ9-Tetrahydrocannabinol (THC). However, CBD does not have the same biological impacts and fails to act as a substitute in animal drug discrimination models for THC (World Health Organization, 2018). CBD is currently recognized by the World Health Organization (WHO) as a substance without abuse or dependence potential that is available unsanctioned online. CBD can be found in numerous different forms including lotions, tablets, gummies, and more. A large concern being that each form of CBD is advertised and sold with highly varying recommended doses.
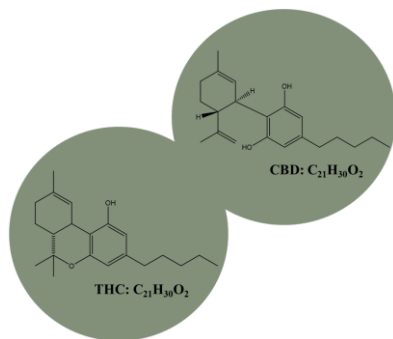

Fig. 1 | Chemical Formula and structure of both CBD and THC.

Cannabis, as of 2023, is labeled the most used drug of abuse in the world[2]. Long-term cannabis use can have negative impacts on the pulmonary, cardiovascular, and central nervous systems; these risks increase if cannabis use begins in phases of adolescence[2]. The large majority of CBD research currently is on adults, and little is known about the effects of CBD in adolescence. However, CBD has been found to have unique anti-epileptic properties and promising application as an adjunctive treatment of drug-resistant epilepsy in adolescence[3]. Recent clinical trials are targeting CBD in application as an

anti-inflammatory, anti-oxidant, anti-anxiolytic, and ant-psychotic treatment.

The applications of CBD for varying neurological disorders and trauma are currently being broadly studied[4]. CBD has been shown to have promising effects in aiding recovery from TBI via protective properties that may counter neurological damage[5]. Research also supports CBD use as adjunctive treatment for anxiety, depression, and schizophrenia[2]. Specifically in treatment of schizophrenia, CBD may be an advantageous treatment because it does not act as a dopamine receptor antagonist[6]. Cannabidiol, like THC, binds to two G protein-coupled receptors (GPCRs): cannabinoid receptors 1 and 2 (CB1, CB2)[7]. However, the exact mechanism of interaction of CBD with these receptors is not yet well understood. Research suggests that that hydrophobic cannabidiol can be transported in the blood by albumin or lipoproteins, though the exact intracellular carrier has not been identified[8].

Cannabinoid signaling systems contribute to the process of restoring homeostasis following change or injury within the body and brain[7]. CBD is of interest in treatment separate from THC due to the compounds non-addictive and non-hallucinogenic characteristics. The mechanism through which CBD effects CB1 receptors may explain the interactions between CBD and alternate compounds, including THC. CBD can act as a negative allosteric modulator for CB1, while not activating the receptor itself[7]. CBD has also been found to act as a partial agonist for CB2 receptors[7].

The therapeutic effects of cannabidiol also include significant interest as an adjunctive treatment in substance abuse disorders (SUDs)[9]. CBD's application in SUD treatment is likely due to its strong anti-anxiolytic properties and potentially its anti-inflammatory effects[10]. Human clinical trials show induced anxiety reduction following 300-600mg oral CBD administration[11]. CBD has also been found to prevent development of alcohol dependent impulsivity in rat models[12].

In a clinical trial with subjects diagnosed with cocaine SUD, CBD administration lead to reduction

in drug-motivated behavior[9]. CBD was given transdermally in a gel preparation to avoid potential conversion to psychoactive cannabinoids via gastrointestinal (GI) break down[9]. CBD detection in plasma was performed following reduction in drug-motivated behavior following transdermal administration. However, CBD in plasma following IP injection is unknown. IP injection of CBD avoids the potential GI breakdown and provides an alternative and possibly more direct form of administration compared to transdermal application.

This pilot study aims to analyze the effects of IP injected CBD on drug seeking behavior as well as CBD levels in the blood following injection. With recent research suggesting application of CBD as a therapeutic for treatment of SUDs along with numerous other disorders, it is important that blood concentrations, sex differences, and possible routes of administration are well understood. Growing public use and access to wide varieties of CBD forms and doses creates increasing need for CBD research and public education to ensure substance safety and use recommendations are correct.

Results

CBD and active lever response for sucrose

Following 10 days of fixed-ratio (FR1) schedule of reinforcement with 10% sucrose administration, rats developed addictive behavior observed as increasing sucrose seeking behavior. On test day 11, thirty-minutes after IP injection of CBD or vehicle, rats that received CBD showed increased active lever presses compared to the vehicle group (Fig. 1) in FR1-2hr session of responding. Increase in active lever pressing suggests CBD may increase sucrose seeking behavior in rats. While this was a small treatment group (n=18), increased responding in both male and female subjects suggest need for further analysis of the effect of CBD on addiction behavior. There may also be sex differences present that were not seen here due to low sample size.
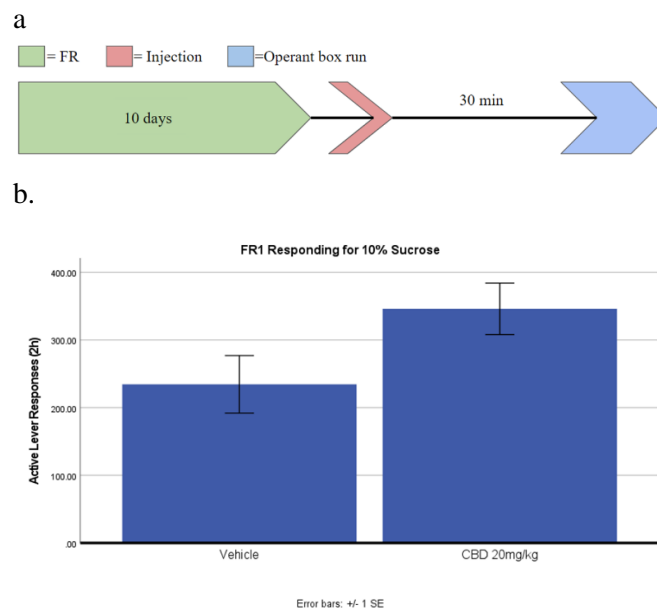
Fig. 2 | CBD effect on FR1 response for 10% sucrose. a. FR1 10-day 2hr/day schedule followed by CBD (20mg/kg body weight) or vehicle (3% Tween 80) IP injection on day 11. Thirty minutes post injection, 2-hour FR1 operant box run repeated. **b.** FR1 responding for 10% sucrose after IP injection of CBD or vehicle. Rats that received CBD had increased lever press response. Error bars are ±1 SE. Active lever response difference is significant for the drug treatment following a t-test, $p<0.05$ (n=18).

CBD detection in plasma

Initial plasma analysis via LC-QTOF prior to salt-assisted liquid-liquid extraction (LLE) contained many proteins much larger than the small molecule target of CBD and resulted in the system column clogging. MSMS of this sample showed high counts of large molecules far above the known 315.2319 m+H mass of CBD (Fig. 3). Samples beyond this point underwent LLE and were successfully analyzed.
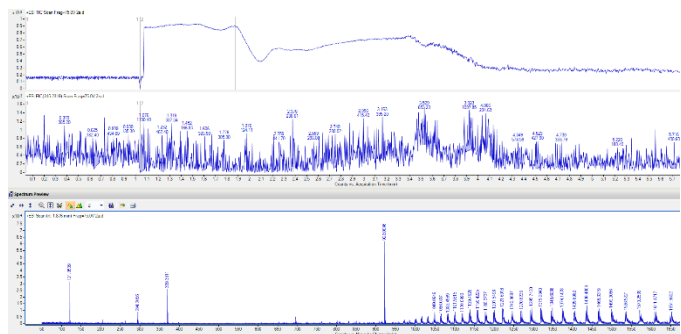
Fig. 3 | TIC Plasma MSMS with large protein contaminants. LC-QTOF total ion count (TIC) read of plasma prior to large protein knock-out.

Following large protein knock-out for better column analysis of small molecule CBD via salt assisted LLE, CBD was detected in the plasma of rats injected with 20 mg/kg body weight CBD in vehicle. Due to having a low sample size (n=5), mechanical difficulties, and QTOF column clogging, CBD was only successfully detected and verified in the plasma of two female Long-Evans rats. Plasma was collected 30-minutes post IP injection and processed via heparinization, centrifugation, and LLE as described below. LC-QTOF analysis shows strong reads with high area counts of CBD in plasma samples. Two samples from each subject were analyzed to ensure proper detection (3a-b, 4a-b). MSMS extracted ion chromatograms (EIC) (Fig. 4 a-d) show area count of the most abundant CBD fragment (193.1222 m/z).
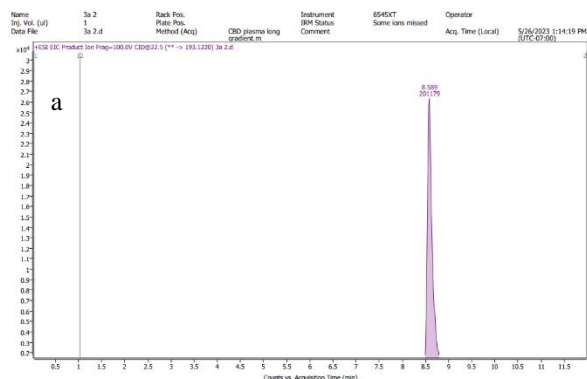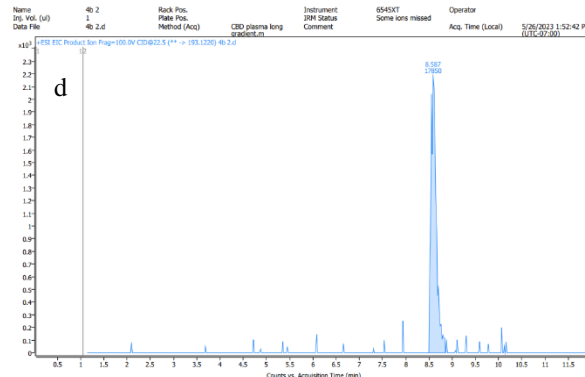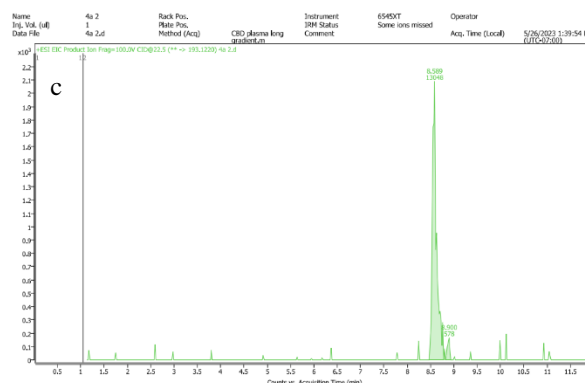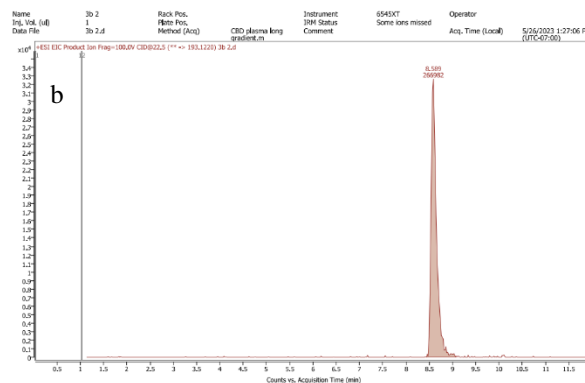

Fig. 4 a-d | MSMS EIC chromatogram of CBD most abundant fragment. a-b. Female subject 3 CBD area counts of 201179.16 and 266982.33 with an acquisition time of 8.6 minutes. **c-d.** Female subject 4 CBD area counts of 13047.77 and 17850.01 with an acquisition time of 8.6 minutes.

As secondary assurance that detected LC-QTOF fragment area counts are cannabidiol, plasma collected without prior CBD IP injection was analyzed as a blank. CBD absence in blank further supports accurate CBD detection in rat plasma samples post IP injection. Standards created with

known concentrations of pure CBD can be used to approximate CBD concentration within plasma samples through standard concentration curve comparison. Standard concentrations varied mildly due to possible standard CBD interaction with 200 proof ethanol as well as 3x dilution with acetonitrile following LLE method. Secondary standards are needed to ensure proper concentration calculations.

Identification #	Area Count	Dilution Factor
3a	201179.16	3
3b	266982.33	3
4a	13047.77	3
4b	17850.01	3

Table 1. | MSMS area count abundance and dilution factors. Area counts from extracted chromatogram (see methods) for CBD fragment 193.1222 m/z for samples a-b for animals 3 and 4. Dilution factor of 3 is incorporated due to 1:3 dilution with acetonitrile during LLE.

Discussion

CBD increases rates of active lever presses in rats following IP injection. CBD in plasma can be detected via LC-QTOF mass spectrometry following LLE. While further research is needed to support these results, as the sample size here is low, a significant increase in both male and female seeking behavior supports the need for continued research on CBD. Increased lever responding for 10% sucrose following CBD administration supports use for targeted increasing rate of response. Targeted increased cue response away from alternate addictive cues may act as a prevention of drug-motivated behavior. For example, CBD administration may increase response to sucrose seeking or social participation and away from cocaine addiction cue response.

Cannabidiol can successfully be detected in plasma following IP injection of 20 mg/kg body weight. With increasing research on CBD application for treatment of various conditions, it is important that levels of CBD entering the blood and reaching the brain are understood. Having the means to analyze CBD concentration in the body will improve research on dose dependency and potentially provide insight into sex differences of dosing recommendations as well as dose target ranges for specific applications.

Acetonitrile use may be a better alternative to 200 proof ethanol in creating a standard concentration curve for analysis of CBD concentration in plasma samples following IP injection. Future directions include CBD standard production with acetonitrile that will allow for comparison of plasma sample area counts vs time of acquisition to determine exact concentration of CBD. Both the behavioral model of the effect of CBD on sucrose seeking and LC-QTOF plasma analysis should be repeated with a greater n to ensure results and analyze possible sex differences. Specifically, sex differences in CBD sensitivity, target dose range, and blood concentrations following 30-minute post IP injection window.

Brain specimens collected post-treatment are being analyzed to determine where the brain CBD is localizing following IP injection as well as how much CBD is residing in the brain. Concentration and localization of CBD in the brain may provide insight into the behavioral effects as well as assess the presence of any possible sex differences in CBD mechanisms.

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Methods

Behavioral pilot

Pure CBD powder (Cayman Chemical Company) was mixed into vehicle solution of 3% Tween 80 at a concentration of 20 mg/mL. CBD powder was added to vehicle and vortexed for 1 minute, heated in 2-minute intervals in a water bath at 85°C, and sonicated for two minutes. Solution was vortexed again before each use and stored refrigerated in absence of light.

Long-Evans rats were selected for behavioral CBD pilot study (n=18). All animals were cared for following proper guidelines and the experimental protocols were approved by Western Washington University Institutional Animal Care and Use Committee (IACUC). Four males and four females were randomly assigned vehicle condition and the remaining five male and five female rats were placed in the CBD condition.

Rats initially responded for sucrose on a FR1 schedule of reinforcement for 10 days at 2hrs/day in order to develop sucrose dependency. All 18 subjects by day 10 developed and maintained sucrose seeking behavior.

On test-day 11, CBD or vehicle (3% Tween 80) was administered at 20 mg/kg body weight via IP-injection 30-minutes pretreatment. Rats were then run on a 2/hr FR1 schedule of reinforcement under the same conditions as days 1-10. Active lever responses were recorded via Med Associates operant boxes. Response rate differences were analyzed via t-test ($p<0.05$).

Plasma and brain collection

Five Long Evan rats (3 female and 2 male) were selected for this study. Age, health, and litter were all controlled for, and protocol was approved by the WWU IACUC. Following proper handling techniques, each rat was given an IP injection of 20 mg/kg CBD based on body weight. Thirty minutes post IP injection rats underwent rapid decapitation. Trunk blood was immediately collected in two

heparin coated Eppendorf tubes and the brain was then extracted. Whole brain collection was immediately placed in isopentane: 2-methyl-butane over dry ice for flash freezing. Brain samples were then stored at -80º F.

Heparinized blood was immediately placed in a refrigerated centrifuge and spun for ten minutes at 4680 RPM. Clear plasma separated from red and white blood cells was then removed and placed in a clean Eppendorf tube via micropipette. For further plasma purification, salt assisted liquid-liquid extraction (LLE) was used to knock out large proteins. 100 μL plasma was combined with 200 μL acetonitrile and vortexed for 60 seconds. 50 mg of a 4:1 salt mixture (magnesium sulfate, sodium chloride, sodium citrate) was added and sample was again vortexed for 60 seconds. LLE sample was then placed in the centrifuge for 10 minutes at 10,000 g and 20°C. Clear plasma was removed from the supernatant and placed in a clean Eppendorf tube.

Above centrifugation and LLE were performed for standard mixture, though desired standard CBD concentration altered the total amount of acetonitrile added. 10 mg of pure CBD (NIDA Drug Supply) was dissolved in 10 mL 200 proof ethanol and vortexed for 60 seconds. This standard then underwent 3 1:100 dilutions with 200 proof ethanol (10 μL initial standard, 990 μL 200 proof ethanol) to get a standard concentration of 100 ng CBD per mL ethanol. Desired standard concentrations then determined volume of stock standards added to 100 μL plasma without prior CBD injection (rat #5). 200 μL acetonitrile minus needed standard was then added for a total volume of 300 μL.

QTOF Plasma analysis

Following salt-assisted LLE of plasma samples and standards an LC-QTOF-MS (Agilent 1290 UHPLC with an AdvanceBio 6545 XT Q-TOF) system was used to quantify CBD concentrations. Separation was attained with an Agilent Eclipse+ C18 RRHD column, a 0.2 ml/min flow rate, and a 12-minute gradient transitioning between water with 0.1% formic acid (solvent A) and acetonitrile with 0.1%

formic acid (solvent B) (Table 2). The system was fitted with an electrospray source with the capillary voltage and nozzle voltage set at 300 and 1500 V respectively. Within the mass spectrometer, the fragmentor voltage was set to 100 V while the skimmer was at 60 V. Analyte confirmation and peak integration were completed with Agilent MassHunter software.

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
2	50	50
8	20	80
9	5	95
10	5	95
10.5	95	5
12	95	5

Table 2. | Extended gradient used for CBD analysis in plasma. Water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) over 12 minutes.

MSMS

MSMS via LC-QTOF was used to ensure expected CBD molecule fragmentation was occurring. Prior MSMS fragmentation of CBD suggests the most abundant fragment is 193.122 m/z[13]. MSMS EIC chromatograms for fragment size 193.1222 were extracted using Agilent MassHunter software.

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