Development of Liquid Chromatography-Tandem Mass Spectrometry Methods of Cannabinoids for Pediatric Patient Samples

A Thesis Submitted to the
College of Graduate and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the College of Pharmacy and Nutrition
University of Saskatchewan
Saskatoon

© Copyright Stephanie Vuong, October 2020. All rights reserved.
Unless otherwise noted, copyright of the material in this thesis belongs to the author.

Abstract

Thirty percent of pediatric epilepsies become resistant to conventional treatments, such as antiepileptic drugs and ketogenic diets. Growing anecdotal evidence of using *Cannabis* for treating epilepsy has prompted parents to acquire *Cannabis* products for their children without the consent or guidance of their pediatrician. Limited scientific based evidence exists for pediatric epilepsy and *Cannabis* therapy. Establishing a standard dosage regimen to ensure the safety and efficacy with *Cannabis* based medicine for pediatric epilepsy requires conducting a pharmacokinetic (PK) study to define the age-dependent pharmacokinetic parameters.

The Cannabidiol and Children with Refractory Epileptic Encephalopathy (CARE-E) open-labelled dose escalation study utilizing a 1:20 Δ^9-tetrahydrocannabinol (THC): cannabidiol (CBD) *Cannabis* herbal extract containing 4% cannabichromene (CBC) will establish a recommended dose for the PK study and define the relationship between the minimum cannabinoid plasma concentration at steady state ($C_{ss,min}$) and dose. A sensitive and selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was utilized to quantify the $C_{ss,min}$. Participant A-04 exhibited a greater than proportional increase in $C_{ss,min}$ relative to the dose (10-12 mg/kg/day), indicating non-linear PK. No THC intoxication was observed during the study. All participants displayed linear pharmacokinetics and seizure frequency reductions at 5-6 mg/kg/day, recommending the 5-6 mg/kg/day dose to be used for the PK study.

Ketogenic diets, a high fat diet used for pediatric epilepsy, may alter the plasma levels of lipoproteins, a major plasma protein in cannabinoid plasma protein binding. The unbound cannabinoid concentration is only able to produce a pharmacological effect; therefore, it is imperative to determine the effects ketogenic diets impart on protein binding to conclude if dosage adjustments are necessary. Cannabinoids may exhibit non-specific binding or buffer solubility issues observed with the commonly used plasma protein binding assays. A novel 3-extraction technique was developed for lipophilic compounds to avoid these issues. A comparative analysis was conducted between commonly used techniques (ultrafiltration and rapid equilibrium dialysis) and the 3-solvent extraction technique, with the 3-solvent extraction technique providing higher cannabinoid recovery and assay

reproducibility, indicating 82.7%, 82.1%, 87.0%, and 93.4% plasma protein binding of 11-OH-THC, CBD, THC, and CBC, respectively.

With legalization of recreational *Cannabis*, there has been growing concern over pregnant women consuming *Cannabis.* Cannabinoids can cross the blood placental barrier and reach the fetal systemic circulation. Increased *Cannabis* use during pregnancy would be a public health concern; therefore, it is crucial to determine the prevalence of prenatal *Cannabis* exposure. This was determined using residual neonate dried blood spot (DBS) screening cards collected from April, May, and June 2018 (pre-legalization) and April, May, and June 2019 (post-legalization). Due to its long half-life 11-nor-9- carboxy- Δ^9-tetrahydrocannabinol (THC-COOH), an inactive THC metabolite, is a suitable drug marker for *Cannabis* exposure. A quantitative LC-MS/MS assay was initially developed, however, factors such as hematocrit effect, chromatographic effect, and sample heterogeneity contributed to inaccurate and imprecise cannabinoid quantification. Alternatively, a qualitative LC-MS/MS assay, which solely utilizes a limit of detection (LOD), was applied, establishing a LOD of 1.47 ng/mL. Currently, we detected THC-COOH in 11 of 220 Saskatchewan residual neonate DBS cards collected pre-legalization, indicating a 5% prenatal *Cannabis* exposure rate.

The recommended dose obtained from the CARE-E dose escalation study and the LC-MS/MS assay will be utilized in single oral dose pharmacokinetic studies in the pediatric population to characterize the required age dependent pharmacokinetic parameters for establishing a standard dosage regimen. The 3-solvent extraction technique will determine the influence ketogenic diets have on the cannabinoid plasma protein binding profile and if dosage adjustments are necessary. Complete analysis of the pre- and post-legalization Saskatchewan, Manitoba, and British Columbia residual neonate dried blood spot samples will establish the prevalence of prenatal *Cannabis* exposure in each province.

Permission to Use

In presenting this thesis/dissertation in partial fulfillment of the requirements for a master's degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis/dissertation in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis/dissertation work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis/dissertation.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Dean of the College of Pharmacy and Nutrition
University of Saskatchewan
Health Sciences Building, 107 Wiggins Road
Saskatoon, Saskatchewan S7N 5E5 Canada

OR

Dean of the College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9 Canada

Acknowledgements

Firstly, I would like to thank my two co-supervisors, Drs. Jane Alcorn and Andrew Lyon. I am grateful for your mentorship and guidance throughout the last four years. Both of you have helped me overcome personal challenges in research and life. You helped me gain confidence in becoming a researcher. I will forever be indebted to you.

I would like to thank my advisory committee members, Drs. Anas El-Aneed and Fang Wu, for providing their invaluable expertise and advice. I would also like to thank my graduate chair, Dr Ildiko Badea, for her support throughout my degree.

I would like to thank my fellow colleagues from the College of Pharmacy and Nutrition, particularly Shelley Yang, Ayat Zagzoog, and Raj Rai. I greatly appreciate your assistance with my research. Thank you for the endless laughter and life advice you have given me. I will treasure our lifelong friendship.

I would like to express my utmost gratitude to Deborah Michel. Thank you for all the support and patience throughout my degree. I appreciate your never-ending encouragement. You have taught me so much and I am forever grateful to you.

Lastly, I would like to acknowledge the College of Pharmacy and Nutrition as well as the College of Medicine for the financial support.

Dedication

I dedicate this thesis to my parents, Toan and Thuy Vuong. Thank you for instilling values that I have carried throughout my whole life. You both inspired me through the challenges you have faced. You taught me to work hard, be kind, and stay optimistic. I am grateful for the love and support you have provided me. I hope I have made you proud. I love you always.

Table of Contents

Abstract i

Permission to Use iii

Acknowledgements iv

Dedication v

Table of Contents vi

List of Tables x

List of Figures xiii

List of Abbreviations xv

1 Introduction 1

2 Literature Review 3

2.1 Refractory Epileptic Encephalopathy 3
2.1.1 What is refractory epileptic encephalopathy? 3
2.1.2 Treatments of Epilepsy 4

2.2 *Cannabis* 6
2.2.1 Endocannabinoid system 6
2.2.2 Cannabinoids 8
2.2.3 Therapeutic use of *Cannabis* 11
2.2.4 Side effects of *Cannabis* use 12
2.2.5 Prenatal *Cannabis* exposure 12
2.2.6 Therapeutic *Cannabis* use in pediatric population 14
2.2.7 Safety concerns with *Cannabis* use in the pediatric population 16
2.2.8 CARE-E study 17
2.2.9 Pharmacokinetics of cannabinoids in adults 18

2.3 Age dependent differences in pharmacokinetics and impact on dosage determination 23
2.3.1 Absorption 23
2.3.2 Distribution 24
2.3.3 Metabolism 24
2.3.4 Excretion 25

2.4 A review of quantitative and qualitative cannabinoid analysis 26
2.4.1 Matrices utilized in cannabinoid analysis 26

2.4.2 Different analytical techniques for quantitative and qualitative cannabinoid analysis 33
2.4.3 Reverse phase liquid chromatography 36
2.4.4 Triple quadrupole mass spectrometry 37

3 Rationale 39

4 Overall purpose of study 39

4.1 Hypotheses 40

4.2 Objectives 40

5 Ethics 41

6 Materials and Methods 41

7 Development and Validation of a LC-MS/MS Method for the Quantification of Bioactive Cannabinoids in Plasma of Pediatric Patients on *Cannabis* herbal extract Therapy 43

7.1 Method Development 43

7.2 Method Validation 48
7.2.1 System Suitability 48
7.2.2 Selectivity 48
7.2.3 Linearity 49
7.2.4 Intra- and Inter-day Accuracy and Precision 49
7.2.5 Ion Ratios 49
7.2.6 Carryover 50
7.2.7 Stability 50
7.2.8 Matrix effect studies 51

7.3 Results 52
7.3.1 System Suitability 53
7.3.2 Selectivity 54
7.3.3 Linearity 54
7.3.4 Inter- and intraday accuracy and precision 55
7.3.5 Ion Ratios 59
7.3.6 Stability 59
7.3.7 Carry Over 62
7.3.8 Matrix Effects 63

8 Analysis of CARE-E pediatric patient samples using the plasma LC-MS/MS method 65

8.1 Dose Escalation Study 65

8.2 Results 66

9 Determination of the Unbound Fraction of CBD, THC, CBC, and 11-OH-THC in Human Plasma 73

9.1 Ultrafiltration technique to determine unbound fraction of cannabinoids 73
9.1.1 Method 73
9.1.2 Results 74

9.2 3-solvent extraction technique to determine unbound, lipoprotein, and remaining bound fraction of cannabinoids 75
9.2.1 Method 75
9.2.2 Results 76

9.3 Rapid equilibrium dialysis (RED) technique for determining the unbound cannabinoid fraction 78
9.3.1 Method 79
9.3.2 Results 80

10 Development of a Dried Blood Spot Assay for the Detection of Prenatal *Cannabis* Exposure in Newborns 83

10.1 Method Development for Quantitative Analysis 84
10.1.1 Method 85
10.1.2 Results 88

10.2 Method Development for Qualitative/Drug Detection Analysis 89
10.2.1 Method 89
10.2.2 Method Validation 90
10.2.3 Detection of cannabinoids in neonate dried blood spot samples in Saskatchewan pre-legalization of recreational *Cannabis* 92

10.3 Results 92
10.3.1 Limit of Detection 93
10.3.2 Prenatal *Cannabis* exposure detection in Saskatchewan June 2018 neonate DBS samples 93

11 Discussion 95

11.1 Context 95

11.2 LC-MS/MS Method Development and Validation 97
11.2.1 Method Development 97
11.2.2 Method Validation 99

11.3 CARE-E Study 102
11.3.1 Non-specific binding and cannabinoid instability issues 103
11.3.2 CARE-E sample analysis 104

11.3.3 Factors affecting pharmacokinetic variability ... 105
11.4 Plasma Protein Binding Assay ... 107
11.5 Dried Blood Spot Assay ... 113
12 Future Work ... 116
12.1 LC-MS/MS optimization ... 116
12.2 CARE-E Dose Escalation Study ... 117
12.3 Pharmacokinetic studies ... 117
12.4 Dried Blood Spot- Prevalence of Prenatal *Cannabis* Exposure ... 118
References ... 120
Appendix ... 142

List of Tables

Table 7.1. Multiple Reaction Monitoring (MRM) conditions for the identification and quantification of CBD, THC, 11-OH-THC, and CBC in human plasma 47

Table 7.2. Mass spectrometer parameters for plasma LC-MS/MS method 48

Table 7.3. System suitability data of 11-OH-THC, CBD, THC, and CBC, showing the mean peak area of five repeated injections of a 50 ng/mL concentration and acceptable CV (%) of less than 6%. 53

Table 7.4. Intraday accuracy and precision of LLOQ, LQC, MQC, and HQC for CBD and THC for each of the 3 days of method validation. Accuracy and precision of observed LLOQ and QC concentrations must be within 20% and 15% of the theoretical concentrations, respectively (n=6). 56

Table 7.5. Intraday accuracy and precision of LLOQ, LQC, MQC, and HQC for 11-OH-THC and CBC for each of the 3 days of method validation. Accuracy and precision of observed LLOQ and QC concentrations must be within 20% and 15% of the theoretical concentrations, respectively (n=6)... 56

Table 7.6. Interday accuracy and precision data of LLOQ, LQC, MQC, and HQC for CBD, THC, 11-OH-THC, and CBC over the 3 days of method validation. Accuracy and precision of observed LLOQ and QC concentrations must be within 20% and 15% of the theoretical concentrations, respectively (n=18)... 57

Table 7.7. Ion ratio precision of CBD, THC, 11-OH-THC, and CBC, comparing the peak areas of the qualifier ions and quantifier ions of intraday (n=12) and interday (n=36) samples during method validation 59

Table 7.8. Comparison of CBD, THC, 11-OH-THC, and CBC in LQC, MQC, and HQC samples unprocessed and processed for 6 hours at room temperature to samples extracted

at 0 hours. Unprocessed and processed samples remained stable, staying within 15% the nominal concentration for all cannabinoids. 60

Table 7.9. Peak area of pre-spike, post-spike, and pure spike LQC samples used to calculate the extraction efficacy, matrix factor, and recovery of CBD and THC undergoing sample extraction in human plasma. 63

Table 7.10. Peak area ratio of pre-spike, post-spike and pure spike LQC samples to demonstrate the internal standard correcting for the variation in CBD and THC during sample extraction in human plasma. 64

Table 8.1. Acceptable tolerance criteria for cannabinoids based on the ion ratio relative intensity 66

Table 8.2. Ion ratio of CARE-E site A participant samples from visits 2-6, with % bias calculated from the reference standard curve ion ratios of CBD (0.38), THC (0.60) and CBC (0.57). 66

Table 8.3. Minimum CBD plasma concentrations (ng/mL) at steady state of the CARE-E site A (Saskatchewan) participants from visit 1-5 during the dose escalation study. 69

Table 8.4. Minimum THC plasma concentrations (ng/mL) at steady state of the CARE-E site A (Saskatchewan) participants from visit 1-5 during the dose escalation study. 70

Table 8.5. Minimum CBC plasma concentrations (ng/mL) at steady state of the CARE-E site A (Saskatchewan) participants from visit 1-5 during the dose escalation study. 70

Table 9.1. Unbound and total concentration of CBD in human plasma using ultrafiltration 74

Table 9.2. Concentrations of 11-OH-THC, CBD, THC, and CBC in isooctane, 2-octanol, and acetonitrile following 3-solvent extraction in human plasma. 77

Table 9.3. Unbound and bound fractions of 11-OH-THC, CBD, THC, and CBC in human plasma following 3-solvent extraction technique. Bound fraction includes lipoprotein and remaining plasma protein bound cannabinoids. 78

Table 9.4. Unbound and total concentration of 11-OH-THC, CBD, THC, and CBC in human plasma using Rapid Equilibrium Dialysis with 4-hour incubation time (n=3) 80

Table 9.5. Comparison of CBD and THC concentrations in PBS and human plasma incubated at 0 and 2 hours, in replicates of 6, and determination of recovery, based on the comparison of CBD and THC concentrations in buffer to plasma (nominal concentration of 50 ng/mL). 81

Table 10.1. Conditions outlining mobile phase gradient for the DBS LC-MS/MS method ... 86

Table 10.2. Multiple Reaction Monitoring (MRM) conditions for the identification of THC-COOH, CBD, and THC in dried blood spots (DBS) 86

Table 10.3. Mass spectrometer parameters for dried blood spot LC-MS/MS method 87

Table 10.4. Detection of THC-COOH, CBD, and THC following analysis of 220 Saskatchewan neonate DBS samples (June 2018) obtained from the Roy Romanow Provincial Lab 94

Table 11.1. Summary of three cannabinoid plasma protein binding assays, detailing the technique used, plasma protein bound fraction, and limitations to the study. 111

List of Figures

Figure 2.1. Chemical structures of phytocannabinoids, endocannabinoids, and synthetic cannabinoids- a) cannabidiol, b) Δ^9-tetrahydrocannabinol, c) 11-hydroxy-Δ^9-tetrahydrocannabinol, d) 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, e) cannabinol, f) cannabichromene, g) 6-α-hydroxy-cannabidiol, h) 6-β-hydroxy-cannabidiol, i) 7-hydroxy-cannabidiol, j) anandamide, k) 2-arachidonoylglycerol, l) HU-210, m) WIN 55, 212-2, and n) JWH-018. 9

Figure 2.2. General schematic diagram displaying a triple quadrupole mass spectrometer, consisting of electrospray ionization (ESI) in positive ionization mode and channel electron multiplier (CEM) detector.......... 38

Figure 7.1. LC-MS/MS method optimization, showing efficient separation and increased sensitivity with modifications to mobile phase composition, flow rate, and sample extraction with a) initial LC-MS/MS conditions of an isocratic method running for 5 minutes with flow rate of 250 µL/min, b) gradient method running for 13.5 minutes with flow rate of 250 µL/min, and c) final LC-MS/MS isocratic method running for 10 minutes with flow rate of 700 µL/min.......... 44

Figure 7.2 Chromatogram depicting the retention times of the quantifier ions for 11-OH-THC (1.83 min), CBD (2.19 min), THC (5.55 min) and CBC (8.50 min). 53

Figure 7.3. Chromatograms displaying the 6 blank individual sources of human plasma used in the method validation to determine the selectivity of the LC-MS/MS method. An unknown peak detected at 3.05 minutes has the same MRM transitions as the CBD, THC, and CBC quantifier ions but not detected in the qualifier ion. The unknown peak does not elute at the same retention times of the cannabinoids, therefore not interfering with the quantitative analysis. 54

Figure 7.4. Standard curves of CBD, THC, 11-OH-THC, and CBC, during the 3 days of method validation, showing linearity with R^2 values $\geq$0.98. CBD, THC, and 11-OH-THC have 8 standard points, ranging from 0.49-125 ng/mL. CBC has 7 standard points, ranging

from 0.98-125 ng/mL. Standard curves are weighted 1/x to improve the regression fit of the low concentration standards. 55

Figure 7.5. Chromatograms comparing the response of CBD, THC, 11-OH-THC, and CBC quantifier ions in ULOQ standard (1a) and double blank (1b). Absence in the response of quantifier ions indicate no carryover of cannabinoids from the ULOQ. Similarly, the response from ULOQ (2a) and double blank (2b) of the internal standards indicated no carryover of the internal standard. 62

Figure 8.1. Minimum plasma concentration of CBD, CBC, and THC at steady state ($C_{SS,min}$) of the seven CARE-E participants during the CARE-E dose escalation study at site A (Saskatchewan). Adapted from Huntsman et al. (2019). Dosage Related Efficacy and Tolerability of Cannabidiol in Children with Treatment-Resistant Epileptic Encephalopathy: Preliminary Results of the CARE-E Study. Frontiers in Neurology. 71

Figure 9.1. General schematic diagram of 3-solvent extraction technique using isooctane, 2-octanol, and acetonitrile to determine unbound, lipoprotein bound, and remaining plasma protein bound fractions of cannabinoids, respectively. 75

Figure 9.2. Diagram displaying the assay and sample chamber of equilibrium dialysis plate (a) and the equilibrium of the unbound drug concentration as it selective diffuses through the cellulose membrane insert (b). Rapid equilibrium dialysis plate is dependent to size, with a membrane cutoff of 8 kDa, restricting the passage of plasma protein bound cannabinoids. 79

Figure 10.1. Chromatogram displaying the LOD (1.47 ng/mL) of CBD, THC (a) and THC-COOH (b), achieving signal-to-noise (s/n) ratio greater than 3 in positive ionization mode. 93

List of Abbreviations

2-AG	2-Arachidonoylglycerol
5-HT_{1A}	Serotonin 1A receptor
6α-OH-CBD	6α-Hydroxy-cannabidiol
6β-OH-CBD	6β-Hydroxy-cannabidiol
7-OH-CBD	7-Hydroxy-cannabidiol
11-OH-THC	11-Hydroxy-Δ^9-tetrahydrocannabinol
AAG	Alpha-1-acid-glycoprotein
ADHD	Attention Deficit Hyperactivity Disorder
AEA	Anandamide
AED	Antiepileptic drugs
API	Atmospheric pressure ionization
ATP	Adenosine triphosphate
AUC	Area under the curve
BCRP	Breast cancer resistance protein
C_{max}	Maximum plasma concentration at steady state
$C_{ss,min}$	Minimum plasma concentration at steady state
CB_1	Cannabinoid type 1 receptor
CB_2	Cannabinoid type 2 receptor
cAMP	Cyclic adenosine monophosphate
CARE-E	Cannabidiol in Children with Refractory Epileptic Encephalopathy
CBC	Cannabichromene
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBM	*Cannabis* based medicine
CBN	Cannabinol
CE	Collision energy
CEM	Channel electron multiplier
CINV	Chemotherapy-induced nausea and vomiting
CRIS	Cannabinoid Research Initiative of Saskatchewan
CSWS	Continuous spike and waves during sleep

CXP	Cell exit potential
CYP	Cytochrome P450
DBS	Dried blood spot
dc	direct current
DP	Declustering potential
DRUID	Driving under the Influence of Drugs, Alcohol, and Medicines
DUI	Driving under the influence
ECS	Endocannabinoid system
EEG	Electroencephalogram
ESES	Electrical status epilepticus in sleep
ESI	Electrospray ionization
FAAH	Fatty acid amino hydrolase
FABP	Fatty acid binding protein
FDA	Food and Drug Administration
$f_{(b)}$	Bound drug fraction
$f_{u(b)}$	Unbound drug fraction
GABA	gamma-Aminobutyric acid
GC	Gas chromatography
GLUT-1	Glucose transporter 1
GPR3	G-protein coupled receptor 3
GPR55	G-protein coupled receptor 55
HCT	Hematocrit
HIV	Human Immunodeficiency Virus
HPLC	High performance liquid chromatography
HQC	High quality control
IgG	Immunoglobulin G
KD	Ketogenic diet
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LGIT	Low glycemic index treatment
LGS	Lennox-Gastaut Syndrome
LLOQ	Lowest limit of quantification
LOD	Limit of detection

LQC	Low quality control
MAGL	Monoacylglycerol
MAP kinase	Mitogen-activated protein kinase
MCT	Medium chain triglyceride
MQC	Middle quality control
MRM	Multiple reaction monitoring
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PDA	Photodiode array detector
P-gp	P-glycoprotein
PK	Pharmacokinetics
PTSD	Post-traumatic stress disorder
QC	Quality control
RED	Rapid equilibrium dialysis
rf	radio frequency
RSD	Relative standard deviation
SUDEP	Sudden Unexpected Death in Epilepsy
SWGTOX	Scientific Working Group for Forensic Toxicology
THC	Δ^9-Tetrahydrocannabinol
THCA	Δ^9-Tetrahydrocannabinolic acid
THC-COOH	11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol
T_{max}	Time to reach maximum plasma concentration at steady state
TRPV1	Transient receptor potential cation channel subfamily V member 1
UGT	Uridine 5'-diphospho-glucoronosyltransferase
ULOQ	Upper limit of quantification
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet

1 Introduction

Cannabis sativa has gained interest as potential treatment for multiple medical conditions, including cancer, HIV, epilepsy, diabetes, among other disorders. The pharmacology of plant-derived cannabinoids, also known as phytocannabinoids, presents large knowledge gaps. The determination of the therapeutic properties of *Cannabis* and cannabinoids, specifically cannabidiol, requires further, extensive research. Recently, *Cannabis*-based medicines (CBM) have gained more attention as an alternative treatment for children with refractory epileptic encephalopathy. Still, doctors hesitantly consider giving their pediatric patients CBM, due to current lack of knowledge on the safety and efficacy of *Cannabis* treatment. However, anecdotal reports of antiepileptic properties of CBM have motivated some parents to acquire *Cannabis* products from unregulated distributors and without the knowledge of their doctor.

Parents expose *Cannabis* to their children without clear knowledge of the safety and efficacy of *Cannabis*. In a therapeutic context, this practice can be harmful as no standard dose of *Cannabis* currently exists for children. To be detailed, a high dose can lead to a child overexposed to *Cannabis*, resulting in unwanted, adverse effects, while a low dose can lead to underexposure, which may result in a child reaching subtherapeutic levels. Exposure to high Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) concentrations may not benefit children, potentially putting them at risk. The ability to quantify phytocannabinoids, and their metabolites in biological matrices is a first step to understanding the safety and efficacy of *Cannabis* exposures in children.

The objectives of my thesis include 1) developing liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for quantifying CBD, THC, 11-OH-THC, and CBC in human plasma and dried blood spots; 2) applying the developed LC-MS/MS method to measure minimum steady state plasma cannabinoid concentrations ($C_{ss,min}$) in pediatric patients with refractory epileptic encephalopathy on *Cannabis* oil therapy in the Cannabidiol and Children with Refractory Epileptic Encephalopathy (CARE-E) study; 3) determining the plasma protein binding profiles of cannabinoids in human plasma; and 4) applying the LC-MS/MS method to determine the prevalence of prenatal

Cannabis exposure pre- and post-legalization of recreational *Cannabis* using residual newborn screen cards. Of note, the CARE-E study is designed to assess the safety and tolerability of CBM in children as well as provide an appropriate starting dose for future pharmacokinetic (PK) studies in the pediatric population.

2 Literature Review

2.1 Refractory Epileptic Encephalopathy

2.1.1 What is refractory epileptic encephalopathy?

Refractory epileptic encephalopathy is an aggressive group of epilepsy disorders with seizures that can lead to neurological impairments, such as cognitive and psychoactive disorders, and behavioral issues.[1] It was recently reported that 6.9 of 1000 children in Canada are diagnosed with epilepsy.[2] Of these children, 30% develop epilepsies that are treatment resistant. Epilepsy occurs due to the irregular electrical discharges from different regions of the brain.[3] Many types of epileptic disorders fall into the category of pediatric refractory epileptic encephalopathy, including Lennox-Gastaut syndrome (LGS), Dravet syndrome, infantile spasms, and continuous spike wave sleep (CSWS/ESES).

Lennox-Gastaut syndrome, a rare form of epilepsy, represents 1-10% of childhood epilepsies, usually occurring in children before the age of 8, most commonly during the ages of 3 and 5 years old.[4] Types of seizures associated with LGS include tonic and atypical absence seizures (seizures that start slow with mild impairment of consciousness), myoclonic (brief jerks of the muscle), generalized tonic-clonic (seizure that affects the whole brain, causing individual to lose consciousness while muscles stiffen and spasm), and focal seizures (affects a region of the brain and the individual can either be conscious or unconscious while seizures occur).[4] LGS can lead to lesions in varying brain regions, potentially resulting in prominent cognitive and social impairments.[4,5]

Dravet syndrome, another rare but severe form of epilepsy, affects 1 in 15,700 people in the United States.[6] Types of seizures associated with Dravet syndrome include clonic/hemiclonic convulsive seizures (uncontrolled jerking), myoclonic seizures, focal seizures, and absence seizures.[7] The disruptive paroxysmal electroencephalogram (EEG) pattern consists of quick, short or isolated spike waves induced by eye closure.[8] Dravet syndrome results in central nervous system effects such as frequent seizures, developmental delays, speech impairment, and motor

issues.[6] Individuals with Dravet syndrome have increased risk of sudden unexpected death in epilepsy (SUDEP).[6]

The prevalence of infantile spasms occurs in 1-4 out of 10000 births. Infantile spasms usually appear at the early stage of childhood; most commonly occurring from 3-5 months of age.[9] These types of seizures occur during the stages of waking up or the early stages of sleep.[9] Infantile spasms develop disorganized EEG patterns, called hypsarrthythmia, consisting of a cluster of high voltage slow wave spikes followed by attenuation of paroxysmal fast activity.[10] Some individuals eventually recover from infantile spasms, but most often individuals remain with cognitive impairments throughout their whole life.[10]

Continuous spike wave sleep (CSWS), also known as electrical status epilepticus in sleep, has a prevalence of 0.5-0.6% in all childhood epilepsies.[11] This rare form of epilepsy consists of epileptic discharges during the slow sleep cycle, with an EEG pattern of constant spikes and waves. However, this pattern stops during the REM cycle.[12] After onset of CSWS, neurocognitive deterioration begins, affecting motor, behavioral, and language development.[11] This form of epilepsy does not result in the formation of lesions.

2.1.2 Treatments of Epilepsy

Many different antiepileptic drugs (AEDs) are available for treatment of epilepsy. Some of the common AEDs prescribed include clobazam, stiripental, topiramate, lamotrigine, levetiracetam, phenytoin, carbamazepine, valproic acid, and clonazepam. Individuals may be prescribed a single AED, or multiple AEDs, depending on the degree of their epileptic disorders. Some AEDs have a narrow therapeutic index, meaning slight changes in dose or blood concentration can elicit toxic effects.[13] This can be harmful, with potential for relapse of seizures or other negative side effects.[13] Often, antiepileptic drugs are ineffective against refractory epileptic encephalopathy.[14] Patients can develop tolerance or resistance to the effects of AEDs.

One possible cause of epileptic encephalopathy results from a metabolic disorder associated with glucose transporter 1 deficiency. The movement of glucose across the

blood brain barrier requires the functionality of glucose transporter 1 (GLUT-1).[15] Any deficiency in GLUT-1 function will result in reductions in glucose levels in the brain, the main biochemical energy source. To compensate for decreased brain glucose levels, epileptic children with a GLUT-1 deficiency may undergo a ketogenic diet, a diet consisting of foods with high fat content. Although firstly introduced in the 1920s, the ketogenic diet did not gain interest as a form of epileptic treatment until the 1990s.[16]

Although the true mechanism of the ketogenic diet remains unknown, ketone bodies may act to replace glucose as the new energy source for the brain.[15] Fatty acids can produce ketone bodies through ketogenesis. Enzymatically, fatty acids from dietary lipids undergo β-oxidation to produce acetyl-CoA, which in turn forms ketone bodies acetoacetate and β-hydroxybutyrate. Due to low carbohydrate levels in the diet, the body reduces its glucose utilization and switches to ketone bodies, produced from fatty acids.[1517] The ketone bodies act as the main source for energy production through cellular metabolism, helping to regulate neuronal function.[16]

Children with epilepsy typically use four types of ketogenic diets – classic ketogenic diet (KD), medium-chain triglyceride ketogenic diet (MCT), modified Atkins diet (MAD), and low glycemic index treatment (LGIT).[18] Classic ketogenic diet consists of excluding carbohydrates while increasing the intake of high-fat foods. Medium-chain triglyceride ketogenic diets contain more ketone bodies per unit of energy in comparison to the other ketogenic diets, which consists of long-chain triglycerides.[18] MCT diets have less restrictions than the classic ketogenic diet, meaning lower levels of fat intake and more allowance for carbohydrate intake.[19] This makes the less restrictive ketogenic diet more appealing and nutritional. The modified Atkins diet consists of a ratio of 1-gram fat to 1 gram of carbohydrates and proteins combined.[20] Lastly, the low glycemic index treatment focuses on restricting the intake of carbohydrates, only allowing the consumption of foods with a glycemic index of 40-60 grams per day.[20]

Many children discontinue the diet due to the inadequate nutritional value and the strict guidelines of the diet, as well as side effects that can occur while on the diet.[18] The side effects include high cholesterol levels, constipation, gastroesophageal reflux, vitamin D deficiency, kidney stones, and osteomalacia.[21] Due to the strict ketogenic diet, children may not receive proper nutrition needed for healthy growth, which can lead to

other health effects. If children decide to pursue a ketogenic diet, vitamin supplementation and adequate hydration must be regulated to help minimize the side effects.[21]

2.2 *Cannabis*

2.2.1 Endocannabinoid system

The endocannabinoid system consists of cannabinoid receptors, endocannabinoids, and enzymes that play a role in the chemical and biological processes associated with endocannabinoids.[22] Cannabinoid receptors, located in the central and peripheral nervous system, consists of two main cannabinoid receptors- cannabinoid type 1 (CB_1) and cannabinoid type 2 (CB_2). CB_1 receptors, predominantly found in the central nervous system, modulate neurotransmission.[23] CB_1 receptors located on the presynaptic terminals of GABAergic and glutamatergic neurons, affect the release of γ-amino butyric acid (GABA) and glutamate neurotransmitters in the human body.[22,23] CB_1 receptors also regulate opioids, dopamine and serotonin.[24] Cannabinoid type 2 receptors, predominantly found in immune cells (such as T cells, B cells, and monocytes), modulate the immune response.[23]

Cannabinoid receptors mainly associate with Gi/Go proteins, inhibiting adenylyl cyclase and activating mitogen-activated protein kinase (MAP kinase).[22] Adenylyl cyclase catalyzes adenosine triphosphate (ATP) into 3',5'-cyclic adenosine monophosphate (cAMP), a second messenger necessary for the stimulation of protein kinase A.[25] Activation of CB_1 receptors also causes the inhibition of voltage-gated calcium (Ca^{2+}) channels and activation of inwardly rectifying potassium (K^+) channels. Their activation results in the extension of the resting membrane potential, which can reduce the length of action potentials.[22] Inhibition of voltage-gated Ca^{2+} channels can prevent the formation of neurotransmitter vesicles, inhibiting neurotransmitter release.

Depending on the type of neurons as well as the brain region expressing CB_1 receptors, different effects can occur. Activation of CB_1 receptors on glutaminergic neurons inhibit glutamate, an excitatory neurotransmitter, leading to a reduction in

neuronal excitation. Inhibition of glutamate release in the hippocampus, basal ganglia, and midbrain will affect memory, movement coordination, and pain response, respectively.[26] Activation of CB_1 receptors on GABAergic neurons will inhibit GABA, an inhibitory neurotransmitter, leading to an increase in neuronal excitation. The mechanism of the inhibition of GABA remains unknown, as many speculate this process would have counteracted the effects of glutamate inhibition, but this is not the case.[26] Cannabinoid receptors are not just exclusive to cannabinoid type 1 and 2 (CB_1 and CB_2 receptors), but they include orphan cannabinoid receptors, such as GPR18, GPR55, and vanilloid type receptors.[27]

Endocannabinoids originate endogenously in the body and are fatty acid cannabinoids derived from arachidonic acid-containing phospholipids. The two main endocannabinoids are arachidonoylglycerol (2-AG) and anandamide (AEA or arachidonoylethanolamide) (Figure 2.1).[28] Raphael Mechoulam identified the first endocannabinoid, AEA, a partial agonist of CB_1 receptors .[28] Both 2-AG, a full agonist of CB_1 receptors, and AEA have an "on demand" synthesis and release, meaning neuron induction must occur for synthesis of endocannabinoids. AEA and 2-AG depend on the release of Ca^{2+}.[29] In the presence of a phospholipase D-like enzyme, N-arachidonoyl-phosphatidyl-ethanolamine hydrolyzes to produce AEA. sn-2-Arachidonate-containing diacylglycerol depends on sn-1-diacylglycerol lipase for the formation of 2-AG.[29] These ligands play an important role in physiological processes, such as emotions, appetite, and pain sensitivity.[30] When activating CB_1 receptors, anandamide can also lower blood pressure, leading to bradycardia.[29] Cannabinoid receptor activation requires small amounts of 2-AG production.[29] Termination of endocannabinoid activity follows after the release from the cannabinoid receptors, transporting into the neurons where they undergo degradation.[29]

An understanding of how endocannabinoids cross the plasma membrane remains unknown; however, studies have suggested that AEA and 2-AG undergo passive diffusion or endocytosis before undergoing intracellular transport for inactivation.[31] Once in the neuron, endocannabinoids require the presence of a carrier protein to mediate transport within the cell.[29] Fatty acid-binding proteins (FABPs) as well as heat shock proteins 70.2 (HSP70.2) act as intracellular carriers for AEA and 2-AG,

solubilizing and transporting them to fatty acid amide hydrolase (FAAH) or monoacylglycerol (MAGL) to be inactivated.[32] FAAH can degrade both AEA and 2-AG, forming arachidonic acid and ethanolamine (from AEA) as well as glycerol (from 2-AG).[29] MAGL selectively degrades 2-AG, forming arachidonic acid and glycerol.[29]

The presence of the phytocannabinoids, THC and CBD, can increase the levels of AEA and 2-AG by binding to FABPs, resulting in inhibition of FABPs.[32] Because FABPs are inhibited, AEA and 2-AG cannot be transferred to FAAH to be inactivated, resulting in an accumulation of circulating endocannabinoids.[32] The accumulation of AEA and 2-AG will prolong the effects associated with the endocannabinoids.

2.2.2 Cannabinoids

Cannabinoids are a class of chemical compounds that share similar structural and/or functional properties of Δ^9-tetrahydrocannabinol (THC) that act on cannabinoid receptors. Cannabinoids categorize into three classes: phytocannabinoids, endocannabinoids, and synthetic cannabinoids. The phytocannabinoids originate from the *Cannabis sativa* plant. To date, researchers have identified 565 compounds in the *Cannabis* plant, including more than 120 cannabinoids. Of these 120 compounds, some of the most discussed phytocannabinoids involve Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), cannabinol (CBN), and cannabichromene (CBC) (Figure 2.1).

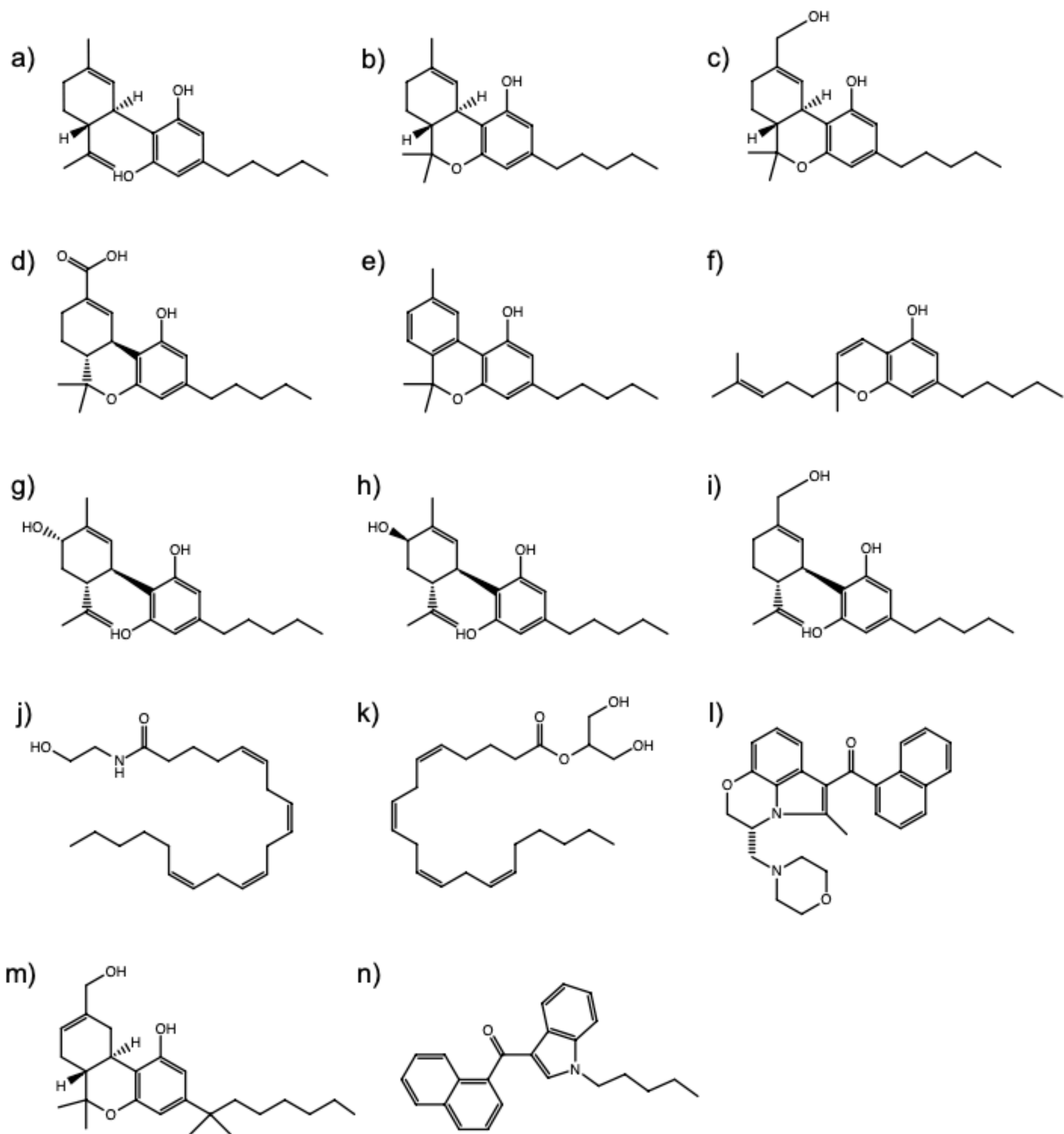

Figure 2.1. Chemical structures of phytocannabinoids, endocannabinoids, and synthetic cannabinoids- a) cannabidiol, b) Δ^9-tetrahydrocannabinol, c) 11-hydroxy-Δ^9-tetrahydrocannabinol, d) 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, e) cannabinol, f) cannabichromene, g) 6-α-hydroxy-cannabidiol, h) 6-β-hydroxy-cannabidiol, i) 7-hydroxy-cannabidiol, j) anandamide, k) 2-arachidonoylglycerol, l) HU-210, m) WIN 55, 212-2, and n) JWH-018.

Although discovered in the 1930s, the isolation of THC, the main psychoactive cannabinoid of *Cannabis sativa*, did not occur until 1964.[33] Tetrahydrocannabinolic acid (THCA), the precursor of THC and primary form found in the *Cannabis* plant, undergoes decarboxylation via light or heat, to produce THC. After consumption, THC rapidly metabolizes to 11-OH-THC, its main secondary psychoactive metabolite.[32] 11-OH-THC further metabolizes to THC-COOH, a non-psychoactive metabolite of THC. Forensic labs often use 11-OH-THC to determine recent *Cannabis* exposure. THC-COOH is a biomarker used in clinical and forensic labs to detect exposure to *Cannabis*. THC-COOH undergoes conjugation to produce THC-COOH-glucuronide, a major water-soluble urinary metabolite of THC. The high concentration of CB_1 receptors found in the central nervous system is responsible for the psychoactive effects, observed mostly in THC, a partial agonist the receptor.[34,35] Researcher assume the psychoactive properties associated with THC stems from the high concentration of CB_1 receptors found in the central nervous system.[35]

Cannabidiol (CBD) is one of the main therapeutic cannabinoids found in *Cannabis sativa*. Although considered a non-psychoactive cannabinoid, CBD exerts antianxiety and other neurological effects.[36] Similarly to THCA, the precursor cannabidiolic acid (CBDA) undergoes decarboxylation to produce CBD. The mechanism of cannabidiol (CBD) still remains unclear as CBD has a low affinity for both CB_1 and CB_2 receptors, although CBD has a greater affinity to CB_2 receptors compared to CB_1 receptors, which may explain the absence of psychoactive properties in CBD.[35]

It is assumed that CBD binds to various non-cannabinoid receptors, such as GPR55 receptors, TRPV1 receptors, 5-HT_{1A} receptors, and more recently discovered GPR3 and GPR6 receptors.[37,38] It is believed that CBD binds to TRPV1 receptors, eliciting anti-inflammatory and antinociceptive effects by desensitizing nociceptors.[39] Additionally, CBD binds to 5-HT_{1A} receptors, serotonin receptors that modulate anxiety, mood, and hunger. Binding of CBD to these receptors may display protective effects against ischemia (inadequate blood supply to the heart) and also produces anxiolytic effects, which reduces anxiety.[39]

Synthetic cannabinoids, man-made chemical compounds, have the same psychoactive effects observed in phytocannabinoids. They are used commonly because

they are not considered to be a controlled substance.[40] The consumption of synthetic cannabinoids has been declining in the United States due to the legalization of medical and recreational *Cannabis*. Common synthetic cannabinoid drug brands found in the market include Spice and K2. These drugs are marketed as herbal blends, directed for the usage as incense.[41] Included in these drugs are synthetic cannabinoids WIN 55, 212-2, HU210, and JWH-018 (Figure 2.1). Studies have shown that HU210, a CB_1 and CB_2 receptor agonist, may reduce gastric acid and pepsin secretion, as well as reduce inflammation in acute pancreatitis.[42] JWH-018, an agonist of both CB_1 and CB_2 receptors, has greater affinity to the cannabinoid receptors, compared to THC. Higher affinity to the CB receptors explains the adverse side effects of JWH-018, such as vomiting, headaches, paranoia, tachycardia, seizures, and hallucinations.[43]

While effects may be similar to some phytocannabinoids, synthetic cannabinoids tend to reach their peak effects more rapidly.[44] Issues exist with these synthetic cannabinoid drugs as they are usually laced with other components that are not stated on the label. In fact, some ingredients do not match what is listed in the mixture, causing uncertainty in the degree of potency and safety of the product.[41]

2.2.3 Therapeutic use of *Cannabis*

Since ancient times *Cannabis* has been used therapeutically for many different health conditions, including chronic pain, nausea/vomiting resulting from chemotherapy, diabetes, anxiety, depression, epilepsy, cachexia resulting from HIV/AIDS and cancer, and other conditions.[45] The use of *Cannabis* for the diverse range of health issues in part relates to the different physiochemical characteristics displayed by many distinctive phytocannabinoids present in the plant *Cannabis sativa*.

Two synthetic THC analogs are currently the only Food and Drug Administration (FDA) approved cannabinoid drug in the United States – Dronabinol (also known as Marinol) and Nabilone. Both Dronabinol and Nabilone are given to patients dealing with nausea and vomiting associated with chemotherapy, and Dronabinol also is used for anorexia and wasting seen in Human Immunodeficiency Virus (HIV) patients.[45] Dronabinol is no longer available in Canada due to its psychoactive effects. Another

cannabinoid drug is Nabiximols, an oral spray containing a mixture of CBD and THC. Nabiximols is approved in Canada for the use in treatment-refractory cancer pain, multiple sclerosis spasms, and central pain.[45]

2.2.4 Side effects of *Cannabis* use

The therapeutic use of *Cannabis* is associated with some important consequences. With the benefits come the side effects, some considered severe and permanently damaging, especially in children. While THC helps relieve the symptoms of spinal cord injury, Parkinson's disease, cachexia, and Crohn's disease, the psychoactive effects tend to deter patients from medical *Cannabis*. These psychoactive effects can include anxiety, dysphoria, memory impairment, and psychomotor and cognitive performance impairment.[33] Additionally, the brain does not reach full maturation until the age of 25. Considering the central nervous system contains CB_1 receptors, long duration of exposure to high THC concentrations may cause long-term detrimental effects.[46]

Although CBD has fewer side effects compared to THC, if taken in high doses, CBD can alter cytochrome P450 (enzymes found in the liver) activity, thus changing hepatic drug metabolism.[35] High CBD doses can also increase tremors associated with Parkinson's disease. Lowering the dose may relieve these side effects but lowering the dose may result in subtherapeutic levels.[35] High CBD doses may temporarily cause immediate low blood pressure followed by lightheadedness.[35]

2.2.5 Prenatal *Cannabis* exposure

Legalization of recreational *Cannabis* use raises concerns of women consuming *Cannabis* during pregnancy. In Spain, detection of cannabinoids in the meconium determined a prevalence of *Cannabis* exposure of 5.3%, comparable to the international prevalence of 4.5%, with *Cannabis* reported as the most used illicit drug by pregnant women in Western countries.[47] Other surveys have reported prevalence of prenatal *Cannabis* exposure between 5-16%.[48] A study conducted in Ontario, Canada reported an increase prevalence of prenatal *Cannabis* exposure from 1.2% in 2012 to 1.8% in

2017.[49] Expectedly, this number will increase with more accessibility after the legalization of recreational *Cannabis*.

Varying numbers reported stem from the challenges in self-reporting pregnant women. These challenges include under-reporting, risk of legal consequences, and possible loss of child custody.[47] *Cannabis* is now considered the number one drug of abuse in pregnant women. Pregnant women perceive *Cannabis* less harmful to the fetus compared to other drugs, such as cocaine, heroin, and methamphetamine.[48] Synthetic cannabinoids are more potent than phytocannabinoids, due to high affinity for the CB receptors.[48]

A study in Colorado reported 69% of the *Cannabis* retailers surveyed promoted the use of *Cannabis* to treat morning sickness, while 36% deemed *Cannabis* use safe during pregnancy.[50] Majority of women while pregnant report using *Cannabis* to help relieve the nausea and vomiting associated with morning sickness, with no understanding of the damage that can be done to the fetus.[50] With legalization, pregnant women have more access to obtain recreational *Cannabis* without their physician's consent. Many women do not know they are pregnant while consuming *Cannabis* products while some women are unaware about the consequences of using *Cannabis* while pregnant.

The endocannabinoid system plays a significant role in reproduction. The ECS contributes to fertilization, oocyte transport, implantation, embryo development, and immune regulation.[51,52] Studies suggest a decrease in FAAH enzymes, which metabolizes endocannabinoid AEA, is linked to spontaneous miscarriages.[52] During embryo development, AEA is highly controlled. High AEA levels during the non-receptive stage and low AEA level during the receptive stage of implantation suggests AEA downregulation important for successful embryo implantation.[52] Exposure to THC can increase AEA levels by inhibiting FABP, potentially leading to unsuccessful fertility.[48]

The uterine endometrium, placenta, and ovaries express cannabinoid receptors. Cannabinoid receptors start developing around 14 weeks of gestation and increase during the third trimester, with the greatest density located in the frontal lobe and cerebellum.[47] One-third of cannabinoids present in the maternal plasma crosses the

placental barrier.[47] In vitro exposure of CBD to placental cells inhibited breast cancer resistance protein (BCRP) and p-glycoprotein (P-gp) efflux transport, enhancing the permeability of other cannabinoids and xenobiotics.[48,53]

In rats, prenatal *Cannabis* exposure resulted in changes to the dopaminergic activity in the corpus striatum, a section of the brain responsible for motor and reward activity.[46,53] Reduced dopamine levels links to increase risk of attention deficit hyperactivity disorder (ADHD) and changes in motor activity. Reduced dopamine levels in the prefrontal cortex and hypothalamus-pituitary axis also make the neonate susceptible to cognitive impairment and emotional dysregulation.[47,48]

There is still a lack of understanding on the effects of *Cannabis* exposure during the early or late stages of pregnancy. One study suggests *Cannabis* use early in pregnancy increases the risk of physical development, such as inadequate fetal growth, lower birth weight, small head circumference, and thicker prefrontal cortex.[47,54] Cannabinoids can decrease the subpopulation of T cells, leading to potential immune dysregulation, an effect prominent throughout adolescence and adulthood.[48] Increased anxiety and depression due to prenatal *Cannabis* exposure is exhibited as early as childhood through adulthood.[47,48] Many effects are not immediately detected and can take years before any neurodevelopmental impairments are displayed. In school age children, those exposed to *Cannabis* prenatally had delayed development with speech and language skills.[48]

With the increase of *Cannabis* accessibility after the legalization of recreational *Cannabis*, more research is needed to determine the long-term effects *Cannabis* use has on the fetus.

2.2.6 Therapeutic *Cannabis* use in pediatric population

Incidence of pediatric and adolescent *Cannabis* exposure has increased with the decriminalization of recreational and medical *Cannabis* use. This increase allowed for greater accessibility of regulated *Cannabis* products for pediatric therapeutic use without the knowledge or guidance of a pediatrician. *Cannabis* based medicine (CBM) has gained interest for its use in treatment resistant disorders, including pediatric

epilepsy. Considerable evidence of successful *Cannabis* use for pediatric treatment exist; however, most are anecdotally based rather than scientifically based. Therapeutic use of *Cannabis* for the pediatric population includes autism, perinatal brain injury, neuroblastoma, chemotherapy-induced nausea and vomiting (CINV), neuropathic pain, post-traumatic stress disorder (PTSD), Tourette syndrome, major depressive disorder, and epilepsy.[55,56]

Cannabis use for pediatric treatment focuses mainly on treatment of pediatric epilepsy. The ECS has shown to play an important role in the mechanism in seizures. During a seizure episode, the excessive glutamate release will cause CB_1 receptors to provide a negative feedback mechanism by reducing glutamate release, ultimately reducing neuronal hyperexcitability.[57] However, individuals with epilepsy appear to have downregulation of CB_1 receptors on glutaminergic axon terminals and upregulation on GABAergic axon terminals, creating an imbalance in the ECS which can contribute to epileptic activities.[57]

Few studies have investigated the efficacy of purified CBD oil for pediatric epilepsy. Devinsky et al. conducted a 12-week open label clinical trial using Epidiolex in 162 participants with doses titrated up to a maximum of 25 mg CBD/kg/day, administered twice daily.[58] Studies using purified CBD oil report administration of higher CBD doses, averaging around 20-25 mg/kg/day. Devinsky reported a median of 36.5% reduction in seizure frequency at 25 mg/kg/day. Additionally, in 2017, Devinsky et al. conducted a 14-week randomized clinical trial using Epidiolex, with a dose of 20 mg CBD/kg/day in 120 participants.[59] Participants receiving CBD treatment reported a median of 38.9% seizure frequency reduction. Thiele et al. also conducted a randomized clinical trial using purified CBD oil at a similar dose of 20 mg CBD/kg/day, reporting a median of 43.9% reduction in seizure frequency.[60] However, participants receiving the placebo reported a 21.8% reduction in seizures, making it difficult to understand the true CBD efficacy.

The entourage effects between cannabinoids and terpenes in CBM may provide similar effects of purified CBD but at lower doses. Terpenes, such as linalool, potentially display anticonvulsant properties by modulating neuronal excitability, specifically decreasing glutamate release.[61] A 20-week open label trial led by McCoy et al.

determined the efficacy of a 1:50 THC:CBD CBM in 20 participants, using a dose of 16 mg CBD/kg/day administered twice daily.[62] The median seizure frequency reduction was reported at 70.6%. Tzadok et al. conducted a retrospective study with 1:20 THC:CBD CBM, with doses ranging from 1-20 mg CBD/kg/day, with majority of participants administered <10 mg CBD/kg/day.[63] 89% of participants reported a reduction in seizures, with 52% obtaining >50% seizure frequency reduction. Hausman-Kedem et al. conducted an observational, longitudinal study to determine efficacy of a 1:20 THC:CBD CBM in 69 participants.[64] Initial maximum dose was set to 50 mg CBD/kg/day administered three times daily. However, participants could only tolerate doses of 4-32 mg CBD/kg/day, with an average of 11.4 mg CBD/kg/day. 56% of participants achieved >50% seizure reduction frequency.

While research on *Cannabis* use for the pediatric population has steadily increased, large gaps of knowledge still exist, such as the safety and efficacy. Published clinical studies provide large disparities with the use of pure CBD or cannabinoid herbal extracts, amounts of CBD and THC in CBM, and dose. Purified CBD oils are less accessible due to higher costs and regulatory restrictions.[65] Purified CBD oils do not contain any THC, avoiding any possible psychoactive effects associated with THC consumption. Practitioners are hesitant to prescribe CBM due to possible THC intoxications. Most CBMs contain low amounts of THC, reducing the adverse side effects associated with the psychoactive cannabinoid. Standardization of the dose and type of CBM is necessary to ensure appropriate safety and efficacy.

2.2.7 Safety concerns with *Cannabis* use in the pediatric population

A majority of participants in clinical trials experienced at least one form of adverse effects. Most adverse effects associated with therapeutic use of CBD are mild to moderate, including somnolence, fatigue, vomiting, nausea, and decrease in appetite.[58-64] Severe, but rare, adverse effects include status epilepticus, diarrhea, pneumonia, increase levels of liver aminotransferase enzymes, and weight loss. The most common adverse effect, somnolence, was associated with participants taking clobazam and CBM concomitantly.

Use of *Cannabis* based medicine as an adjuvant treatment to AEDs can lead to drug-drug interactions. As a CYP2C19 inhibitor, CBD increases N-desmethylclobazam, an active metabolite of clobazam, potentially causing overexposure to clobazam.[65,66] The majority of adverse effects decreased with dose reduction of CBM or dose adjustment/removal of AEDs.

2.2.8 CARE-E study

The Cannabidiol in Children with Refractory Epileptic Encephalopathy (CARE-E) study, led by Dr. Richard Huntsman, a pediatric neurologist from University of Saskatchewan, and Richard Tang-Wai, a pediatric neurologist from University of Loma Linda, investigated the use of CBM to treat pediatric epilepsies nonresponsive to anticonvulsant drugs and ketogenic diet.[67] The study used purified Cannimed herbal extract with a 1:20 ratio of Δ^9-tetrahydrocannabinol (THC):cannabidiol (CBD) with the primary goal of evaluating the safety and tolerability of cannabidiol rich CBM for children with epilepsy.

This study demonstrated that the CBM has low side effects in children. The researchers will conduct a larger clinical trial to demonstrate the efficacy of CBD as a treatment for epilepsy with approval from Health Canada. Other objectives of the CARE-E study include evaluation of the pharmacokinetics of CBD and Δ^9-THC, the efficacy of the CBM (measured by the decrease in duration and frequency of seizures), and the quality of life in participants dealing with refractory epileptic encephalopathy. The CARE-E study aims to provide evidence that CBD enriched CBM produces high therapeutic effects with low side effects.

Seven participants have completed the CARE-E dose escalation study in Saskatchewan.[2] All participants reported a reduction in seizure frequency starting at a dose of 5-6 mg/kg/day CBD-rich CBM, with four participants having $>$50% seizure frequency reduction. At 10-12 mg/kg/day, all participants have an average seizure frequency reduction of 74%, with five participants having $>$50% seizure frequency reduction. Of those 5 participants, 3 became seizure free. All participants continuously

maintained seizure reductions 3 months after weaning from the CBD rich CBM. The complete CARE-E study has not yet been published.

The CARE-E study also monitored the safety and tolerability of the CBM. All participants reported side effects from the CBD rich CBM, including nausea, vomiting, diarrhea, increased appetite, and difficulty sleeping. However, these side effects were considered minor and did not increase with increasing doses of the CBD rich CBM. No participants reported adverse effects related to THC consumption.

2.2.9 Pharmacokinetics of cannabinoids in adults

2.2.9.1 Absorption

The route of administration can influence the time it takes for a drug to be effective, as well as the concentration of the drug found in the body. The most common route of administration of *Cannabis* is inhalation via smoking. Cannabinoids quickly absorb through the lungs, where it can diffuse from the alveoli directly to the bloodstream, and subsequently distribute to the brain.[68] Because of the direct diffusion into the blood, cannabinoids can reach the target organs quickly, with pharmacological effects occurring in seconds to minutes.[68] The peak effects of THC associated with inhalation can last for 15 to 30 minutes, before slowly decreasing and persisting for an additional 2 to 3 hours.[69] Inhalation of THC and CBD exhibit high absorption; however, bioavailability varies due to factors such as puff duration, puff intervals, breath hold time, and depth inhalation.[68] Inhalation bioavailability reported for THC varies from 10-35%. CBD inhalation bioavailability is approximately 31%.[70] Inhalation bioavailability is much higher than oral bioavailability due to first pass effects (i.e. the loss of drug prior to reaching the systemic circulation) observed with oral administration. Maximum plasma concentration at steady state (C_{max}) and area under the curve (AUC) values higher in frequent smokers compared to occasional smokers, but time at which C_{max} is observed (T_{max}) remains the same.[70]

Oromucosal administration undergoes rapid absorption though the mucosal membrane, providing higher plasma cannabinoid concentrations than oral administration but lower concentrations compared to inhalation administration.[70] A portion of the dose may undergo oral absorption, which may reduce cannabinoid

bioavailability.[71] Increased cannabinoid bioavailability is observed with food administration, with mean AUC and C_{max} values 3-5 times higher than without food intake.[70]

The absorption of cannabinoids varies with oral administration due to first pass metabolism. For instance, THC can pass through the liver and undergo first pass metabolism to form 11-OH-THC, the main psychoactive metabolite. Additionally, CBD undergoes extensive first pass metabolism.[72] Onset of pharmacological effects of THC occurs around 30-90 minutes, with peak effects at 2 to 3 hours, before decreasing and lasting for 4 to 12 hours.[69] Blood concentrations of ingested THC reach 25-30% of levels following inhalation.[23]

Oral is the principal route of CBD administration. Varying oral bioavailability is observed with CBD, ranging from 13-19%.[53] Low bioavailability is due to poor solubility, first pass metabolism, and gastrointestinal degradation.[70,73] Oral bioavailability can increase with increase in lipid formulation due to improved solubility or absorption via the lymphatic system.[17] Transporters may play a role on the oral bioavailability of cannabinoids, such as P-gp efflux transporters, which are located in intestines, blood-brain barrier, and blood-placental barrier. As a P-gp inhibitor, CBD may increase permeability of P-gp substrates, such as THC.[53,74] T_{max} of orally administered CBD varies from 1-4 hours, compared to inhalation T_{max} of 0.6hr.[70] Oral T_{max} values higher than inhalation, providing benefits of longer periods of relief.

2.2.9.2 Distribution

The physicochemical characteristics of a drug determines how a drug is distributed in the body. Both CBD and THC are considered highly lipophilic compounds; therefore, they will be highly distributed in the highly vascularized and lipid-like tissues like adipose, heart, brain, mammary glands, liver, lungs, and the spleen.[34,68] In the brain, higher 11-OH-THC levels are present compared to THC (only 1% of THC in systemic circulation reaches the brain), suggesting 11-OH-THC contributes significantly to the psychoactive effects associated with THC.[34] THC is a substrate of P-gp transporter, undergoing efflux from the blood-brain barrier, limiting its permeability.[74]

Efflux transport can greatly affect THC effects by reducing brain concentrations. Due to high lipophilicity, cannabinoids will deposit into fatty tissues, leading to a slow and extended release of cannabinoids into the plasma over time. This, with the addition of enterohepatic recirculation, contributes to a larger volume of distribution of cannabinoids and a long half-life (CBD $t_{1/2}$= 1-5 days; THC $t_{1/2}$= 4.1 days).[34,70] In adults, volume of distribution of CBD and THC ranges from 2.5 – 10 L/kg.[34,72]

In general, THC and CBD display >95% binding to plasma proteins.[72] This means that only 1-5% of total THC and CBD concentration is unbound and can exert pharmacological effects. Human serum albumin (600 μM in blood) is the most common plasma protein and is known to have 8 different binding sites with ability to bind endogenous and xenobiotic compounds.[75] Serum albumin binds strongly to lipophilic and acidic drugs.[76] This may include cannabinoids such as THCA and CBDA, cannabinoids that form THC and CBD through decarboxylation. Metabolites of THC, such as 11-OH-THC and THC-COOH, as well as their glucuronide forms bind largely to albumin.[77] α_1-Acid glycoprotein (AAG) binds tightly to basic and neutral drugs.[76] As neutral cannabinoids, THC and CBD would likely bind to α_1-acid glycoproteins (12-30 μM). In some cases, diseases can affect albumin and AAG binding by decreasing the levels of albumin and AAG in the body or the binding ability or increasing AAG concentrations.[75]

Cannabinoids do not actually bind to lipoproteins but instead partition into the lipid portion of the lipoprotein particle.[76] More lipophilic drugs are found in the membrane lipids in comparison to water. In epileptic patients, immunoglobulin G (IgG), a major type of antibody, levels exist considerably higher than those in normal individuals.[78] However, many cannabinoids have shown to suppress the levels of immunoglobulins, decreasing the immune response of the body.[79]

Only the unbound fraction of cannabinoids ($f_{u(b)}$) is pharmacologically active or has the ability to produce an effect. Considering the physicochemical characteristics of CBD and THC, plasma protein binding is high, therefore alterations of lipids and lipoproteins due to ketogenic diets may influence the cannabinoid $f_{u(b)}$.[80] Increases in dietary fat intake may increase blood lipoprotein levels.[80] Lipoproteins are a dynamic group of protein-lipid complexes that cannabinoids extensively bind to, ultimately

modifying the cannabinoid $f_{u(b)}$.[81] Knowing the impact of $f_{u(b)}$ and bound fraction ($f_{(b)}$) of cannabinoids can assist in dosage determinations.

Cannabinoids exhibit non-specific binding, adsorption to certain plastics and membrane filters found in conventional plasma protein binding techniques, such as ultrafiltration and rapid equilibrium dialysis.[82] Non-specific binding will cause inaccurate quantification of cannabinoids and will result in the underestimation of cannabinoid $f_{u(b)}$.

2.2.9.3 Elimination

The liver is the main contributor to the metabolism of THC and CBD, although the brain, small intestines, heart and lungs also play a role but with a lower contribution.[34] Cytochrome P450 (CYP) enzymes are located in the liver, intestine, kidney, lungs, heart, brain, and skin, with the highest expression found in the liver and intestines. When ingested, THC has a bioavailability of 6% due to first pass metabolism, which is considerably lower than inhalation, which has a bioavailability of 27%.[83] Under CYP metabolism, THC undergoes oxidation to produce 11-OH-THC.[83] As the primary metabolite, 11-OH-THC is considered to be more potent than its parent compound.[23] The main cytochrome P450 isoforms that play a role in the metabolism of THC to 11-OH-THC are CYP2C9, CYP2C19, and CYP3A4.[74] Additionally, 11-OH-THC can be metabolized into the inactive 11-nor-9-carboxy-THC (THC-COOH) by alcohol dehydrogenase or aldehyde oxygenase.[69] Finally, THC-COOH can further undergo glucuronidation to be excreted in the urine.[83]

Due to its high lipophilicity, THC tends to remain in the body for a long duration. The elimination half-life of THC can range from 20-30 hours, with full elimination occurring in 13-30 days, depending on the frequency of *Cannabis* use.[23,34] The majority of cannabinoids are excreted through the feces (~ 65%), while only 20% is excreted through the urine.[69] Because THC is a highly lipophilic compound, it undergoes tubular reabsorption, therefore a low concentration of unchanged THC is found in the urine.[34] 11-OH-THC is mainly excreted through feces while the inactive metabolite, THC-COOH, is excreted mainly through the urine as THC-COOH glucuronide via glucuronidation by UGT1A1 and UGT1A3.[69,84] Considered to undergo rapid metabolism and excretion, 11-

OH-THC has a detection window of 3 days.[74] The excretion half-life of THC-COOH is 44-60 hours in the urine, displaying a much slower excretion rate.[85] Due to a lower clearance, THC-COOH is considered a suitable diagnostic marker for the detection of *Cannabis* use.

Over 100 metabolites have been identified for CBD.[86] The main metabolic enzymes involved in CBD metabolism include CYP450 enzymes, glucuronosyltransferases, and sulfotransferases. Particularly, CYP1A1, CYP1A2, CYP2C19, CYP2D6, CYP3A4, CYP3A5, and CYP2A9 (minor) are the phase I enzymes responsible for producing the main monohydroxylated metabolites, 6-alpha-hydroxy cannabidiol (6α-OH-CBD), 6-beta-hydroxy-cannabidiol (6β-OH-CBD), 7-hydroxy-cannabidiol (7-OH-CBD), and 7-carboxy-cannabidiol (7-COOH-CBD). The most abundant metabolite formed is 7-COOH-CBD.[73,86]

At doses greater than 10 mg/kg, CBD may become dose-dependent, displaying nonlinear pharmacokinetics.[73] Plasma concentrations of CBD disproportionally increase with dose, due to saturation of CYP enzymes. Interestingly, CBD can undergo self-inhibition at high doses by inhibiting CYP2C and CYP3A enzymes, resulting in higher plasma concentrations than expected with linear pharmacokinetics.[87] Metabolites 6α-OH-CBD and 6β-OH-CBD may also contribute to the inhibition of CYP3A enzymes.[73]

Furthermore, CBD and its hydroxylated metabolites can undergo phase II metabolism via the glucuronidation (major) or sulfation pathway, though majority of the glucuronidated metabolites derive from the parent cannabinoid.[86] Enzymes UGT1A9, UGT2B7, and UGT2B17 play a major role in the glucuronidation of CBD, producing urinary metabolites CBD-glucuronide, 6-OH-CBD glucuronide and 7-OH-CBD glucuronide.[84,88] The majority of CBD is excreted through feces, where a high amount of free CBD can be detected.[88,89] Chronic oral administration of CBM displays a half-life 2-5 days.[70]

When co-administered with THC, CBD can competitively inhibit THC metabolism through competitive interaction of CYP enzymes.[86] Previous studies have reported that a high concentration of CBD partially inhibits CYP metabolism of THC, resulting in slight decrease of the formation of 11-OH-THC through first pass metabolism and increase of THC levels in the plasma.[72]

2.3 Age dependent differences in pharmacokinetics and impact on dosage determination

Little is known about the oral pharmacokinetics of cannabinoids in children. Children cannot be considered miniature adults, as there are many physiological differences between adults and children.[90] High variability is found in the pharmacokinetics of children in general, due to the rapid physiological changes during development.[91] Developmental maturation of physiological and biomedical processes is known to result in differences between children and adult drug pharmacokinetics.[90] Despite research on the pharmacokinetics of cannabinoids in adults, such data cannot be extrapolated to children, due to differences in body and organ size, route of administration, and physiological characteristics.

2.3.1 Absorption

Few studies discuss the differences of absorption in children, as most of the absorption effects of xenobiotics tend to focus on newborns and infants. The pH of the gastrointestinal tract does not reach adult values until the age of 3.[92] Basic drugs are more rapidly absorbed in a child compared to adults.[83] Intestinal transit time is considered to be shorter in children up to the age of 12 years compared to adults.[91] Immature gastrointestinal mucosal epithelium observed in infants can increase the bioavailability of xenobiotics.[93] Gut microflora does not reach similar levels of adulthood until adolescence. In fact, the gut microflora levels are different in infants, children, and adults.[92] Because of the immature gastrointestinal mucosal epithelium and the reduction of gut microflora, intestinal permeability is greater, enhancing the absorption of xenobiotics through the intestines and increasing the risk for toxic plasma concentrations in a child.[92] Reductions in first pass metabolism due to immature development of the CYP and phase II enzymes seen in infants and toddlers also can result in higher concentration of unchanged drug in the systemic circulation.[94] However, as CYP enzyme expression reach adult values by the age of 10, first pass metabolism

becomes comparable to adult capacity. This results in oral bioavailability variation throughout childhood.

2.3.2 Distribution

The body composition of an individual can have a major impact on how a drug distributes in the body. Body fat, total body water, plasma protein levels, and muscle mass can determine the volume of distribution in an individual.[91] In general, infants have a lower level of plasma proteins than older children. Additionally, plasma proteins in children mostly have a lower binding capacity.[92] This means that there will be more unbound drug fraction distributing in the blood circulation and can diffuse to the target organs or be metabolized and excreted. However, plasma protein levels reach adult levels within a few years.[91]

Changes in volume of distribution can impact drug pharmacokinetics. In infants, body water makes up 74% of the total body weight, decreasing to 55-60% when they reach adulthood.[90] The high percentage of body water in infants results in a higher volume of distribution of water-soluble drugs as compared with adults.[34]

Studies have shown that the blood brain barrier is not fully developed at birth, which makes the brain more vulnerable to xenobiotics.[92] In fact, the brain does not fully mature until the age of 25.[95] The brain of a child is proportionally large compared to their body, which can increase the distribution of xenobiotics to the brain.[92] Studies have shown at 4-6 years of age, the central nervous system volume reaches 80-90% of the CNS volume in adults, yet body surface area reaches adult values at 16-18 years of age.[92] With the addition of the under developed brain, this can result in different brain distribution characteristics relative to the adult.

2.3.3 Metabolism

Drug metabolism is an important process in detoxifying an active drug into an inactive metabolite that can be readily excreted from the body. Newborns and infants have low CYP mediated metabolism and low renal excretion, which can result in a drug exerting a greater potential of pharmacological or toxic effect.[93] However, through early

childhood, CYP metabolism capacity can exceed the capacity of an average adult, which can either increase detoxification of several drugs or cause the drug to undergo a greater degree of bioactivation and create a more toxic compound.[93] It is not until adolescence when enzyme activity reaches the same level as adulthood.[91]

While most cytochrome P450 enzymes do reach full functional capacity early on in life, there are a few isoforms that very slowly develop. For instance, CYP1A2 enzymes, which make up 13% of enzymes found in the liver, are known to metabolize antidepressants, antipsychotics, and polycyclic aromatic hydrocarbon (PAH) compounds. These enzymes reach 81% of adult values at 2 years of age.[92] The major enzyme isoform in humans, CYP3A4, metabolizes about 50% of drugs.[92] This isoform only reaches 50% adult values at 4-5 months of age and a developmental switch between the fetal CYP3A7 and adult CYP3A4 is observed in the first months of life.[91] Although CYP3A enzymes have a major role in metabolism, these enzymes develop early in life and may not produce major concerns if substrate overlap exists between CYP3A7 and CYP3A4.

Another P450 enzyme important in xenobiotic metabolism is CYP2C19. This enzyme contributes to the metabolism of anticonvulsant and non-steroidal anti-inflammatory drugs.[92] This isoform does not reach comparable adult capacity until the age of 10.[90] Enzymes involved in glucuronidation do not reach full functional capacity until 3 months to 3 years of age.[92] This reduces the ability of a drug to become more water soluble and more readily excreted through the urine. The liver, the most important organ for metabolism, and the kidney, the most important organ for excretion, of infants are still immature, which can affect the functionality of metabolism and excretion. As maturation of these organs progresses, metabolism and renal clearance increase.[93]

2.3.4 Excretion

Excretion is an important process to ensure that drugs and their metabolites are removed from the body, discontinuing the effects produced from these compounds. Immature drug excretion processes result in drug accumulation, which can increase the effects of drugs. Renal function increases with age due to an increase in renal blood

flow and decrease in renal vascular resistance.[96] Renal blood flow increase is dependent on the increase in cardiac output.[78] It takes about 2 to 3 years for renal function to reach maturation. [91]

The three main processes of renal excretion all reach full functional capacity at different rates. Glomerular filtration reaches full functional capacity by 6 months.[91] Tubular reabsorption does not reach adult values until 1-3 years.[92] Tubular secretion does not reach full adult functional capacity until 7 months, but exceeds adult values in children and adolescents.[92] With the different developmental rates of glomerular filtration, tubular reabsorption, and tubular secretion, this could cause difficulty in determining the renal excretion rate of a drug in children. An understanding the oral pharmacokinetics of drugs in children will aid in the development of age appropriate dosing regimens.

2.4 A review of quantitative and qualitative cannabinoid analysis

2.4.1 Matrices utilized in cannabinoid analysis

2.4.1.1 *Cannabis* plant components

Cannabinoids

Recreational *Cannabis* and *Cannabis*-based medicinal products come in various forms, including dried flower, fresh plant, oil extracts, edibles, and vapes. Often, *Cannabis* plant materials are analyzed for cannabinoid identification, quantification of cannabinoid concentration, potency, purity and quality control testing of regulated *Cannabis* products.[97] Countries regulate the maximum amounts of cannabinoids, especially THCA and THC, present in *Cannabis* strains and products.[94,98] The *Cannabis* industry often monitors original cannabinoid constituents (THCA, CBDA, and CBGA), which requires avoidance of decarboxylation during sample extraction and analysis.[97,99]

In addition to cannabinoids, other components found in the *Cannabis* plant, such as terpenes, mycotoxins, trace metals and pesticides, are often quantified for pharmacological and toxicological purposes.

Terpenes

Terpenes are responsible for the scent and flavor of *Cannabis* plants and play a role in fragrance preference for certain *Cannabis* strains.[100] Additionally, terpenes may possess therapeutic effects, though this is debatable amongst cannabinoid researchers. Termed “entourage effect”, cannabinoids and terpenes may provide a synergistic effect and aid in treating anxiety, depression, epilepsy, etc.[100,101] Characterization and quantification of the 200 identified terpenes present in different *Cannabis* strains may provide more information on the possible effects of *Cannabis* use.[101]

Mycotoxins

Improper storage and humid environmental conditions can create a favorable situation for growth of fungi and bacteria on *Cannabis* plants.[102] Fungi can produce secondary metabolites, termed mycotoxins. Aflatoxin, derived from the *Aspergillus* species, poses significant risks to humans, as it is considered to be carcinogenic.[102,103] Therefore, quantification of mycotoxins in *Cannabis* plants is pertinent to ensuring the safety of consumers. Under the *Cannabis* Act, regulated by Health Canada, all *Cannabis* plants must undergo aflatoxin testing, recommending limits set by the European Pharmacopeia of 2-12 μg/kg aflatoxin B1 and total of 4-15 μg/kg for aflatoxin B1, B2, G1, and G2.[103]

Trace metals

Trace metals, found in soil, rocks, pesticides, and fertilizer, can deposit and accumulate in *Cannabis* plants, potentially exposing consumers to metal toxicity.[102] Following the European Pharmacopeia and United States Pharmacopeia, Health Canada has set acceptable limits of arsenic, cadmium, lead, and mercury at sub μg/g in all *Cannabis* plant products.[104]

Pesticides

Pesticides aid in deterring unwanted pests from *Cannabis* plants, although pesticides pose a risk to humans, animals, and the environment.[105] Quantification of pesticides is necessary to ensure levels are well within the regulatory requirements. Acceptable pesticide limits depend on the type of *Cannabis* product. Regulated by Health Canada, *Cannabis* industries must test for 96 pesticides, including myclobutanil, imidacloprid, deltamethrin, and carbofuran, with allowance of 0.02-3 μg/g pesticide in dried *Cannabis* flowers, 0.01-2.5 μg/g in oil, and 0.01-1.5 μg/g in fresh plants.[104,105]

2.4.1.2 Biological matrices

Th most commonly used biological matrices for cannabinoid analysis include whole blood, plasma, serum, urine, saliva and hair. These biological matrices are often used for forensic/toxicological testing, therapeutic drug monitoring, and pharmacokinetic studies. Blood (either whole blood, plasma, or serum) provides the most reliable quantification of cannabinoid concentration in pharmacokinetic studies and toxicological testing.[106]

Urine

Recent *Cannabis* intake can be identified by detecting THC and THC-COOH and the urinary metabolites, THC glucuronide, and THC-COOH glucuronide, making it suitable for drug abuse analysis, driving under the influence (DUI) testing, and workplace testing.[107] Urine analysis also provides information on cannabinoid metabolism.[84-89] The use of urine is less invasive than venous blood collection. With chronic *Cannabis* use THC, 11-OH-THC, and THC-COOH may be detected in urine for up to 24 days.[108] With occasional *Cannabis* use THC and THC-COOH urinary metabolites can detect intake within 6 hours of *Cannabis* consumption while THC, 11-OH-THC, CBD, and CBN have no to little detection in urine.[107]

Oral Fluids/Saliva

Oral fluid testing is the most advantageous for roadside and workplace testing as it provides a non-invasive collection that can be collected on-site.[109,110] Metabolite THC-COOH provides an ideal marker for active drug use, as it is not present in *Cannabis* products (only present after metabolism) and has prolonged excretion.[111] Measurements of THC in oral fluids must be taken with precaution, to avoid THC contamination from oral fluid collection immediately after *Cannabis* consumption.[109]

Both THC and THC-COOH may be detectable in oral fluid up to 48 hours and 29 days, respectively.[110] Detected only after *Cannabis* exposure, CBD and CBN may be possible markers for recent *Cannabis* exposure.[112] Excluding the potential cannabinoid contamination immediately after *Cannabis* from residual cannabinoids in the oral cavity, oral fluid cannabinoid concentrations are comparable to plasma cannabinoid concentrations.[111] Wide inter-individual variability exists for oral fluid detection due to amount and time duration of *Cannabis* consumption and body weight.[110] Dry mouth, a common side effect with *Cannabis* consumption, can interfere and provide difficulty in collecting enough oral fluid volume in a timely manner.[112,113]

Hair

Hair has been used as a biological matrix for forensic cannabinoid testing, particularly to detect for chronic use of *Cannabis*.[114] The route of administration is important in reliably analyzing hair for *Cannabis* consumption. Oral administration of *Cannabis* provides no detectable hair levels of THC or THCA, yet external contamination of THC and THCA due to side-stream smoke can result in false positives of *Cannabis* consumption.[115,116] External contamination can be reduced by extensive washing of hair follicles prior to sample extraction; however, washing may not remove external contamination completely.[114,115] THC-COOH provides more reliable testing as metabolism occurs within the body, therefore a low possibility of external contamination.[115,116]

Blood

Blood specimens, including whole blood, plasma, and serum, are the most commonly used matrices for cannabinoid quantification and identification. These matrices are widely used in pharmacokinetic studies, tolerability and safety studies, therapeutic drug monitoring, workplace drug testing, DUI testing, and post-mortem analysis. Plasma cannabinoid concentrations provide information on total body cannabinoid exposure, necessary for determining pharmacokinetic characteristics of cannabinoids.

Due to high plasma protein binding and low erythrocyte partitioning, blood-to-plasma cannabinoid ratio for THC, 11-OH-THC, and THC-COOH is approximately 0.39, 0.53 and 0.37, respectively.[117] Based on the blood-to-plasma cannabinoid concentration ratio, cannabinoids are primarily isolated in the plasma. Use of whole blood for cannabinoid quantification may provide less sensitivity. Although whole blood analysis does not require prior plasma separation, plasma and serum samples provide better sensitivity in quantification of cannabinoids, as they contain less blood components and therefore less matrix effect or interference. Whole blood is susceptible to hemolysis, the rupturing of red blood cells, resulting in the release of hemoglobin and potassium into the plasma water.[118] Plasma and serum are processed to remove blood cells, minimizing partial matrix effect. However, the main contributors of matrix effect in plasma and serum are plasma proteins and phospholipids.

Plasma cannabinoid concentrations are more stable compared to whole blood cannabinoid concentrations. Whole blood cannabinoid concentrations vary in storage time periods. THC and CBD whole blood concentrations significantly decreased after 3 months when stored at -20°C.[119] Both 11-OH-THC and THC-COOH whole blood concentrations were stable until 26 weeks at -20°C. CBD, THC, 11-OH-THC, and THC-COOH plasma concentration were all stable until 1 year at -20°C.[119] If samples require reanalysis in the future, plasma provides better stability over blood.

The Driving under the Influence of Drugs, Alcohol, and Medicines (DRUID) analysis requires the use of whole blood for forensic testing.[117] However, blood analysis

requires on-site collection to preserve the cannabinoid concentration, as levels of cannabinoids can decrease rapidly.[106] Venous blood collection requires sampling in a sanitary environment, which can delay collection time and potentially cause cannabinoid levels to reach well below the cut-off limit.[120] Additionally, blood venous collection is invasive, has storage complications, and requires trained personnel for sampling.[120,121]

Dried blood spot

Dried blood spot (DBS) sampling has gained interest as an alternative for forensic testing. In fact, many US forensic laboratories implement the use of DBS for determining cannabinoid concentration in DUI testing. These labs have developed assays, using THC and 11-OH-THC as biomarkers for recent *Cannabis* exposure, due to their short half-lives, and THC-COOH as a biomarker for *Cannabis* exposure, as it usually remains in the body for several days.[120,121] It is less invasive, requires low volume of blood, and provides safer transport and storage conditions compared to the traditional venous collection.[120,122,123] DBS sampling also does not require complex preparation, such as addition of anticoagulant or plasma separation.[124] DBS samples can be stored at room temperature for an extended period of time while analytes remain stable.[122]

However, DBS has major disadvantages in quantitative analysis. Hematocrit (HCT) effect can interfere with accurate quantification of cannabinoids in DBS analysis. HCT, the volume of red blood cells in the blood, influences the viscosity of blood, affecting how the blood spot spreads on the card.[125] Individuals have different HCT levels, therefore blood spot spread and the volume of blood that dries per unit of area of the card will vary. This variation can cause additional extraction issues related to matrix effects.[125] DBS also undergo a chromatographic effect; more cells are concentrated in the center of the blood spot. Inconsistencies in distribution of the blood spot will cause issues with determining the punch area on the DBS card for sampling.

DBS testing was developed in 1963 and initially targeted neonate screening, the so-called heel prick tests, for early detection of congenital disorders, such as phenylketonuria, sickle cell disease, and now cystic fibrosis.[126] All newborns in Canada complete panels of heel prick tests typically within 24-48 hours after birth. These DBS

specimens are collected by pricking the heel of the newborn with a lancet and spotting the blood onto Whatman dried blood spot cards.

Canadian provinces vary in terms of DBS storage time periods for future use. Quebec, Newfoundland, and Nova Scotia require neonate DBS storage for 1-5 years while British Columbia and Alberta store for 5-10 years. Saskatchewan, Manitoba, Ontario, and New Brunswick store DBS samples for at least 21 years.[127] Researchers may access the cards if approved by research ethics boards; however, it raises concerns from the public on the nature of information that may come from the research.[127] Nonetheless, research for the secondary use of DBS samples usually requires specimen deidentification. The neonate heel prick tests are mandatory in Canada through provincial legislation because the testing allows for rapid identification of affected neonates, initiation of treatments to prevent developmental delay, mental retardation, disease exacerbation or death.

Plasma protein binding analysis

Unbound drug fraction ($f_{u(b)}$), an essential pharmacokinetic parameter, is the only fraction of the drug that exerts pharmacological effects. Plasma protein binding assays, such as ultrafiltration, ultracentrifugation, and equilibrium dialysis can determine the $f_{u(b)}$ of a drug.[128] Very few studies regarding plasma protein binding exist in the cannabinoid research area, with most plasma protein binding studies conducted, solely on THC and its metabolites, in the 1970s and 1980s.[81,129,130] Plasma protein binding profiles of THC, 11-OH-THC, and THC-COOH have been generalized for all cannabinoids, which must be cautioned, as plasma protein binding depends on the degree of lipophilicity and presence of a charge. Increase of plasma protein binding correlates to increase of lipophilicity.[128]

Additionally, highly lipophilic compounds exhibit non-specific binding due to adsorption to the surfaces of plastic and membrane filters commonly found in the conventional plasma protein binding techniques such as ultrafiltration and rapid equilibrium dialysis.[128] Cannabinoids display poor solubility to buffer solutions, an additional challenge with Rapid Equilibrium Dialysis (RED) analysis. Sethi et al. have developed a plasma protein binding assay that avoids the use of aqueous solvents and

materials associated with non-specific binding.[131] This novel technique extracts the unbound, lipoprotein bound, and remaining plasma protein bound using isooctane, 2-octanol, and acetonitrile, respectively.

Isooctane, a non-polar solvent, creates a soluble environment for free cannabinoids. Isooctane is not miscible with water, producing a two-layer phase. Proteins are insoluble in isooctane; therefore, only the unbound fraction of cannabinoids is able to diffuse into the isooctane.[131] Lipoproteins are soluble in 2-octanol, allowing cannabinoids bound to lipoproteins to diffuse into the solvent.[132] Similar to isooctane, 2-octanol is immiscible with water. Other plasma proteins, such as albumin and α-glycoprotein, are not soluble in 2-octanol and will remain in the aqueous phase. Acetonitrile is miscible with water, forming a homogenous environment. The organic solvent will reduce the hydration layer around the remaining plasma proteins, misconfiguring the protein and releasing the bound cannabinoid.[133]

The type of cannabinoid, matrix, and cannabinoid concentrations requires an understanding of each analytical technique, along with the advantages and disadvantages, to determine a suitable approach from accurate quantification and identification of cannabinoids.

2.4.2 Different analytical techniques for quantitative and qualitative cannabinoid analysis

Increased demand for *Cannabis* production prompts regulation of products deemed safe for consumption. Additionally, with arising potential therapeutic uses of *Cannabis*, researchers need to ensure the safety, tolerability, and efficacy of *Cannabis* products. Pharmaceutical and biomedical cannabinoid analyses require accurate quantitative (measurement) or qualitative (identification) assessment.

2.4.2.1 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy can determine the chemotype of certain *Cannabis* plants, or the variation of cannabinoid constituents present in the

plant.[134] NMR can also confirm the structures of lesser known cannabinoids and newly discovered synthetic cannabinoids. This is an advantage over gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) techniques with limited mass spectral libraries.[135] However, isolation of cannabinoids prior to NMR analysis can create difficulty, requiring extensive sample extraction, large volumes of sample, and specialized training.[135]

2.4.2.2 Gas chromatography

Gas chromatography (GC) is used for the quantitative and qualitative analysis of both *Cannabis* plant material and biological matrices. However, GC requires the use of high temperatures in the injector to convert sample to gas phase prior to chromatographic process. This can obstruct accurate measurement and detection of acidic cannabinoids, such as THCA and CBDA, as they can decarboxylate to their neutral cannabinoid forms, THC and CBD, from the high temperatures.[136-138] Derivatization of acidic cannabinoids, either silyation or esterification, can prevent decarboxylation, maintaining the acidic cannabinoids in plant or biological samples.[138] However, determination of total THC concentration (sum of THCA and THC concentration) quantification may benefit from GC analysis.[136]

GC analysis requires volatility of analytes to properly undergo chromatographic separation, providing challenges for analysis of several synthetic cannabinoids.[139] Low volatile synthetic cannabinoids undergo incomplete volatility and may require longer elution times, impeding high throughput analysis.[140]

2.4.2.3 Liquid chromatography

Another commonly used technique is high performance liquid chromatography (HPLC). Unlike GC, HPLC does not require the conversion of gas phase analytes via high temperatures, making it a suitable technique for acidic cannabinoids. The most commonly used technique for HPLC cannabinoid analysis is reverse phase (C-8 and C-18 columns) liquid chromatography. The use of nonpolar stationary phase provides efficient separation of cannabinoids, a class of compounds with varying polarity.

However, reverse phase columns do not provide chiral separation, an issue for many cannabinoids that exist as enantiomers.[141] Chiral columns can provide specificity between enantiomers. pH of mobile phase is important for stable retention times of acidic cannabinoids, with pH at least 2 units lower than the pKa of the acidic cannabinoids.

The most common detectors coupled to HPLC for cannabinoid analysis include ultraviolet (UV) detection, photodiode array (PDA) detection, fluorescence, and mass spectrometry. Typically, cannabinoids are present at high concentrations in *Cannabis* based products, making it more suitable to use HPLC-UV or HPLC-PDA compared to mass spectrometry.[122,142,143] Biological cannabinoid samples typically use mass spectrometry as it provides sensitivity for quantification and identification of low cannabinoid concentrations. Multiple cannabinoid analysis requires a technique with high selectivity and specificity. Many cannabinoids have similar polarity characteristics, proving problematic for HPLC-UV.[141] Cannabinoids can co-elute, resulting in overlapping peaks that will obstruct accurate peak area measurement. MS can differentiate cannabinoids based on mass-to-charge (m/z) ratios, allowing for greater selectivity. The high selectivity will lower the background noise and in turn provide greater sensitivity.

Pharmacokinetic studies (cannabinoid metabolite formation, tolerability studies, therapeutic drug monitoring), cannabinoid stability studies, and toxicological testing requires quantification of low cannabinoid concentrations.[144-146] Tandem mass spectrometry (coupling two mass analyzers) will provide selectivity by scanning for the m/z ratio of the ionized analyte (precursor ion) and its fragmentations (product ions). MS requires the use of stable isotope internal standards for accurate quantification. Internal standards can be expensive and may not be available for many of the lesser known cannabinoids.[141] Nonetheless, LC-MS/MS provides the greatest sensitivity and selectivity for high throughput pharmaceutical cannabinoid analysis in biological matrices.

2.4.3 Reverse phase liquid chromatography

Liquid chromatography is a technique used to separate analytes from other analytes or endogenous/exogenous components found in the matrix, based on the physicochemical properties and the interaction with the mobile phase and stationary phase. It is important to separate analytes from each other or from other components in the matrix to reduce any potential interferences in quantitative analysis.

Reverse phase is the most commonly used form of liquid chromatography, consisting of using a nonpolar stationary phase and polar mobile phase. The column, consisting of the nonpolar stationary phase, interacts with and adsorbs to the analyte.[147] Retention of the compound on the non-polar column is based on polarity. Non-polar analytes, such as THC and CBC, interact longer with the column, resulting in longer retention, while polar analytes, such as 11-OH-THC, have less interaction and elute off the column faster. Adjusting the mobile phase conditions and selecting an appropriate column is necessary to achieve optimal conditions for separation of cannabinoids.

The mobile phase consists of two different solvents, comprised of aqueous (mobile phase A) and organic (mobile phase B) solvents. The type of solvents and pH modifiers used in LC-MS/MS analysis is critical. The type of mobile phase B plays a major role in the elution of your analyte. Acetonitrile and methanol are the two most commonly used organic solvents for mobile phase, with acetonitrile having higher elution strength compared to methanol, resulting in shorter retention times.[147] However, this can be unsuitable if analysis involves multiple analytes, as overlapping peaks may occur.

pH environment can dictate the degree of ionization of the analyte. pH levels below the pKa of the analyte may result in the analyte remaining in the nonionized form, leading to greater retention.[148] pH levels above the pKa will produce ionized forms of the analyte. Ions have no or minimal interaction with the stationary phase, therefore will elute from the column earlier. pH levels within 2 units of the pKa can shift the analyte between the ionized and nonionized form, which will greatly impact the shift in retention time.[148] Commonly used pH modifiers/buffers include ammonium formate, ammonium acetate, formic acid, and acetic acid. By controlling the pH with pH modifiers or buffers

can provide the method with better separation, retention and peak shape of the analytes.

2.4.4 Triple quadrupole mass spectrometry

Mass spectrometry is a technique used commonly for the quantification and detection of drugs and biological material. Triple quadrupole mass spectrometry (Figure 2.2) is considered to be the ideal instrumentation to use for quantitative analysis due to its sensitivity and selectivity for both high and low mass molecules, consisting of two mass analyzers, used to select and scan for specific mass-to-charge ratios.[149]

The first stage of mass spectrometry is the ionization of the analyte. For the detector to produce a signal from the analyte, the analyte must undergo desolvation and ionization.[147] Electrospray Ionization (ESI), a commonly used atmospheric pressure ionization (API) technique, ionizes the analyte in the condensed phase by applying a voltage to the sample as it passes through the capillary of the electrospray probe, producing either positively or negatively charged ions.[147] Polar, volatile solvents are necessary for ESI, due to better desolvation and lower surface tension.

The mobile phase containing the analyte undergoes nebulization from a nebulizer gas applied down the probe, producing a fine aerosol of charged droplets. Temperature and a heater gas will aid in the desolvation of the analyte suspended in condensed phase, resulting in a gas phase ion. Once converted to a gas phase ion, now termed as a precursor ion, it will travel through the curtain plate employed with curtain gas, helping to prevent solvents and neutral molecules from entering the quadrupoles. As the precursor ions travel through the orifice plate, a declustering potential is applied to help gas phase ions dissociate from the charge droplets.[150] Finally, an ion guide helps focus the precursor ion into a tighter pathway, helping to increase the sensitivity of ions detected.

The first and third quadrupole, the two mass analyzers, consist of a direct current (dc) and radio frequency (rf) potential, alternating between positive and negative potential, creating a spiral trajectory pathway.[150] In the first quadrupole, the mass-to-

charge (m/z) ratio of the precursor ion is selected, only allowing corresponding analytes to travel down the quadrupole.

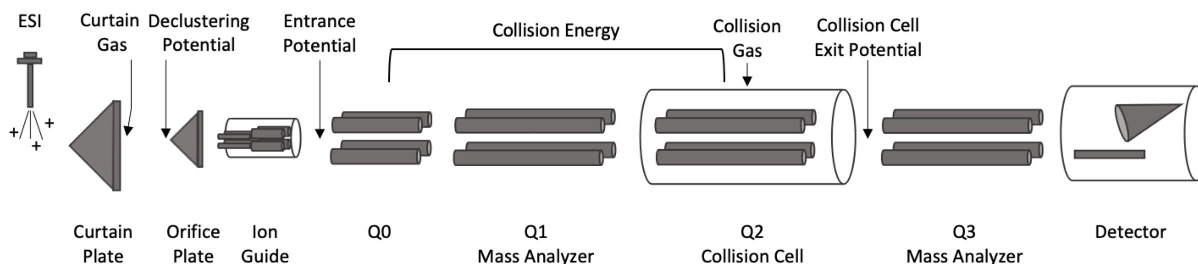

Figure 2.2. General schematic diagram displaying a triple quadrupole mass spectrometer, consisting of electrospray ionization (ESI) in positive ionization mode and channel electron multiplier (CEM) detector.

Collision energy is applied between Q0 and Q2 to create a potential difference, causing the precursor ion to convert that energy to internal vibrational energy. The excited, unstable precursor ion travels to the collision cell, colliding with a collision activated dissociation gas (typically nitrogen or argon gas) to undergo fragmentation, forming product ions.[150] After scanning for the selected product ions in the third quadrupole, a cell exit potential is applied to accelerate the ions to exit the third quadrupole to the detector. The product ions produce an electrical signal, amplified by the channel electron multiplier (CEM), a commonly used mass spectrometer detector.[95] The electrical signal produced by the ions is represented as peak intensity on a chromatogram.

A technique, termed multiple reaction monitoring, is commonly used in triple quadrupole to increase the selectivity of the LC-MS/MS method by scanning for multiple product ions from a single or multiple precursor ions.[150] Typically, the most abundant product ion, known as the quantifier ion, is used to measure the response and concentration of the analyte.[149] The second product ion chosen is the qualifier ion, which confirms the mass spectrometer is scanning for the intended analyte.

3 Rationale

The CARE-E study will require collecting small volumes of plasma from pediatric patients using CBD rich CBM formulation for refractory epileptic encephalopathy to determine minimum plasma cannabinoid concentrations at steady state ($C_{ss,min}$), the lowest plasma concentration at steady state prior to administration of the next dose. CBM administered to the pediatric participants contains low THC levels. It is important to quantify THC concentrations in the plasma samples to determine if participant reports adverse, psychoactive effects associated with THC consumption. Quantification of $C_{ss,min}$ requires development of a sensitive LC-MS/MS method.

Several children with refractory epileptic encephalopathy administered *Cannabis* based medicine often are on a ketogenic diet. Ketogenic diets may alter lipoprotein production, potentially altering the plasma protein binding of cannabinoids. Understanding the plasma protein binding profiles and the effects of a ketogenic diet necessitates the development of an LC-MS/MS method that can accurately capture the unbound and bound cannabinoid fractions.

Pharmacokinetic studies involve multiple blood sampling time points, typically over a time period of 24-48 hours, to capture the time course of total body exposure. Collection of large blood volumes and invasiveness of serial blood sampling in pediatric patients raises concerns on the practicality of using plasma for pharmacokinetic studies. Development of a DBS assay can provide a less invasive and lower sample volume collection for pediatric pharmacokinetic studies. As well, development of a DBS assay allows for assessment of maternal *Cannabis* exposure through the analysis of archived DBS samples obtained for mandatory neonatal screening testing.

4 Overall purpose of study

The overall purpose is to develop selective and sensitive LC-MS/MS analytical assays to quantify cannabinoids and metabolites in pediatric plasma, neonate dried blood spot, and plasma protein binding samples.

4.1 Hypotheses

1. Minimum steady state cannabinoid concentrations will increase proportionally with increases in dose of a 1:20 THC:CBD CBM administered daily to children diagnosed with refractory epileptic encephalopathy.

2. The 3-solvent extraction technique will provide more accurate quantification of the unbound and bound fractions of CBD, THC, 11-OH-THC, and CBC with no to minimal non-specific binding, compared to the traditional plasma protein binding assays.

3. Legalization of recreational *Cannabis* will result in an increased prevalence of cannabinoid detection in residual neonatal dried blood spot samples.

4.2 Objectives

This study aims to:

1. Develop and validate an LC-MS/MS method for the quantification of bioactive cannabinoids in plasma of pediatric patients on *Cannabis* 1:20 THC:CBD herbal extract therapy.

2. Quantify minimum cannabinoid concentration at steady state ($C_{ss,min}$) in plasma samples of pediatric patients in the CARE-E study.

3. Conduct a comparative analysis for the determination of plasma protein binding characteristics of CBD, THC, 11-OH-THC, and CBC using ultrafiltration, rapid equilibrium dialysis, and a newly adapted 3-solvent extraction technique.

4. Develop and validate a dried blood spot assay to determine the prevalence of prenatal *Cannabis* exposure in newborns pre- and post-legalization of recreational *Cannabis*.

5 Ethics

The CARE-E (15-192) and Prevalence of Prenatal *Cannabis* (17-319) Studies were approved by the University of Saskatchewan Biomedical Research Ethics Board (Appendix I). Consent for the enrollment of pediatric patients was obtained through their legal guardians for the CARE-E Study. Collection of provincial neonate DBS samples and venous blood collection from participants was approved for the prenatal *Cannabis* study.

6 Materials and Methods

<u>Chemicals and Reagents</u>

CBD (cat no. C-045), THC (cat no. T-005), 11-OH-THC (cat no. H-027), THC-COOH (cat no. T-010), and CBC (cat no. C-143) stock standards were purchased from Cerilliant (Round Rock, TX) at a concentration of 1 mg/mL, dissolved in methanol. CBD-d3 (cat no. C-084), THC-d3 (cat no. T-003), 11-OH-THC-d3 (cat no. H-04), and THC-COOH-d3 (cat no. T-004) internal standards were also purchased from Cerilliant (Round Rock, TX) at a concentration of 0.1 mg/mL dissolved in methanol. CBC-d9 (cat no. 21294) was purchased from Cayman Chemicals (Ann Arbor, MI). All stock and internal standards were stored at -20°C until use. Fisher Scientific Optima™ LC/MS grade acetonitrile (cat no. A9551), LC/MS grade water (cat no. W64), LCMS grade methanol (cat no. A45564), and LCMS grade formic acid (cat no. A11750) were purchased from ThermoFisher Scientific (Whitby, ON).

Human Plasma

Citrated human plasma used was obtained from the Canadian Blood Services (Human Biomedical Ethics approval was obtained for using this blood). Pharmacokinetic studies used lithium heparin plasma. Whole blood used for the dried blood spot method development and validation was obtained from volunteers, with ethics approval, and collected in lithium heparin blood collection tubes.

Materials

Eppendorf™ Protein LoBind microcentrifuge tubes (cat no. 022431081), disposable borosilicate glass tubes, Pasteur pipettes, 15 mL polypropylene centrifuge tubes, and SureOne micropipette tips were purchased from Fisher Scientific (Hampton, NH). Agilent 2 mL amber autosampler vials (Santa Clara, CA) were used to store stock and internal standards as well as for LC-MS/MS analysis. Agilent 200 µL HPLC flat bottom inserts (Santa Clara, CA) were used for LC-MS/MS analysis. Pasteur pipettes (cat no. 13-678-6G) were purchased from Fisher Scientific. Thermolyne™ Dri-bath™ was purchased from Thermo Fisher Scientific. BD Vacutainer® Barricor™ (Mississauga, ON) blood sample collection tubes were gifted through Becton Dickinson (Franklin Lakes, NJ). Centrifree Amicon Ultrafiltration Devices with Ultracel PL membranes (cat no. 4104) were purchased from Millipore (Billerica, MA). Rapid Equilibrium Dialysis plate (cat no. 90006) was purchased from Thermo Fisher Scientific. Beckman® Coulter Allegra® 25R Refrigerate Benchtop Centrifuge with rotor A-15 was used for the ultrafiltration study. Whatman 903 Protein Saver cards were purchased from VWR (Radnor, PA). Captiva EMR-Lipid 1 mL 96-well plates (cat no. 5190-1001), Captiva 1 mL 96-well deep collection plates (cat no. A69600100), and vacuum collar (cat no. A796) were purchased from Agilent.

Instrumentation

LC-MS/MS analysis was conducted using an Agilent 1290 Infinity liquid chromatography instrument with binary pumps (Santa Clara, CA) coupled to a SCIEX QTrap 6500 mass spectrometer (Concord, ON). The Agilent Zorbax Eclipse XDB-C18 Narrow Bore 2.1 x 75mm, 3.5-micron column and Zorbax Eclipse XDB-C8 Narrow Bore 2.1 x 12.5 mm, 5-micron guard column were purchased from Agilent (Santa Clara, CA).

Software

SCIEX Analyst® 1.6.2 and 1.7 was used to perform instrumental control, compound optimization, parameter optimization and data acquisition. SCIEX MultiQuant™ 3.0.1 was used to perform quantitation analysis.

7 Development and Validation of a LC-MS/MS Method for the Quantification of Bioactive Cannabinoids in Plasma of Pediatric Patients on *Cannabis* herbal extract Therapy

7.1 Method Development

The initial conditions of the method originated from peer-reviewed literature. The LC-MS/MS method was previously developed by two graduate students, Ahmed Almousa and Muath Helal, in the Alcorn lab group. Initially, the LC-MS/MS method consisted of a 5-minute isocratic sample run time, held at 10% mobile phase A and 90% mobile phase B with a flow rate of 250 μL/min. The LLOQ was set to 0.98 ng/mL.

As depicted in Figure 7.1a, the 11-OH-THC and CBD peaks overlapped. Although the two cannabinoids have different MRM transitions, both potentially interfered with each other, causing a potential matrix effect. It is important to ensure good separation between each analyte to avoid confounding interferences. Method optimization can be completed by modifications to mobile phase composition, flow rate, and sample extraction.

The initial method (isocratic method of 250 μL/min of 10% mobile phase A and 90% mobile phase B for 5 minutes) resulted in overlapping 11-OH-THC and CBD peaks. To resolve this issue, a gradient method was substituted for isocratic delivery of the mobile phase (Figure 7.1b). After optimization, the gradient conditions for LC-MS/MS analysis were as followed: 80% B from 0-3.5 minutes, increasing to 90% B until 10 min, and returning to 80% B over 10.5-13.5 min.

Initially, the flow rate of the LC-MS/MS assay was set to 250 μL/min. Use of higher flow rate, 700 μL/min, allowed a return to isocratic conditions, with mobile phase composition optimized to 77.5% B. These changes allowed the sample run time to shorten to 10 minutes and maintained adequate separation of 11-OH-THC and CBD (Figure 7.1c).

Modifications to the sample extraction method further increased sensitivity. Following protein precipitation, 700 μL of supernatant was dried using a dry bath with filtered air and reconstituted with 200 μL mobile phase (22.5% A: 77.5% B v/v). Peak

intensity of 11-OH-THC, CBD, THC, and CBC at 0.98 ng/mL, with the initial LC-MS/MS conditions, were 2.6 x 10^3, 6.9 x 10^3, 3.8 x 10^3, and 2.1 x 10^3, respectively. After the addition of drying and reconstituting to the sample extraction method, peak intensities increased to 1.1 x 10^4, 2.1 x 10^4, 9.2 x 10^3, and 6.2 x 10^3 for the respective cannabinoids. Increase of peak intensity allowed for a lower LLOQ of 0.49 ng/mL.

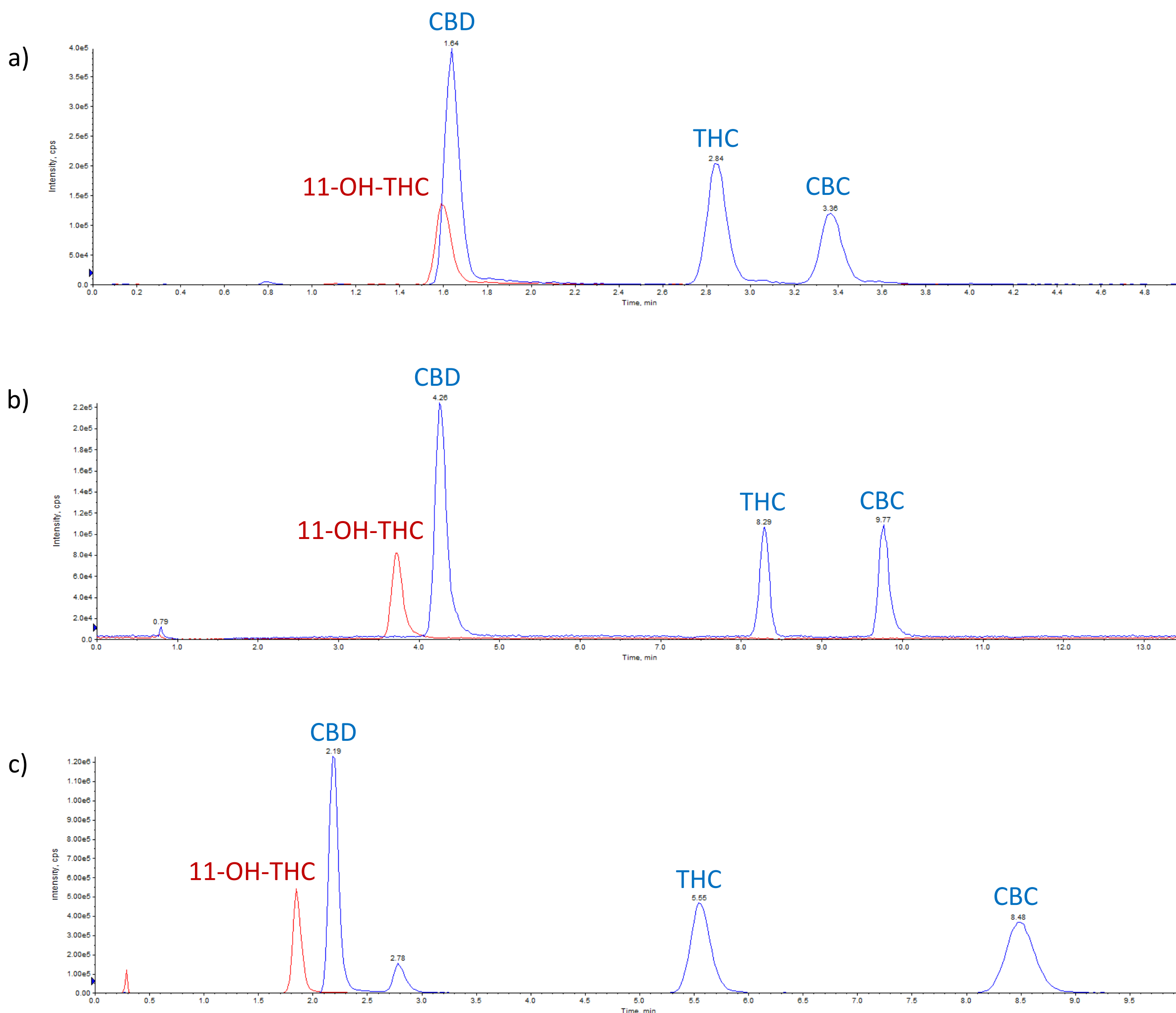

Figure 7.1. LC-MS/MS method optimization, showing efficient separation and increased sensitivity with modifications to mobile phase composition, flow rate, and sample extraction with a) initial LC-MS/MS conditions of an isocratic method running for 5 minutes with flow rate of 250 μL/min, b) gradient method running for 13.5 minutes with flow rate of 250 μL/min, and c) final LC-MS/MS isocratic method running for 10 minutes with flow rate of 700 μL/min.

Stock and working standard solution preparation

Stock solution of 5000 ng/mL was prepared by adding 20 µL of 1 mg/mL CBD, THC, 11-OH-THC, THC-COOH, CBC, and CBN each to 3880 µL methanol. Eight working standard concentrations were prepared, 9.77, 19.53, 39.06, 78.12, 156.25, 625, 1250, and 2500 ng/mL. The lowest limit of quantification (LLOQ) was determined from a signal-to-noise ratio ≥ 5. Four working standards, ranging from 156.25-2500 ng/mL, were prepared by parallel dilution from the original stock solution with methanol. The remaining 4 working standards, 9.8-78.12 ng/mL, were prepared by serial dilution from the 625 ng/mL working standard.

Quality control concentrations were determined according to the standard curve. The lowest quality control (LQC) must be within 3 times the LLOQ, the middle quality control (MQC) approximately 50% of the standard curve range, and the highest quality control (HQC) within 75% of the upper limit of quantification (ULOQ). The LQC for CBD, THC, 11-OH-THC, and CBC was 1.40 ng/mL. The MQC and HQC for CBD, THC, 11-OH-THC, and CBC were 50.56 ng/mL and 101.2 ng/mL, respectively. All standards and quality controls were prepared in amber autosampler vials, wrapped with parafilm, and stored at -20°C.

Internal standard preparation

100 µg/mL CBD-d3, THC-d3, 11-OH-THC-d3, THC-COOH-d3, and CBC-d9 stock solutions were diluted in methanol to prepare a 100 ng/mL working internal standard solution. Internal standards were prepared in amber autosampler vials, wrapped in parafilm, and stored at -20°C. On day of analysis, 10 µL of 100 ng/mL was added to 600 µL acetonitrile for protein precipitation, with a final internal standard concentration of 1.64 ng/mL.

Sample preparation procedure

For standards and quality controls, 10 µL of analyte was added to 190 µL blank human plasma. For the double blank and blank samples, 10 µL of methanol was added to 190 µL blank human plasma. 10 µL of methanol and 600 µL of cold acetonitrile was added to the double blank sample. 610 µL of cold (4°C) acetonitrile spiked with internal

standard (1.64 ng/mL) was added to the blank, standard and quality control samples in Eppendorf Lo-bind microcentrifuge tubes. All samples were mixed thoroughly by vortex mixing. Samples were centrifuged at 14000 rpm for 10 minutes at 4°C. 700 μL of supernatant was transferred to borosilicate culture tubes and dried with filtered air at 35°C for 20 minutes using dry bath, followed by reconstitution with 200 μL of mobile phase (77.5% mobile phase B: 22.5% mobile phase A; refer to liquid chromatography conditions below). Samples were then transferred to HPLC inserts in amber autosampler vials and readied for mass spectrometric analysis.

Liquid chromatography conditions

Method development and validation experiments utilized the Agilent 1290 Infinity LC System with a Zorbax Eclipse XDB-C18 Narrow-Bore 2.1 x 75 mm 3.5-micron column and Zorbax Eclipse XDB-C8 Narrow-Bore 2.1 x 12.5 mm 5-micron guard column, both controlled at 30°C, with an isocratic flow rate of 700 μL/min for 10.5 minutes. Mobile phase comprised of 77.5:22.5 (% v/v) of methanol and water containing 0.1 mM ammonium formate. The autosampler temperature was controlled at 4°C with a sample injection volume of 5 μL.

Mass spectrometry conditions

The mass spectrometric instrument consisted of a SCIEX QTrap® 6500 triple quadrupole mass spectrometer (Washington, DC), equipped with TurboIonSpray® electrospray (ESI) interface, with ionization spray voltage set to 5500 V in positive ionization mode. Multiple reaction monitoring (MRM) conditions and mass spectrometer conditions, outlined in Table 7.1 and 7.2, were optimized. Triple quadrupole mass spectrometry offered in unit resolution, with nitrogen gas used for all gases.

Table 7.1. Multiple Reaction Monitoring (MRM) conditions for the identification and quantification of CBD, THC, 11-OH-THC, and CBC in human plasma

	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Declustering Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)
CBD Quantifier	315.1	193.2	50	61	25	12
CBD Qualifier	315.1	259.2	50	61	19	14
CBD-D3	318.1	196.1	50	56	29	12
THC Quantifier	315.1	193	50	91	31	12
THC Qualifier	315.1	259.2	50	96	31	10
THC-D3	318.1	196.1	50	96	31	10
11-OH-THC Quantifier	331.1	193.1	50	66	33	12
11-OH-THC Qualifier	331.1	105.0	50	66	41	12
11-OH-THC-D3	334.1	196.1	50	66	41	12
CBC Quantifier	315.1	193.2	50	61	25	12
CBC Qualifier	315.1	259.2	50	61	19	14
CBC-D9	324.4	202.1	50	71	27	16

Table 7.2. Mass spectrometer parameters for plasma LC-MS/MS method

Curtain Gas (CUR)	Collision Activated Dissociation (CAD)	Ionization Spray Voltage (V)	Temperature (°C)	Nebulizer Gas	Heater Gas	Entrance Potential (V)
50	9	5500	700	50	50	10

7.2 Method Validation

Method validation involved the assessment of selectivity, linearity, intra- and inter-day accuracy and precision, carryover, stability of analytes, and matrix effect conducted over 3 days, following the 2018 FDA Bioanalytical Method Validation Guidance for Industry.[151]

7.2.1 System Suitability

System suitability was analyzed in five repeated injections of a 50 ng/mL standard in methanol to determine the performance and reproducibility of the LC-MS/MS assay and instrumentation. Mean peak area and standard deviation of each cannabinoid were calculated to determine the coefficient of variance (CV). No system suitability criterion was established in the FDA Bioanalytical Method Validation Guidance for Industry. However, MDS Pharma Services (MDSPS) has established a cut-off CV of 6%.[152]

7.2.2 Selectivity

Selectivity was analyzed in 6 individual lots of blank human plasma to determine the absence of co-eluting compounds with the analytes of interest. Plasma sources could be pooled if no interferences are detected.

7.2.3 Linearity

To determine linearity, standard curves (ranging from 0.49 - 125 ng/mL) were prepared each day of method validation. Standard curves were assessed with weighted linear regression analysis of 1/x. Following FDA bioanalytical method validation guidelines, the coefficient of determination (R^2), analysis of how close the observed standard curve points are to the fitted regression line, must be greater than or equal to 0.98. Accuracy of the LLOQ and remaining standard curve points require observed concentration within ±20% and 15% of the nominal concentrations, respectively.

7.2.4 Intra- and Inter-day Accuracy and Precision

Intra- and inter-day accuracy and precision were assessed by analysis of 6 replicates of the LLOQ, LQC, MQC, and HQC concentrations over the course of 3 separate days. FDA guidelines accept intra- and inter-day accuracy and precision of LLOQ and QC observed concentrations within ± 20% and 15% of the nominal concentrations, respectively. Accuracy was calculated using equation 7.1:

$$Accuracy\ (\%) = \frac{Observed\ concentration}{Nominal\ concentration} \times 100\% \qquad \text{Equation 7.1}$$

Intra- and interday precision of replicates were calculated using percent relative standard deviation (RSD), as stated in the equation 7.2:

$$Precision\ (RSD\%) = \frac{Standard\ deviation\ of\ replicates}{Mean\ concentration\ of\ replicates} \times 100\% \qquad \text{Equation 7.2}$$

7.2.5 Ion Ratios

Ion ratios, determined by the peak area intensity of the qualifier ion relative to the peak area intensity of the quantifier ion (Equation 7.3), confirm the presence of specific cannabinoids and assess for any interferences that may produce inaccurate quantification. All standard curve and quality control samples from method validation

were used to evaluate consistency of the ion ratios for each cannabinoid. No criteria on the acceptable ion ratio precision has been established in the FDA guidelines.

$$Ion\ ratio = \frac{Peak\ area\ of\ qualifier\ ion}{Peak\ area\ of\ quantifier\ ion} \qquad \text{Equation 7.3}$$

7.2.6 Carryover

To determine potential carryover of analyte from the previous injection, an ULOQ of 125 ng/mL sample was injected followed by an injection of a double blank (plasma sample with no analyte or internal standard) Carryover analyte should be less than 20% of the peak area of LLOQ and less than 5% of the peak area of internal standard.

7.2.7 Stability

Short-term stability (unprocessed and processed)

Occasionally, clinical samples may be left at room temperature in the laboratory setting for several hours, leading to potential instability or degradation of the analyte. Stability tests determine whether or not the analyte was stable in these conditions and if unstable, conditions need optimization to ensure the analyte was not compromised. Short-term stability was assessed by storing processed (extracted) and unprocessed samples on bench-top at room temperature for 6 hours. 6 replicates of LQC (1.40 ng/mL), MQC (50.6 ng/mL), and HQC (101.2 ng/mL) were analyzed for both unprocessed and processed conditions.

Long-term stability

Long-term stability covers the time period where reanalysis of study samples may be needed. To assess long-term stability of the analytes, 6 replicates of LQC, MQC, and HQC were prepared and stored at -80°C for analysis at 1, 2, 3, 6, and 12 months from the start of method validation. Samples were stored in the dark to prevent photodegradation of cannabinoids.

Freeze thaw stability

Freeze thaw stability was conducted using 3 sets of 6 replicates of the LQC (1.40 ng/mL), MQC (50.6 ng/mL), and HQC (101.2 ng/mL) to determine if analytes of interest were stable under a cycle of varying temperature conditions. All sets were stored at -80°C. After 24 hours, all samples were thawed at room temperature, with only one set analyzed and the remaining sets placed in -80°C for another 24 hours then thawed at room temperature. A maximum of three freeze thaw cycles were performed. First day of accuracy and precision samples were used as time 0 for freeze thaw stability.

Autosampler stability

Autosampler stability determines the stability of analytes of interest under the HPLC autosampler conditions, such as resident time and temperature. To assess autosampler stability of CBD, THC, 11-OH-THC and CBC, the standard curve and replicates of the LLOQ and QCs from the previous day of method validation were stored in the autosampler at 4°C and reinjected 24 hours after first injection.

7.2.8 Matrix effect studies

Matrix effect studies investigates the extraction efficiency, matrix factor, and recovery of the analyte based on sample preparation and the matrix. These models investigate how well the analyte can be recovered through sample extraction (extraction efficiency and recovery) and the possible interference of endogenous compounds in the matrix (matrix factor).

Six replicates of pre-spike, post-spike, and pure spike at the LQC (1.40 ng/mL) and HQC (101.2 ng/mL) concentrations were used to determine extraction efficacy, matrix factor, and recovery of analytes of interest. For pre-spike, 10 µL of working standard was added to 190 µL human plasma. Samples were subsequently processed following the sample extraction procedure. For post-spike, 10 µL of methanol was added to 190 µL human plasma. 610 µL of cold acetonitrile and internal standard was added to the samples. Following protein precipitation, samples were centrifuged at 14000 rpm for 10 min at 4°C. 8.7 µL of working standard was added to 691.3 µL of

supernatant. Samples were then dried using filtered air and reconstituted with mobile phase. For pure-spike, 10 μL of working standard and 8.7 μL of internal standard was added to 182.7 μL of mobile phase.

Extraction efficiency, matrix factor, and recovery were calculated from the peak area ratio of the analytes, using Equations 7.4-7.6. Extraction efficiency is calculated using pre-spike samples (fortified with analyte prior to extraction) and pure-spike samples (fortified with analyte in mobile phase), indicating any loss/interference of cannabinoids during extraction in the presence of endogenous compounds. Matrix factor is calculated using post-spike samples (fortified with analyte after extraction) and pure spike samples, determining potential interferences between endogenous compounds and cannabinoids in the absence of extraction limitations. Recovery is calculated by comparing pre-spike samples and pure-spike samples, where post-spike samples represent 100% recovery of cannabinoids. Comparison of peak area ratios between the analyte and internal standard were assessed to determine if the internal standard compensated for any cannabinoid loss through sample preparation.

$$Extraction\ efficiency\ (\%) = \frac{Peak\ area\ of\ pre-spike}{Peak\ area\ of\ pure\ spike} \times 100\% \qquad \text{Equation 7.4}$$

$$Matrix\ factor\ (\%) = \frac{Peak\ area\ of\ post-spike}{Peak\ area\ of\ pure\ spike} \times 100\% \qquad \text{Equation 7.5}$$

$$Recovery\ (\%) = \frac{Peak\ area\ of\ pre-spike}{Peak\ area\ of\ post-spike} \times 100\% \qquad \text{Equation 7.6}$$

7.3 Results

We have developed a sensitive, specific, robust, precise, and accurate LC-MS/MS method for the quantification of bioactive cannabinoids in human plasma. We obtained a standard curve ranging from 0.49 ng/mL - 125 ng/mL for 11-OH-THC, CBD and THC, and 0.98 ng/mL - 125 ng/mL for CBC, with acceptable linearity. The lowest limit of quantification (LLOQ) for CBD, THC, and 11-OH-THC was 0.49 ng/mL and 0.98 ng/mL

for CBC. Retention times for 11-OH-THC, CBD, THC, and CBC were 1.83 min, 2.19 min, 5.55 min and 8.50 min, respectively (Figure 7.2).

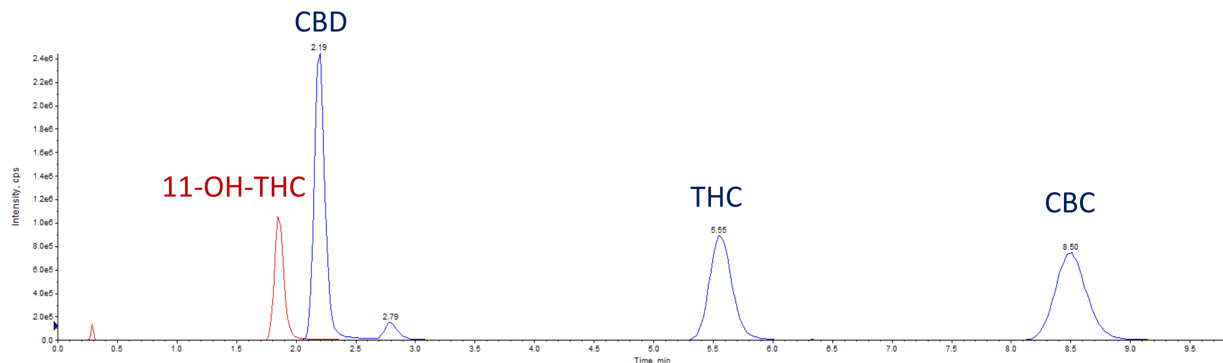

Figure 7.2. Chromatogram depicting the retention times of the quantifier ions for 11-OH-THC (1.83 min), CBD (2.19 min), THC (5.55 min) and CBC (8.50 min).

7.3.1 System Suitability

Analysis of the repeated five injections of a 50 ng/mL 11-OH-THC, CBD, THC, and CBC methanol solution showed consistency in the peak areas obtained. Day to day variation could be seen in the mean peak areas (Table 7.3), which could be influenced by many factors, such as temperature, humidity, multiuser instrumentation, etc. However, all cannabinoid intraday CV values ranged from 0.76 to 3.77% throughout the three days of method validation, all within the MDSPS CV criteria of less than 6%.

Table 7.3. System suitability data of 11-OH-THC, CBD, THC, and CBC, showing the mean peak area of five repeated injections of a 50 ng/mL concentration and acceptable CV (%) of less than 6%.

	Day 1		Day 2		Day 3	
	Peak Area (mean ± SD)	CV (%)	Peak Area (mean ± SD)	CV (%)	Peak Area (mean ± SD)	CV (%)
11-OH-THC	2330039.95 ± 83463.98	3.58	3555647.04 ± 48003.97	1.35	3365363.41 ± 32497.63	0.97
CBD	5811036.66 ± 124100.97	2.14	8762831.02 ± 74006.13	0.84	7994063.74 ± 84676.89	1.06
THC	6558648.54 ±	3.77	9867563.42 ±	1.52	9597970.53 ±	1.83

	247358.61		150350.10		176077.13	
CBC	4810827.32 ± 133232.21	2.77	6676194.91 ± 57839.33	0.87	6541854.78 ± 49998.69	0.76

7.3.2 Selectivity

Analysis of the 6 individual blank human plasma lots showed no co-eluting peaks at the same retention times of 11-OH-THC, CBD, THC, and CBC (Figure 7.3). A peak with the same MRM transitions as the quantifier ions of CBD, THC, and CBC was detected; however, did not interfere with the quantification of the cannabinoids as it eluted at 3.05 minutes. Additionally, the peak was not present in the qualifier ion transition for CBD, THC, and CBC.

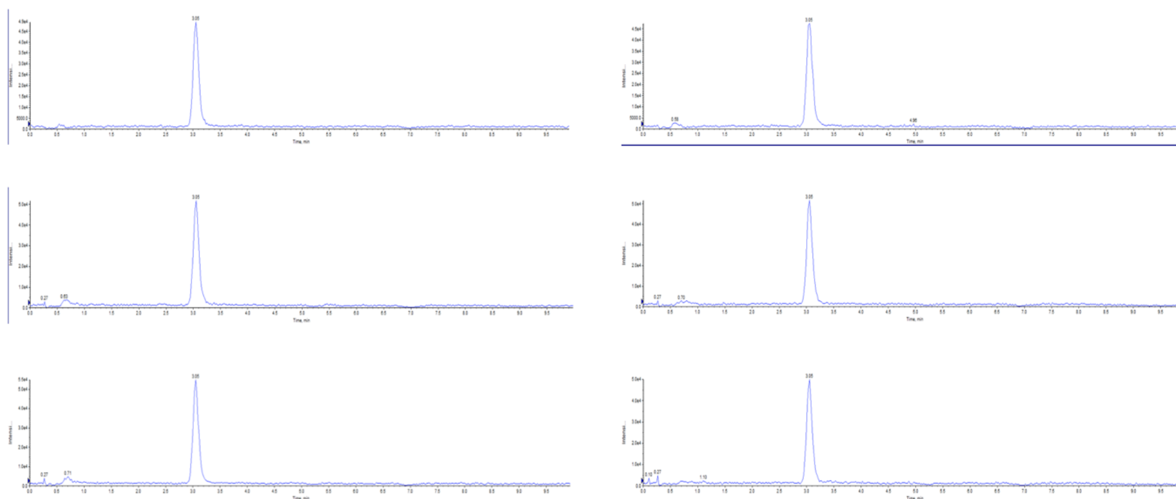

Figure 7.3. Chromatograms displaying the 6 blank individual sources of human plasma used in the method validation to determine the selectivity of the LC-MS/MS method. An unknown peak detected at 3.05 minutes has the same MRM transitions as the CBD, THC, and CBC quantifier ions but not detected in the qualifier ion. The unknown peak does not elute at the same retention times of the cannabinoids, therefore not interfering with the quantitative analysis.

7.3.3 Linearity

Standard curves prepared in pooled human plasma, with concentrations of CBD, THC, and 11-OH-THC ranged from 0.49-125 ng/mL and 0.98-125 ng/mL for CBC,

displayed linearity ($R^2 \geq 0.98$) during the 3 days of method validation (Figure 7.4). The lowest R^2 value achieved during method validation was 0.9969 in 11-OH-THC. The slopes of CBD, THC, 11-OH-THC, and CBC demonstrated consistency throughout method validation, with averages of 0.1416 ± 0.0047, 0.1602 ± 0.0057, 0.2092 ± 0.0093, and 0.1665 ± 0.0064, respectively. All standard curve points were within the acceptable criteria of accuracy.

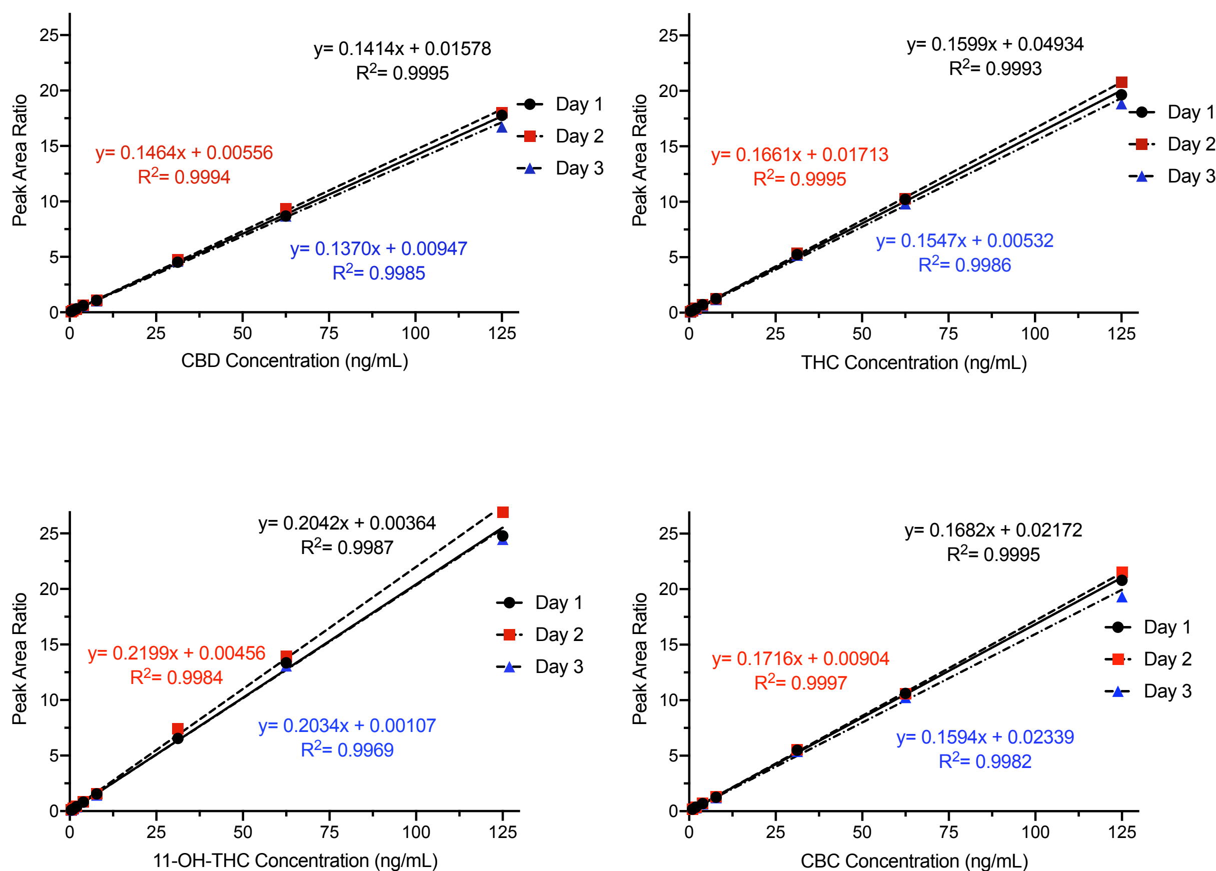

Figure 7.4. Standard curves of CBD, THC, 11-OH-THC, and CBC, during the 3 days of method validation, showing linearity with R^2 values ≥0.98. CBD, THC, and 11-OH-THC have 8 standard points, ranging from 0.49-125 ng/mL. CBC has 7 standard points, ranging from 0.98-125 ng/mL. Standard curves are weighted 1/x to improve the regression fit of the low concentration standards.

7.3.4 Inter- and intraday accuracy and precision

All LLOQ and QC replicates over the course of 3 days remained within acceptable criteria. Intraday accuracy and precision data of CBD and THC were displayed in Table 7.4 while 11-OH-THC and CBC were displayed in Table 7.5. Interday

accuracy and precision of all cannabinoids over the 3 days were displayed in Table 7.6. The lowest accuracy and most variation in precision were shown in the LLOQ of all four cannabinoids, particularly in CBD.

Table 7.4. Intraday accuracy and precision of LLOQ, LQC, MQC, and HQC for CBD and THC for each of the 3 days of method validation. Accuracy and precision of observed LLOQ and QC concentrations must be within 20% and 15% of the theoretical concentrations, respectively (n=6).

		CBD			THC		
(ng/mL)	Day of Analysis	Observed Concentration (mean ± SD; ng/mL)	Precision (RSD%)	Accuracy (%)	Observed Concentration (mean ± SD; ng/mL)	Precision (RSD%)	Accuracy (%)
0.49 (LLOQ)	1	0.43 ± 0.03	6.98	85.62	0.47 ± 0.04	8.51	93.47
	2	0.44 ± 0.04	9.09	89.97	0.48 ± 0.07	14.58	97.76
	3	0.42 ± 0.03	7.14	86.54	0.44 ± 0.02	4.55	90.53
1.40 (LQC)	1	1.47 ± 0.06	4.08	101.15	1.48 ± 0.03	2.03	106.02
	2	1.39 ± 0.07	5.04	99.48	1.46 ± 0.06	4.11	104.59
	3	1.41 ± 0.03	2.13	101.04	1.41 ± 0.07	4.96	100.90
50.56 (MQC)	1	51.57 ± 0.99	1.92	101.99	50.26 ± 0.59	1.17	99.40
	2	50.59 ± 0.55	1.09	98.99	50.60 ± 0.60	1.19	100.09
	3	53.10 ± 0.52	0.98	105.03	53.53 ± 0.91	1.70	105.88
101.20 (HQC)	1	114.26 ± 1.75	1.53	112.90	114.74 ± 2.21	1.93	113.38
	2	99.37 ± 1.14	1.15	98.19	99.21 ± 3.20	3.23	98.03
	3	104.05 ± 2.46	2.36	102.81	107.72 ± 4.24	3.94	106.44

Table 7.5. Intraday accuracy and precision of LLOQ, LQC, MQC, and HQC for 11-OH-THC and CBC for each of the 3 days of method validation. Accuracy and precision of observed LLOQ and QC concentrations must be within 20% and 15% of the theoretical concentrations, respectively (n=6).

		11-OH-THC			CBC		
(ng/mL)	Day of Analysis	Observed Concentration (mean ± SD; ng/mL)	Precision (RSD%)	Accuracy (%)	Observed Concentration (mean ± SD; ng/mL)	Precision (RSD%)	Accuracy (%)

0.49 (LLOQ)	1	0.47 ± 0.02	2.26	101.55	-	-	-
	2	0.46 ± 0.05	10.87	93.85	-	-	-
	3	0.47 ± 0.04	8.51	96.25	-	-	-
0.98 (LLOQ)	1	-	-	-	0.94 ± 0.04	4.26	98.83
	2	-	-	-	0.94 ± 0.04	4.26	95.93
	3	-	-	-	0.87 ± 0.06	6.90	83.63
1.40 (LQC)	1	1.53 ± 0.05	3.27	109.39	1.35 ± 0.05	3.70	96.25
	2	1.45 ± 0.05	3.45	103.53	1.39 ± 0.05	3.60	99.33
	3	1.38 ± 0.06	4.35	98.63	1.24 ± 0.05	4.03	88.79
50.56 (MQC)	1	50.82 ± 1.99	3.92	100.52	50.82 ± 0.89	1.75	100.51
	2	51.70 ± 0.37	0.72	102.25	51.45 ± 0.51	0.99	101.77
	3	53.55 ± 0.71	1.33	105.91	54.15 ± 0.53	0.98	107.09
101.20 (HQC)	1	113.58 ± 4.37	3.85	112.23	116.29 ± 2.55	2.19	114.91
	2	100.56 ± 3.53	3.51	99.37	99.85 ± 2.05	2.05	98.66
	3	102.93 ± 2.33	2.26	101.71	109.17 ± 4.25	3.89	107.88

Table 7.6. Interday accuracy and precision data of LLOQ, LQC, MQC, and HQC for CBD, THC, 11-OH-THC, and CBC over the 3 days of method validation. Accuracy and precision of observed LLOQ and QC concentrations must be within 20% and 15% of the theoretical concentrations, respectively (n=18).

Cannabinoid	Theoretical Concentration (ng/mL)	Observed Concentration (mean ± SD; ng/mL)	Precision (RSD %)	Accuracy (%)
CBD	LLOQ (0.49)	0.42 ± 0.04	9.52	86.67
	LQC (1.40)	1.43 ± 0.06	4.20	101.89
	MQC (50.56)	51.75 ± 1.26	2.43	102.36

	HQC (101.20)	105.89 ± 6.63	6.26	104.63
THC	LLOQ (0.49)	0.45 ± 0.05	11.11	91.71
	LQC (1.40)	1.45 ± 0.06	4.14	103.84
	MQC (50.56)	51.46 ± 1.65	3.21	101.79
	HQC (101.20)	107.22 ± 7.24	6.75	105.95
11-OH-THC	LLOQ (0.49)	0.48 ± 0.06	12.50	97.22
	LQC (1.40)	1.45 ± 0.08	5.52	103.85
	MQC (50.56)	52.02 ± 1.65	3.17	102.89
	HQC (101.20)	105.69 ± 6.70	6.34	104.43
CBC	LLOQ (0.98)	0.90 ± 0.08	8.89	91.13
	LQC (1.40)	1.34 ± 0.10	7.46	94.79
	MQC (50.56)	51.95 ± 1.76	3.39	103.12
	HQC	108.04 ± 7.99	7.40	107.15

	(101.20)

7.3.5 Ion Ratios

Assessment of the ion ratios for the standard curve and quality control samples of CBD, THC, 11-OH-THC, and CBC during method validation determined precision of ion ratios (Table 7.7). Consistency was identified within and between days of analysis, with average ion ratios of 0.33, 0.60, 0.94, and 0.50 for CBD, THC, 11-OH-THC, and CBC, respectively.

Table 7.7. Ion ratio precision of CBD, THC, 11-OH-THC, and CBC, comparing the peak areas of the qualifier ions and quantifier ions of intraday (n=12) and interday (n=36) samples during method validation

	Day 1		Day 2		Day 3		Interday	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)
CBD	0.35 ± 0.02	6.67	0.32 ± 0.03	9.04	0.33 ± 0.03	9.40	0.33 ± 0.03	8.87
THC	0.60 ± 0.04	6.43	0.61 ± 0.06	9.92	0.59 ± 0.06	10.70	0.60 ± 0.06	9.31
11-OH-THC	0.92 ± 0.06	6.31	0.94 ± 0.10	10.94	0.96 ± 0.07	7.29	0.94 ± 0.08	8.47
CBC	0.50 ± 0.03	6.44	0.52 ± 0.04	6.93	0.49 ± 0.05	10.18	0.50 ± 0.04	8.17

7.3.6 Stability

Short-term stability

The unprocessed samples at LQC, MQC, and HQC concentrations of CBD, THC, and 11-OH-THC remained stable after 6 hours at room temperature (Table 7.8). One LQC replicate of CBC displayed 20% loss after 6 hours. This decrease in concentration could have been a pipetting error as the remaining 5 replicates remained within 15% of the nominal concentration (1.40 ng/mL). The processed samples at LQC, MQC, and HQC concentrations for all four cannabinoids remained stable after 6 hours.

Table 7.8. Comparison of CBD, THC, 11-OH-THC, and CBC in LQC, MQC, and HQC samples unprocessed and processed for 6 hours at room temperature to samples extracted at 0 hours. Unprocessed and processed samples remained stable, staying within 15% the nominal concentration for all cannabinoids.

		Concentration at 0 hr (ng/mL; mean ± SD)	Unprocessed concentration at 6 hr (ng/mL; mean ± SD)	Processed concentration at 6 hr (ng/mL; mean ± SD)
CBD	LQC (1.40 ng/mL)	1.39 ± 0.07 (99.29%)	1.45 ± 0.03 (103.57%)	1.36 ± 0.04 (97.14%)
	MQC (50.56 ng/mL)	50.59 ± 0.55 (100.60%)	49.70 ± 0.75 (98.30%)	52.29 ± 0.83 (103.42%)
	HQC (101.20 ng/mL)	99.37 ± 1.14 (98.19%)	111.73 ± 1.32 (110.41%)	101.23 ± 0.82 (100.03)
THC	LQC (1.40 ng/mL)	1.46 ± 0.06 (104.29%)	1.41 ± 0.09 (100.71%)	1.44 ± 0.05 (102.86%)
	MQC (50.56 ng/mL)	50.60 ± 0.60 (100.08%)	48.57 ± 0.52 (96.06%)	51.26 ± 1.00 (101.38%)
	HQC (101.20 ng/mL)	99.21 ± 3.20 (98.03%)	109.13 ± 1.42 (107.84%)	98.64 ± 1.67 (97.47%)
11-OH-THC	LQC (1.40 ng/mL)	1.45 ± 0.05 (103.57%)	1.46 ± 0.07 (104.29%)	1.40 ± 0.07 (100.00%)
	MQC (50.56 ng/mL)	51.70 ± 0.37 (102.25%)	49.62 ± 0.96 (98.14%)	52.42 ± 1.30 (103.68%)
	HQC	100.56 ± 3.53	112.58 ± 1.20	101.61 ± 2.33

	(101.20 ng/mL)	(99.37%)	(111.25%)	(100.41%)
CBC	LQC (1.40 ng/mL)	1.39 ± 0.05 (99.29%)	1.23 ± 0.06 (87.86%)	1.29 ± 0.08 (92.14%)
	MQC (50.56 ng/mL)	51.45 ± 0.51 (101.76%)	48.85 ± 0.55 (96.62%)	51.78 ± 0.89 (102.41%)
	HQC (101.20 ng/mL)	99.85 ± 2.05 (98.67%)	110.13 ± 1.25 (108.82%)	100.70 ± 1.05 (99.51%)

Long-term stability

Although long-term stability required the assessment of stability up to 12 months, only 1, 2, and 3 months had been analyzed due to missed analysis for 6 and 12 months. LQC, MQC, and HQC samples remained stable up to at least 3 months, within ±15% of the nominal concentration of CBD, THC, 11-OH-THC, and CBC. Analysis at 6 and 12 months is currently ongoing.

Freeze thaw stability

The first and third set of freeze thaw stability samples at LQC, MQC, and HQC levels remained stable after their respective freeze thaw cycles, within ±15% of the nominal concentration of CBD, THC, 11-OH-THC, and CBC. All replicates of HQC for THC and CBC from the second set of freeze thaw samples displayed concentrations 15% greater than the nominal HQC concentration (101.2 ng/mL). All CBD and 11-OH-THC concentrations at the HQC level in the second set of freeze thaw stability remained within the acceptable criteria.

Autosampler stability

After 24 hours in the autosampler at 4°C, all six replicates at the LLOQ, LQC, MQC, and HQC levels following reinjection remained stable and within ±15% of the nominal concentrations with the exception of one HQC replicate of CBC. This indicates

that samples could be stored in the autosampler for 24 hours during high throughput analysis.

7.3.7 Carry Over

Carryover was not observed for both the quantifier ions of CBD, THC, 11-OH-THC, and CBC as well as the respective deuterated internal standards, finding no detectable peaks in the double blank, following an injection of the ULOQ standard (Figure 7.5). This indicated that samples with higher concentrations of the cannabinoids would not impact the accurate quantification of subsequently injected samples. LC-MS/MS method does not require injection of a double blank between each sample.

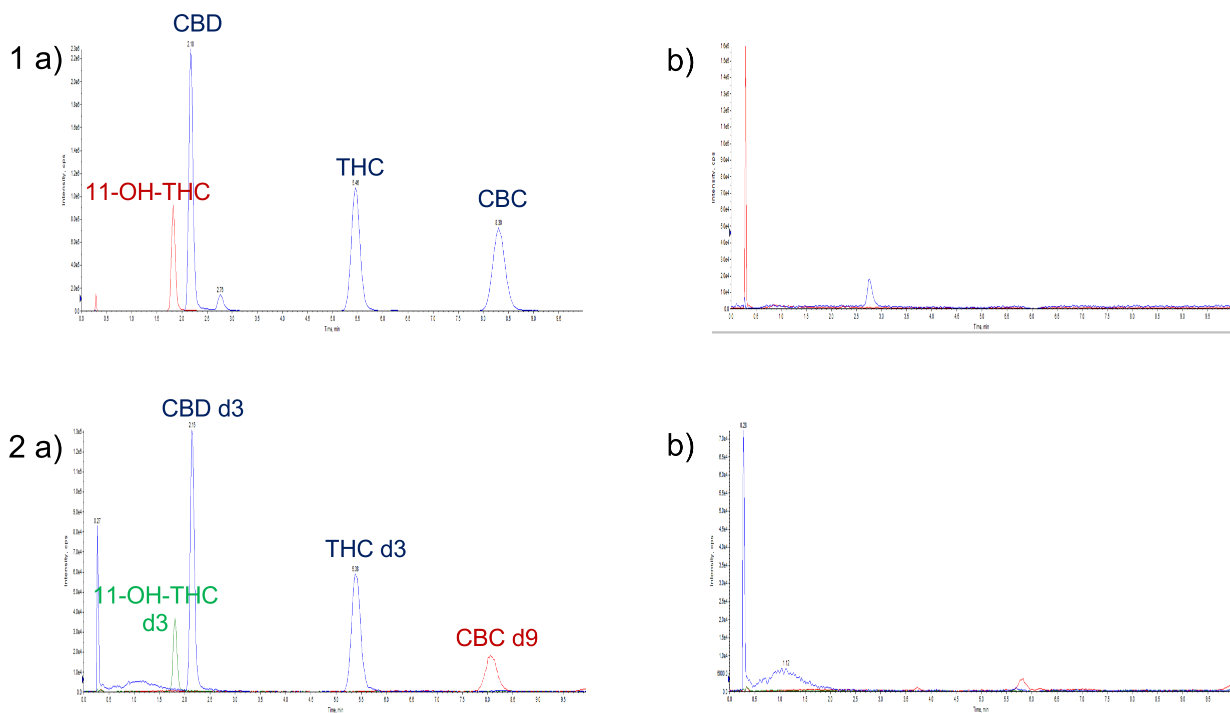

Figure 7.5. Chromatograms comparing the response of CBD, THC, 11-OH-THC, and CBC quantifier ions in ULOQ standard (1a) and double blank (1b). Absence in the response of quantifier ions indicate no carryover of cannabinoids from the ULOQ. Similarly, the response from ULOQ (2a) and double blank (2b) of the internal standards indicated no carryover of the internal standard.

7.3.8 Matrix Effects

CBD and THC showed extensive matrix factors of, 50.37% and 42.35%, respectively, in human plasma (Table 7.9). CBD and THC exhibited low extraction efficiency, with 51.54% in CBD and 37.38% in THC. CBD and THC expressed high recovery, ranging from 88.25-102.33% recovery of CBD and THC in the presence of matrix effect. The extensive matrix factor indicated ion suppression, likely due to incomplete removal of plasma proteins and phospholipids during sample extraction. Protein precipitation may not fully precipitate plasma proteins and does not remove phospholipids from the matrix. Drying and reconstituting the samples to concentrate the analyte would also increase the concentration of other components in the sample.

The addition of a deuterated internal standard corrected for the matrix effect caused by the endogenous plasma components (Table 7.10). The peak area ratio between the analyte and internal standard was displayed in Table 7.10, indicating improvements of extraction efficacy and matrix factor with the inclusion of the internal standard. The matrix effect did not affect the sensitivity of the method.

Table 7.9. Peak area of pre-spike, post-spike, and pure spike LQC samples used to calculate the extraction efficacy, matrix factor, and recovery of CBD and THC undergoing sample extraction in human plasma.

	Pre-spike Peak Area (mean ± SD)	Post-spike Peak Area (mean ± SD)	Pure Spike Peak Area (mean ± SD)	Extraction Efficacy (%)	Matrix Factor (%)	Recovery (%)
LQC (CBD)	148974.27 ± 13794.92	145582.57 ± 10948.96	289054.73 ± 17092.73	51.54	50.37	102.33
LQC (THC)	143481.13 ± 10646.39	162576.00 ± 12911.48	383867.69 ± 112493.90	37.38	42.35	88.25

Table 7.10. Peak area ratio of pre-spike, post-spike and pure spike LQC samples to demonstrate the internal standard correcting for the variation in CBD and THC during sample extraction in human plasma.

	Pre-spike Peak Area Ratio (mean ± SD)	Post-spike Peak Area Ratio (mean ± SD)	Pure Spike Peak Area Ratio (mean ± SD)	Extraction Efficiency (%)	Matrix Factor (%)	Recovery (%)
LQC (CBD)	0.17 ± 0.01	0.18 ± 0.02	0.20 ± 0.003	88.31	93.43	94.52
LQC (THC)	0.24 ± 0.01	0.27 ± 0.03	0.27 ± 0.01	87.92	99.63	88.24

8 Analysis of CARE-E pediatric patient samples using the plasma LC-MS/MS method

The CARE-E study included 7 participants, ages 1-10 years, and were from Saskatoon and under the supervision of Dr. Richard Huntsman.[2] All participants were diagnosed with refractory epileptic encephalopathy. For the CARE-E dose escalation study, participants were administered a 1:20 THC:CBD CBM purchased from Cannimed. This CBM also contained 4% CBC.

8.1 Dose Escalation Study

Patients who were enrolled into a dose escalation study were administered an increasing dose of the CBD rich CBM at their monthly visit. In addition to measuring plasma cannabinoid concentrations, the research group also monitored seizure frequency, safety and tolerability, and interactions with the patients' current anticonvulsant therapy.

The dose escalation study included 7 monthly visits with the pediatrician. At first visit, baseline seizure frequency and blood collection were received. Starting at visit 2, patients were introduced to the CBM, with increasing doses of 2-3 mg/kg/day, 5-6 mg/kg/day, 8-9 mg/kg/day, and 10-12 mg/kg/day of CBD at visits 3, 4, and 5, respectively. Before the administration of the next dose, blood was collected to quantify the minimum plasma cannabinoid concentration at steady state ($C_{SS,min}$). At visit 6, participants were weaned off the CBM and monitored for changes in seizures at visit 7.

Plasma samples were analyzed using the validated human plasma LC-MS/MS method. Samples were quantified within 3 months of collection, to prevent loss from stability issues, with the exception of the visit 5 sample of participant A-05, which was analyzed 8 months after blood collection.

Standard curve ion ratios were averaged and used as the reference ion ratio to calculate the relative tolerance of experimental samples. Relative tolerance for the degree of ion ratios (Table 8.1) were set by the European Commission.[153] Ion ratios within the relative tolerance range confirms the presence of cannabinoids. Samples with

accuracy values outside the relative tolerance range indicate interference of the cannabinoids, which could lead to inaccurate interpretations of cannabinoid plasma concentrations. The ion ratio accuracy of the experimental samples was calculated following Equation 8.1:

$$Ion\ ratio\ bias\ (\%)\ =\frac{Ion\ ratio_{sample}-Ion\ ratio_{standard\ curve}}{Ion\ ratio_{standard\ curve}} \qquad \text{Equation 8.1}$$

Table 8.1. Acceptable tolerance criteria for cannabinoids based on the ion ratio relative intensity

Ion ratio	Relative tolerance
> 0.50	± 20%
0.20 - 0.50	± 25%
0.10 - 0.20	± 30%
< 0.10	± 50%

8.2 Results

All plasma samples displayed ion ratios within the relative tolerance criteria and indicated no endogenous or exogenous interference with CBD, THC, and CBC (Table 8.2). 11-OH-THC plasma concentrations were not quantifiable and therefore not presented in the data below.

Table 8.2. Ion ratio of CARE-E site A participant samples from visits 2-6, with % bias calculated from the reference standard curve ion ratios of CBD (0.38), THC (0.60) and CBC (0.57).

Participant ID	Dose (mg CBD/kg/day)	CBD ion ratio (% bias)	THC ion ratio (% bias)	CBC ion ratio (% bias)
A-01	0	N/A	N/A	N/A
	2-3	0.38 (0.98%)	0.59 (1.05%)	0.63 (10.85%)
	5-6	0.38 (0.98%)	0.64 (7.34%)	0.60 (5.57%)
	8-9	0.38 (0.98%)	0.62 (3.98%)	0.65 (14.37%)

	10-12	0.38 (0.98%)	0.60 (0.63%)	0.46 (19.06%)
A-02	0	N/A	N/A	N/A
	2-3	0.40 (4.23%)	0.67 (12.37%)	0.59 (3.81%)
	5-6	0.36 (6.19%)	0.59 (1.05%)	0.59 (3.81%)
	8-9	0.39 (1.63%)	0.64 (7.34%)	0.60 (5.57%)
	10-12	0.37 (3.58%)	0.63 (5.66%)	0.60 (5.57%)
A-03	0	N/A	N/A	N/A
	2-3	0.44 (14.66%)	N/A	0.49 (13.78%)
	5-6	0.37 (3.58%)	0.59 (1.05%)	0.48 (15.54%)
	8-9	0.37 (3.58%)	0.61 (2.31%)	0.46 (19.06%)
	10-12	0.37 (3.58%)	0.60 (0.63%)	0.66 (16.13%)
A-04	0	N/A	N/A	N/A
	2-3	0.40 (4.23%)	N/A	0.62 (9.09%)
	5-6	0.38 (0.98%)	0.61 (2.31%)	0.47 (17.30%)
	8-9	0.37 (3.58%)	0.58 (2.73%)	0.59 (3.81%)
	10-12	0.37 (3.58%)	0.60 (0.63%)	0.54 (4.99%)
A-05	0	N/A	N/A	N/A
	2-3	0.39 (1.63%)	N/A	0.47 (17.30%)
	5-6	0.37	0.63	0.49

		(3.58%)	(5.66%)	(13.78%)
	8-9	0.38 (0.98%)	0.65 (9.01%)	0.68 (19.65%)
	10-12	0.38 (0.98%)	0.61 (2.31%)	0.67 (17.89%)
A-06	0	N/A	N/A	N/A
	2-3	0.38 (0.98%)	0.60 (0.63%)	0.46 (19.06%)
	5-6	0.37 (3.58%)	0.63 (5.66%)	0.62 (9.09%)
	8-9	0.37 (3.58%)	0.61 (2.31%)	0.68 (19.65%)
	10-12	0.38 (0.98%)	0.60 (0.63%)	0.54 (4.99%)
A-07	0	N/A	N/A	N/A
	2-3	0.29 (14.07%)	N/A	0.52 (3.31%)
	5-6	0.32 (5.19%)	N/A	0.51 (1.32%)
	8-9	0.35 (3.70%)	N/A	0.53 (5.30%)
	10-12	0.33 (2.22%)	0.68 (18.11%)	0.52 (3.31%)

CBD displayed the highest concentration of the three cannabinoids (Table 8.3). Participant A-04 had the highest CBD concentration, with a $C_{ss,min}$ of 124.67 ng/mL at dose 10-12 mg CBD/kg/day. At visit 5 (10-12 mg CBD/kg/day), CBD $C_{ss,min}$ concentrations varied between 24.20-124.67 ng/mL. Though commonly used in high concentrations to treat pediatric epilepsy, each individual responded to CBD differently. Participants A-03, A-06, and A-07 all became seizure free early in the dose escalation study, yet their CBD plasma concentrations were in mid-range compared to the other

participants.[22] Participant A-04 had the highest CBD plasma concentration but had a 63% seizure reduction frequency.

Participant A-05 showed a significant decrease in plasma cannabinoid concentrations at 10-12 mg CBD/kg/day. This particular sample was analyzed 8 months after blood sample collection, which was out of the long-term stability range of 3 months. Plasma samples were stored in polystyrene tubes after blood collection. Cannabinoids show extensive non-specific binding in polymer plastics. The combination of cannabinoid long term instability and non-specific binding may have contributed to loss of cannabinoids.

Most participants displayed linear or dose-independent pharmacokinetics, with changes in $C_{ss,min}$ proportional to the dose (Figure 8.1). However, participant A-04 expressed potential non-linear pharmacokinetics, evident between visits 4 (8-9 mg CBD/kg/day) and visit 5 (10-12 mg CBD/kg/day). Participant A-04 seizure frequency reduction increased from ~50% to 75% between visits 4 and 5.

Table 8.3. Minimum CBD plasma concentrations (ng/mL) at steady state of the CARE-E site A (Saskatchewan) participants from visit 1-5 during the dose escalation study.

Participant ID	Dose (mg CBD/kg/day)				
	0	2-3	5-6	8-9	10-12
A-01	N/D[1]	25.32	48.19	75.95	93.07
A-02	BLQ[2]	11.73	22.39	22.21	42.41
A-03	BLQ	9.80	24.44	79.89	78.53
A-04	BLQ	11.07	41.52	54.40	124.67
A-05	BLQ	10.98	19.20	55.17	24.20
A-06	N/D	19.51	21.76	58.78	73.50
A-07	BLQ	8.38	14.80	27.67	54.78

[1]N/D: Levels analyzed but not detectable
[2]BLQ: Levels below limit of quantification

All participants, except A-01 and A-04 displayed THC $C_{ss,min}$ concentrations below 4 ng/mL, the threshold set as the minimum level for effect (Table 8.4). Four of the participants had no detectable or quantifiable THC $C_{ss,min}$ concentrations until visit 3 (5-6 mg CBD/kg/day). Participant A-07, who became seizure free after visit 3, had no detectable THC levels until after visit 5 (10-12 mg CBD/kg/day).[22] Possibly due to low

amounts of THC present in the CBD rich CBM, THC levels had less variation compared to the other two cannabinoids. At visit 5 (10-12 mg CBD/kg/day), THC $C_{ss,min}$ concentrations ranged from 1.28-4.34 ng/mL.

Table 8.4. Minimum THC plasma concentrations (ng/mL) at steady state of the CARE-E site A (Saskatchewan) participants from visit 1-5 during the dose escalation study.

Participant	Dose (mg CBD/kg/day)				
ID	0	2-3	5-6	8-9	10-12
A-01	N/D[1]	1.20	2.09	3.84	4.34
A-02	BLQ[2]	0.81	1.27	1.59	2.58
A-03	BLQ	N/D	0.72	2.40	2.67
A-04	BLQ	BLQ	1.46	1.74	4.09
A-05	N/D	N/D	1.43	2.10	1.28
A-06	N/D	0.49	0.52	3.54	3.06
A-07	N/D	BLQ	N/D	N/D	1.13

[1]N/D: Levels analyzed but not detectable
[2]BLQ: Levels below limit of quantification

Two participants who became seizure free, A-06 and A-07, had the highest CBC $C_{ss,min}$ concentrations (Table 8.5). Participant A-04 had a disproportional increase of CBC between visits 4 and 5 (Figure 8.1). CBC $C_{ss,min}$ concentrations at visit 5 (10-12 mg CBD/kg/day) varied between 4.88-32.35 ng/mL.

Table 8.5. Minimum CBC plasma concentrations (ng/mL) at steady state of the CARE-E site A (Saskatchewan) participants from visit 1-5 during the dose escalation study.

Participant	Dose (mg CBD/kg/day)				
ID	0	2-3	5-6	8-9	10-12
A-01	N/D[1]	3.50	4.07	6.34	14.30
A-02	N/D	2.63	3.65	2.95	4.88
A-03	N/D	2.45	5.12	7.98	10.44
A-04	N/D	2.09	8.86	11.17	27.30
A-05	N/D	3.39	6.34	10.38	10.43
A-06	N/D	9.35	4.91	21.72	28.84
A-07	N/D	6.04	10.34	21.18	32.35

[1]N/D: Levels analyzed but not detectable

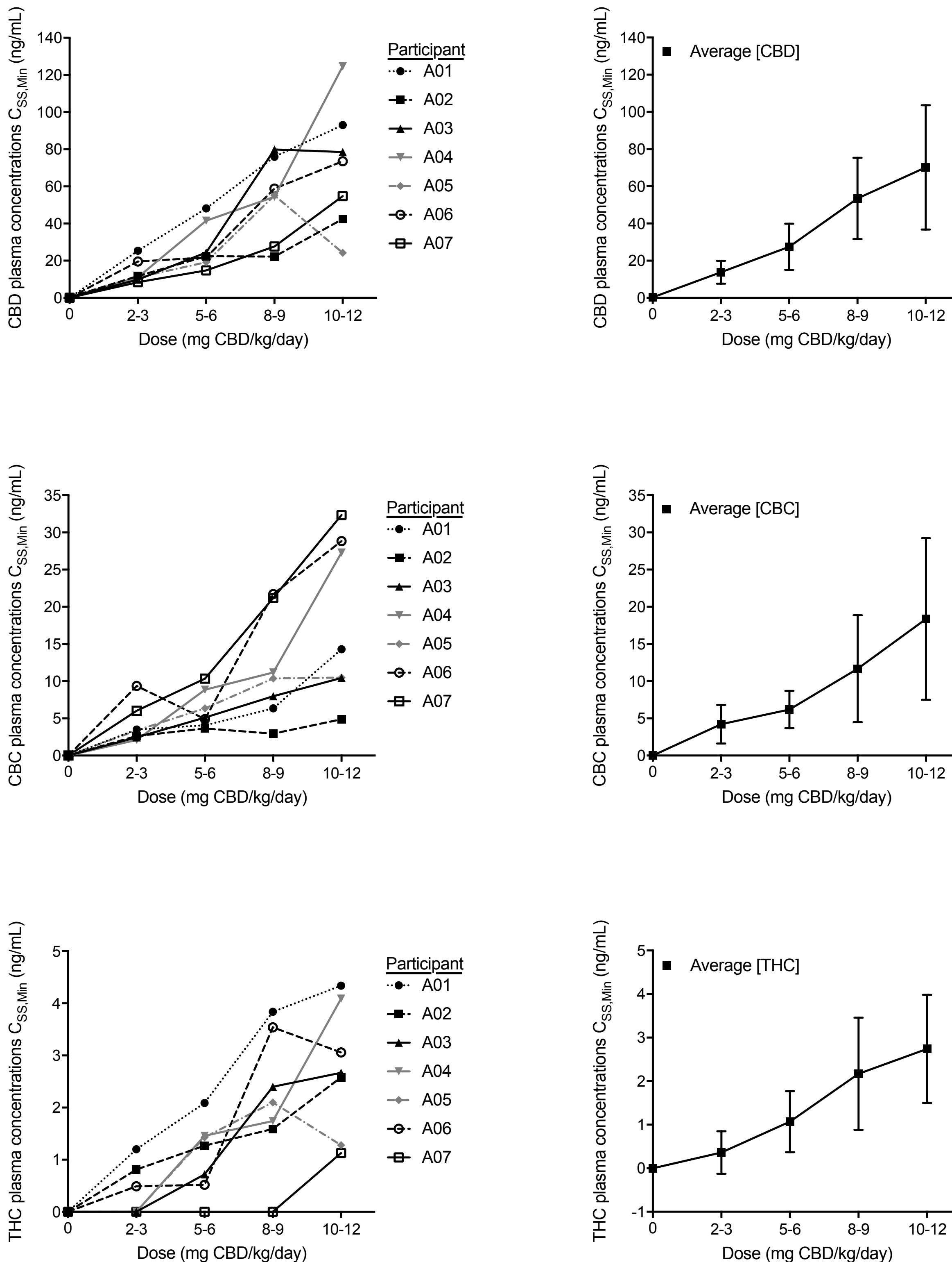

Figure 8.1. Minimum plasma concentration of CBD, CBC, and THC at steady state ($C_{SS,min}$) of the seven CARE-E participants during the CARE-E dose escalation study at site A (Saskatchewan). Adapted from Huntsman et al. (2019). Dosage Related Efficacy and Tolerability of Cannabidiol in Children with

Treatment-Resistant Epileptic Encephalopathy: Preliminary Results of the CARE-E Study. *Frontiers in Neurology.*

9 Determination of the Unbound Fraction of CBD, THC, CBC, and 11-OH-THC in Human Plasma

Cannabinoids display high non-specific binding to material found in commonly used plasma protein binding devices, such as polystyrene and cellulose membrane filters. Non-specific binding would result in the underestimation of the cannabinoid $f_{u(b)}$. A 3-solvent extraction technique had been developed to determine the plasma protein binding of 11-OH-THC, CBD, THC, and CBC while avoiding potential non-specific binding. A comparative analysis of the 3-solvent extraction technique, ultrafiltration, and Rapid Equilibrium Dialysis (RED) will determine the efficacy of the newly developed plasma protein binding assay.

Ketogenic diets, a conventional treatment for refractory epileptic encephalopathy, could alter lipoprotein production. The literature suggested cannabinoids extensively were bound to lipoproteins.[81] Increase of lipoprotein production may change the unbound fraction ($f_{u(b)}$) of cannabinoids, ultimately changing the therapeutic response of cannabinoids. A thorough understanding of the effects of ketogenic diets, a conventional treatment for refractory epileptic encephalopathy, on the unbound fraction of cannabinoids would ensure appropriate interpretation of total cannabinoid concentrations following quantitative analysis with our LC-MS/MS method.

9.1 Ultrafiltration technique to determine unbound fraction of cannabinoids

9.1.1 Method

To determine the efficacy of the ultrafiltration technique, 3 total CBD concentrations were targeted – 500 ng/mL, 1500 ng/mL, and 3000 ng/mL. 75 µL of working standard was added to 1425 µL blank human plasma. 1 mL of spiked human plasma was transferred into the ultrafiltration device to determine the unbound cannabinoid fractions. 200 µL from the remaining plasma sample was transferred to a 2 mL Eppendorf Lo-bind microcentrifuge tube and extracted following the sample extraction procedure to determine the total CBD concentration. The ultrafiltration sample

was centrifuged using the Beckman Coulter centrifuge with the conditions of 1000xg at 25°C for 3 minutes. These conditions allowed ~ 5% of the sample volume (50 µL) to be filtered through. The 50 µL filtrate was transferred to a new Eppendorf Lo-bind microcentrifuge tube and diluted in human plasma to a final volume of 200 µL. Cold acetonitrile and internal standard (610 µL), with a concentration of 1.63 ng/mL, was added to sample and centrifuged for 10 minutes at 14000 rpm and 4°C. 700 µL of supernatant was dried under filtered air at 37°C and reconstituted using 200 µL mobile phase.

Analysis was conducted using the liquid chromatography tandem mass spectrometry (LC-MS/MS) method developed and validated in objective 1. The unbound fractions of CBD and THC was determined by measuring the concentration of the sample in the filtrate cup.

9.1.2 Results

Results show that the unbound cannabinoid fraction was not detected in the filtrate, even at high cannabinoid concentrations (Table 9.1). Targeted concentrations were calculated to ensure that the unbound cannabinoid concentrations would fall into the standard curve. Concentrations measured for the unbound fraction were significantly below the LLOQ, confirming high non-specific binding of the highly lipophilic cannabinoids to the plastic and membrane filter of the ultrafiltration devices.

Table 9.1. Unbound and total concentration of CBD in human plasma using ultrafiltration

Theoretical CBD Concentration (ng/mL)	Unbound CBD Concentration (ng/mL)	Observed Total CBD Concentration (ng/mL)
500	ND[1]	547.85
1500	ND[1]	1521.40
3000	ND[1]	3141.85

[1]ND: Not Detectable

9.2 3-solvent extraction technique to determine unbound, lipoprotein, and remaining bound fraction of cannabinoids

One technique used radiolabeled compounds with the unbound fraction of drug being extracted using a 3-step organic solvent extraction.[131] This technique was used for highly lipophilic compounds with high plasma protein binding characteristics and had issues with binding to plastics. Sethi et al. used radiolabeled compounds and measured with liquid scintillation.[131] However, because the radiolabeled cannabinoids were difficult to synthesize, the 3-step organic solvent extraction technique was modified for liquid chromatography tandem mass spectrometry analysis, using non-radiolabeled cannabinoids (Figure 9.1).

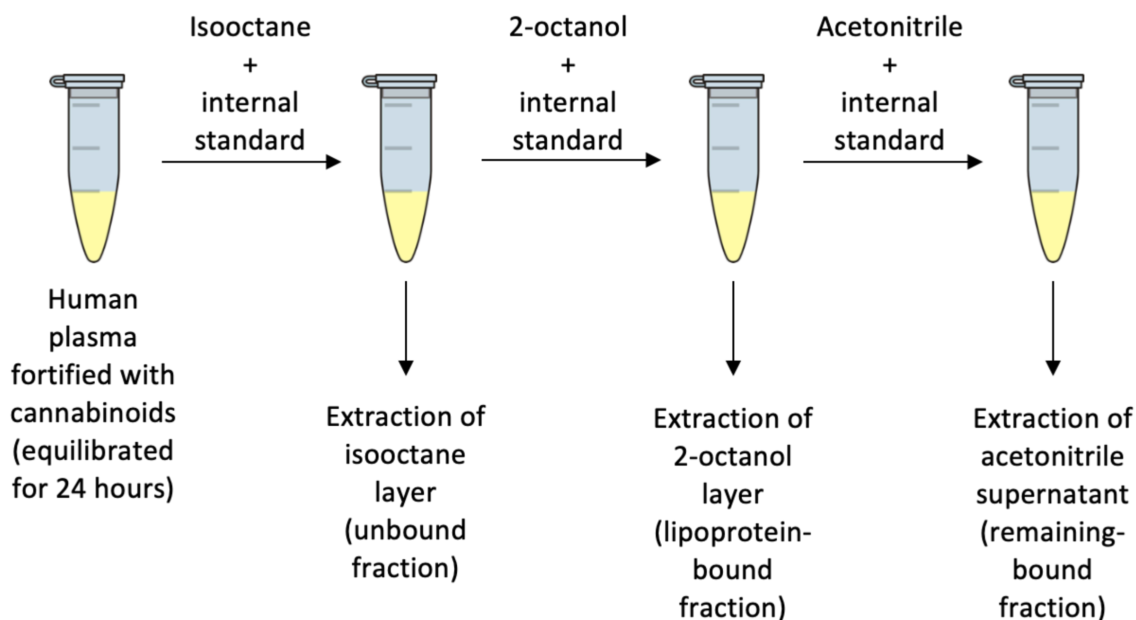

Figure 9.1. General schematic diagram of 3-solvent extraction technique using isooctane, 2-octanol, and acetonitrile to determine unbound, lipoprotein bound, and remaining plasma protein bound fractions of cannabinoids, respectively.

9.2.1 Method

The proposed 3-step organic solvent technique (Figure 9.1) required spiking 190 µL blank pooled human plasma with 10 µL analyte in 2 mL Eppendorf LoBind

microcentrifuge tubes. Samples were vortex mixed for 30 seconds. Samples were equilibrated on the orbital shaker for 24 hours at 37°C with a speed of 175 rpm. Samples were wrapped in foil to avoid photodegradation of cannabinoids.

610 µL isooctane and internal standard was added to plasma and gently inverted 30 times to avoid denaturation of plasma proteins. After inverting, samples were centrifuged for 5 minutes at 3000 rpm at 4°C for separation of the organic and aqueous phases. 500 µL of the isooctane layer was extracted into borosilicate culture tubes. After removal of isooctane, 610 µL 2-octanol and internal standard were added to the plasma, gently inverted, and centrifuged. 500 µL of the 2-octanol layer was extracted from the plasma sample. Due to high viscosity, 2-octanol was difficult to remove from the aqueous phase. Samples were placed in dry ice to freeze the plasma layer to obtain the 2-octanol phase without disrupting the aqueous phase. Following 2-octanol extraction, 610 µL acetonitrile and internal standard were added to the plasma, vortex mixed, and centrifuged at 14000 rpm for 10 minutes at 4°C. 500 µL of the supernatant was extracted from the plasma sample. The acetonitrile portion would contain the remaining bound cannabinoid fraction.

Each portion was dried at 37°C using filtered air and reconstituted with 200 µL mobile phase. Samples were analyzed using the validated plasma LC-MS/MS assay.

9.2.2 Results

The 3-solvent extraction technique repeated over 3 days in replicates of 6 at 25, 50, and 500 ng/mL provided consistent values, determining the reproducibility and reliability of the method.

11-OH-THC, at all three concentrations, displayed approximately 82.7% plasma protein binding, 20% bound to lipoproteins (Table 9.2). CBD presented approximately 82.1% plasma protein binding with 17.9% bound to lipoproteins while THC displayed 87.0% plasma protein binding, with 16.7% lipoprotein bound. Lastly, CBC displayed approximately 93.4% plasma protein binding and 13.3% lipoprotein binding.

Free, lipoprotein-bound, and remaining plasma protein bound fractions at 25, 50, and 500 ng/mL exhibited consistent values, indicating plasma proteins have not

reached saturation of binding sites. The sum of the free cannabinoid, lipoprotein-bound, and remaining plasma protein bound concentrations compared closely to the nominal concentration of each cannabinoid, indicating high recovery of the cannabinoids during the 3-solvent extraction technique.

The degree of lipophilicity of the cannabinoid correlated to the protein bound fraction. 11-OH-THC and CBD, the least lipophilic, displayed approximately 82.7% and 82.1% plasma protein binding, respectively, while CBC, the most lipophilic, displayed approximately 93.4% plasma protein binding (Table 9.3).

Table 9.2. Concentrations of 11-OH-THC, CBD, THC, and CBC in isooctane, 2-octanol, and acetonitrile following 3-solvent extraction in human plasma.

Cannabinoid	Theoretical Concentration (ng/mL)	Free Cannabinoid Isooctane Concentration (mean ± SD; ng/mL)	Lipoprotein-Cannabinoid 2-Octanol Concentration (mean ± SD; ng/mL)	Protein-Cannabinoid Acetonitrile Concentration (mean ± SD; ng/mL)	Total Observed Concentration (mean ± SD; ng/mL)
11-OH-THC	25	4.3 ± 1.2 (18.4%)	4.9 ± 0.5 (20.9%)	14.2 ± 0.3 (60.7%)	23.4 ± 0.5
	50	8.0 ± 0.4 (17.4%)	8.6 ± 0.5 (18.7%)	29.4 ± 0.5 (63.9%)	46.0± 0.4
	500	68.7 ± 5.8 (16.1%)	86.5 ± 8.8 (20.3%)	270.6 ± 12.8 (63.6%)	425.8 ± 15.9
CBD	25	4.5 ± 0.4 (17.0%)	5.5 ± 0.5 (20.8%)	16.5 ± 0.6 (62.2%)	26.5 ± 0.5
	50	10.8 ± 0.8 (20.0%)	8.4 ± 1.1 (15.6%)	34.8 ± 1.0 (64.4%)	54.0 ± 0.8
	500	84.8 ± 10.4 (16.8%)	87.9 ± 10.1 (17.4%)	331.8 ± 17.1 (65.8%)	504.5 ± 19.3
THC	25	3.3 ± 0.4 (12.4%)	5.1 ± 0.5 (19.2%)	18.2 ± 0.9 (68.4%)	26.6 ± 0.6
	50		7.8 ± 1.1	38.4 ± 2.3	53.9 ± 1.9

		7.7 ± 0.5 (14.3%)	(14.5%)	(71.2%)	
	500	67.0 ± 5.4 (12.5%)	89.0 ± 7.1 (16.5%)	382.4 ± 27.5 (71.0%)	538.4 ± 29.2
	25	1.8 ± 0.3 (6.5%)	4.2 ± 0.4 (15.3%)	21.5 ± 1.0 (78.2%)	27.5 ± 0.8
CBC	50	3.8 ± 0.1 (7.1%)	6.6 ± 0.7 (12.3%)	43.2 ± 1.7 (80.6%)	53.6 ± 1.2
	500	32.6 ± 1.4 (6.1%)	67.4 ± 7.3 (12.5%)	437.4 ± 26.0 (81.4%)	537.4 ± 33.9

Table 9.3. Unbound and bound fractions of 11-OH-THC, CBD, THC, and CBC in human plasma following 3-solvent extraction technique. Bound fraction includes lipoprotein and remaining plasma protein bound cannabinoids.

Cannabinoid	Unbound (%)	Bound (%)
11-OH-THC	17.3	82.7
CBD	17.9	82.1
THC	13.0	87.0
CBC	6.6	93.4

9.3 Rapid equilibrium dialysis (RED) technique for determining the unbound cannabinoid fraction

Rapid Equilibrium Dialysis (RED) could determine the unbound and bound fractions of cannabinoids without the interference of non-specific binding. Equilibrium dialysis consists of two chambers – a sample chamber and assay chamber (Figure 9.2). The plasma sample was introduced to the sample chamber. Phosphate buffered saline

(PBS) solution was added to the assay chamber. A cellulose membrane insert, with a 8000 molecular weight cut-off, located between the two chambers, selectively allowed only the diffusion of unbound fraction. An incubation period of 4 hours was required to ensure plasma protein binding reached equilibrium.

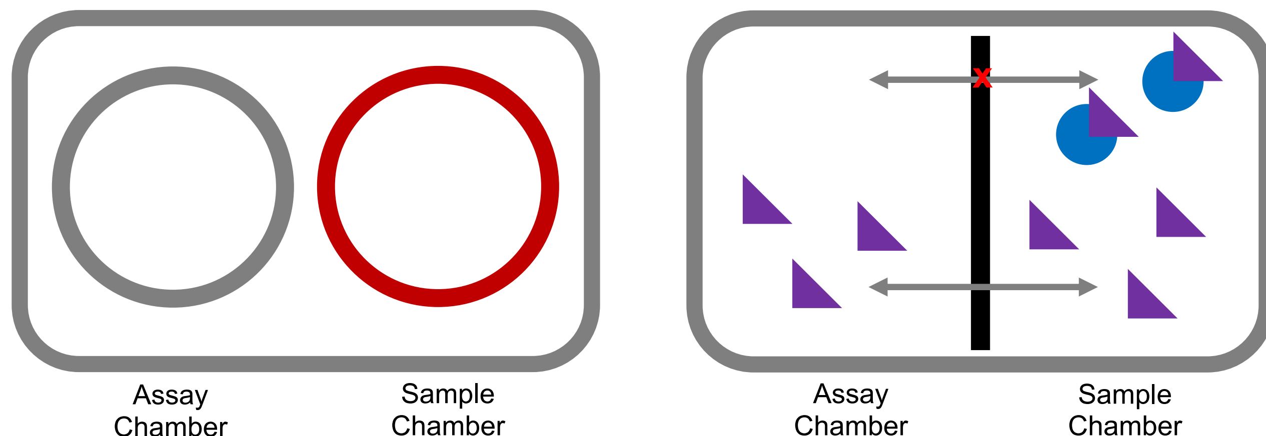

Figure 9.2. Diagram displaying the assay and sample chamber of equilibrium dialysis plate (a) and the equilibrium of the unbound drug concentration as it selective diffuses through the cellulose membrane insert (b). Rapid equilibrium dialysis plate is dependent to size, with a membrane cutoff of 8 kDa, restricting the passage of plasma protein bound cannabinoids.

9.3.1 Method

Following the 3-solvent extraction technique, 1000, 5000, and 10000 ng/mL concentrations of CBD, THC, 11-OH-THC, and CBC were used for RED analysis. 15 µL of analytes was added to 285 µL blank pooled human plasma. 200 µL of sample was transferred to the sample chamber of the RED plate. 400 µL of PBS was added to the assay chamber. Samples equilibrated for 4 hours in an incubated orbital shaker at 37°C and 175 rpm.

After equilibration, 100 µL of plasma and buffer sample were transferred to 2 mL Eppendorf Lo-bind microcentrifuge tubes. 100 µL of blank pooled human plasma and phosphate buffer saline solution (PBS) were added to the buffer and plasma samples, respectively, to provide similar matrices. 610 µL cold acetonitrile and internal standard were added for protein precipitation and centrifuged at 14000 rpm, 4°C for 10 minutes. 700 µL of supernatant was transferred to borosilicate culture tubes, dried under filtered

air at 37°C for 30 minutes, and reconstituted with 200 μL mobile phase. Samples were analyzed using the validated plasma LC-MS/MS method.

To determine the recovery of cannabinoids in PBS, 10 μL of 1000 ng/mL working solution was added to 190 μL of PBS or plasma in replicates of 6. Two types of buffers, Hyclone phosphate buffer saline (PBS) and Gibco Dulbecco's phosphate buffer saline (DPBS) were used to determine if different buffer compositions influenced cannabinoid recovery. Samples in PBS and plasma were incubated in 2 mL Eppendorf Lo-bind microcentrifuge tubes for 0 and 2 hours. 100 μL of sample was transferred to new Eppendorf microcentrifuge tubes. 100 μL of plasma or buffer was added to the tubes to provide similar matrices. Samples followed the same procedure as the RED technique described above. Plasma was used as a control to calculate cannabinoid recovery in PBS.

9.3.2 Results

The rapid equilibrium dialysis technique was not suitable for plasma protein binding analysis of cannabinoids. Loss of unbound cannabinoids during the 4-hour incubation period resulted in poor recovery (Table 9.4). The total cannabinoid plasma concentration closely matched the theoretical final concentration, indicating no non-specific binding to the plastic and membrane filter of the RED plate for cannabinoids found in the plasma compartment.

Table 9.4. Unbound and total concentration of 11-OH-THC, CBD, THC, and CBC in human plasma using Rapid Equilibrium Dialysis with 4-hour incubation time (n=3)

Cannabinoid	Theoretical Plasma Total Concentration (ng/mL)	RED Buffer Unbound Concentration (Mean ± SD; ng/mL)	RED Plasma Total Concentration (Mean ± SD; ng/mL)
11-OH-THC	50	11.2 ± 0.4 (26.5%)	42.2 ± 0.7
CBD	50	1.3 ± 0.4 (2.7%)	48.9 ± 0.3
THC	50	ND[1]	46.5 ± 0.3

CBC	50	ND	56.3 ± 1.5

[1]ND: Not Detected

A comparison test conducted between PBS and plasma determined the recovery of cannabinoids in PBS. The procedure followed similar steps to the RED technique, with the exception of the samples incubated in Eppendorf Lo-bind microcentrifuge tubes. Recovery was calculated by comparing the buffer cannabinoid concentrations to plasma cannabinoid concentrations, following Equation 9.1.

$$Recovery\ (\%) = \frac{[Cannabinoid]_{buffer}}{[Cannabinoid]_{plasma}} \times 100\% \qquad \text{Equation 9.1}$$

At 0 hours, both types of buffers presented no loss of cannabinoids, closely comparable to the plasma concentrations of CBD and THC (Table 9.5). At 2 hours, extensive loss of cannabinoids in both the Hyclone PBS and Gibco DPBS was observed, with approximately 65-76% and 79-88% loss in CBD and THC, respectively. Additionally, Hyclone PBS had a lower recovery of CBD and THC compared to the Gibco DPBS. At 0 and 2 hours, CBD and THC concentrations remained consistent, indicating cannabinoids were stable in human plasma at 2 hours.

Table 9.5. Comparison of CBD and THC concentrations in PBS and human plasma incubated at 0 and 2 hours, in replicates of 6, and determination of recovery, based on the comparison of CBD and THC concentrations in buffer to plasma (nominal concentration of 50 ng/mL).

	Incubation Time (hr)	CBD Concentration (mean ± SD; ng/mL)	CBD Recovery (%)	THC Concentration (mean ± SD; ng/mL)	THC Recovery (%)
Hyclone PBS	0	50.6 ± 2.4	92.7	54.7 ± 2.7	96.6
Gibco DPBS	0	51.4 ± 0.6	94.1	56.6 ± 1.1	99.8
Plasma	0	54.6 ± 0.3	---	56.7 ± 2.7	---

Hyclone PBS	2	13.0 ± 0.9	23.8	6.9 ± 0.5	12.4
Gibco DPBS	2	18.7 ± 1.3	34.2	11.8 ± 1.0	21.1
Plasma	2	54.6 ± 2.0	---	55.6 ± 2.7	---

10 Development of a Dried Blood Spot Assay for the Detection of Prenatal *Cannabis* Exposure in Newborns

With the legalization of recreational *Cannabis* in October 2018, there is concern of increased *Cannabis* use in pregnant women. Prevalence reports of prenatal *Cannabis* exposure can be obtained through various assessments, such as self-reporting and meconium testing. Self-reporting from mothers provides challenges and inconsistencies that can lead to inaccurate determination of the prevalence of prenatal *Cannabis* exposure.[47] These challenges include under-reporting, stigmatization of *Cannabis* use, risk of legal consequences, and guilt of knowing the risks to their newborn. Meconium, the first pass of stool from a newborn, is often used for *Cannabis* detection.[47] However, meconium can only detect *Cannabis* exposure from the third trimester. Meconium is also not readily available for screening. Meconium passing can often be delayed if newborns experienced distress during labor. Some newborns may pass their meconium during labor, which provides difficulty in sample collection. *Cannabis* detection in neonates requires reliable assessment to determine the prevalence of prenatal *Cannabis* exposure.

Dried blood spot (DBS) sampling is a technique that requires only a small volume of blood, which is advantageous over venepuncture blood sampling from pediatric participants. The use of DBS samples allows for analysis of neonate samples and ability to screen newborns for exposure to *Cannabis* through their mothers. DBS tests are done during newborn screening for inborn errors of metabolism, typically within the first 24 hours of birth.[127] This technique requires spotting blood on a filter paper, allowing it to dry before extracting the blood sample from the paper. Sample extraction process represents a challenge to DBS assay development to assure sensitive quantification of cannabinoids from very small volume blood samples.

The CRIS research group are currently conducting a study to determine the prevalence of prenatal *Cannabis* exposure pre- and post-legalization of recreational *Cannabis*. To avoid seasonal bias, 500 DBS samples will be randomly collected and deidentified from April, May, and June of 2018 (pre-legalization) and 2019 (post-

legalization) in Saskatchewan, Manitoba, and British Columbia. A LC-MS/MS method is required to detect THC-COOH, CBD, and THC in the neonate DBS samples.

To assess prevalence of prenatal *Cannabis* exposure, a number of neonate DBS samples collected will represent the entire population of the province and subsequently analyzed for detection of THC-COOH, CBD, and THC. Prevalence of prenatal *Cannabis* exposure was determined by the following Equation 10.1:

$$Prevalence\ (\%) = \frac{Number\ of\ DBS\ samples\ with\ detected\ cannabinoids}{Number\ of\ DBS\ samples\ analyzed} \times 100\% \quad \text{Equation 10.1}$$

10.1 Method Development for Quantitative Analysis

THC-COOH, the main cannabinoid of focus for the DBS assay, provided great difficulty with stability and accuracy in our plasma LC-MS/MS assay. Due to drying samples with filtered air, oxidation of THC-COOH occurred, causing the loss of the analyte. A comparison between drying and reconstituting versus no drying resulted in the loss of the analyte with drying the sample.

THC-COOH has a carboxylic acid functional group, which makes it more suitable to use negative ion mode for the mass spectrometer. However, the other cannabinoids analyzed worked more effectively in the positive ion mode. Experimental polarity switching was used to capture all cannabinoids. Sample run started off with negative ion mode for 1.8 min then switched to positive ion mode for the remainder of the run. However, THC-COOH and CBD had close retention times, making it difficult to create an experimental polarity switch.

Subsequently, a period polarity switch was conducted by switching between negative ionization mode and positive ionization mode constantly throughout the run. This resulted in the loss of points on the peak, with only 4 points detected for most peaks. For a peak to be quantifiable and properly integrated, 12-20 points require capturing per peak. With the MRM conditions for THC-COOH set in positive ionization mode and the polarity mode constantly switching between positive and negative ionization, the mass spectrometer could not capture enough data points for THC-COOH, which resulted in an unquantifiable peak.

The 2-pH rule indicated the pH of the solvent needs to be less than 2 units of the pKa of the analyte in order for the analyte to be predominantly in the unionized form. The pKa of THC-COOH is 4.69. The pH value of 0.1 mM ammonium of water and methanol was around 5. This indicated that the predominant form of THC-COOH was in ionized form. Ionized analytes do not interact well with the stationary phase in the column, which led to the analyte favoring the mobile phase and resulted in a lower retention time.

Additionally, pH values within the pKa mobile phase was modified by replacing the ammonium formate with 0.1% formic acid. This lowered the pH value to 2.3 and resulted in the unionized form of THC-COOH as the predominant form. The mobile phase modification allowed THC-COOH to interact with the stationary phase of the column and created a more stable retention time.

10.1.1 Method

Stock and working standard solution preparation

Stock solution of 50000 ng/mL was prepared by adding 100 µL of 1 mg/mL CBD, THC, THC-COOH, 11-OH-THC, CBC, and CBN each to 1400 µL LCMS grade methanol. Working standards for the standard curve, prepared by parallel dilution, ranged from 294.2-50000 ng/mL. The LLOQ working standard was 294.2 ng/mL. The LQC, MQC, and HQC standards for all cannabinoids were 500, 5000, and 40000 ng/mL, respectively. All samples were prepared in amber autosampler vials, wrapped in parafilm to avoid evaporation, and stored at -20°C until day of analysis.

Internal standard preparation

100 µg/mL CBD-d3, THC-d3, 11-OH-THC-d3, THC-COOH-d3, and CBC-d9 stock solutions were diluted in methanol to prepare a 100 ng/mL working internal standard solution in amber autosampler vials, wrapped in parafilm, and stored at -20°C.

Liquid chromatography conditions

Method development and validation experiments utilized the Agilent 1290 Infinity LC System with a Zorbax Eclipse XDB-C18 Narrow-Bore 2.1 x 75 mm 3.5-micron

column and Zorbax Eclipse XDB-C8 Narrow-Bore 2.1 x 12.5 mm 5-micron guard column, both controlled at 50°C, with a flow rate of 700 µL/min for 6 minutes. Gradient mobile phase comprised of methanol and water containing 0.1% formic acid, with gradient conditions listed in Table 10.1. The autosampler temperature was controlled at 4°C with a sample injection volume of 5 µL.

Table 10.1. Conditions outlining mobile phase gradient for the DBS LC-MS/MS method

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0 - 0.5	25	75
0.5 - 3.0	10	90
3.0 - 3.1	25	75
3.1 - 6.0	25	75

Mass spectrometry conditions

The mass spectrometric instrument consisted of a SCIEX QTrap® 6500 triple quadrupole mass spectrometer (Washington, DC), equipped with TurboIonSpray® electrospray (ESI) interface, set to 5500 V in positive ionization mode. Retention times for THC-COOH, CBD, and THC were 1.80 min, 1.87 min, and 2.75 min respectively. Multiple reaction monitoring (MRM) conditions and mass spectrometer parameters, outlined in Table 10.2 and 10.3, were optimized.

Table 10.2. Multiple Reaction Monitoring (MRM) conditions for the identification of THC-COOH, CBD, and THC in dried blood spots (DBS)

	Q1 Mass (Da)	Q3 Mass (Da)	Dwell Time (msec)	Declustering Potential (DP)	Collision Energy (CE)	Cell Exit Potential (CXP)
THC-COOH 1	345.263	299.200	150	101	27	16
THC-COOH 2	345.263	193.100	150	101	35	10

THC-COOH 3	345.263	119.000	150	101	35	16
THC-COOH D3	348.153	302.200	150	121	27	16
CBD 1	315.139	193.200	150	61	25	12
CBD 2	315.139	259.200	150	61	19	14
CBD D3	318.165	196.100	150	56	29	12
THC 1	315.130	193.100	150	91	31	12
THC 2	315.130	259.201	150	91	27	14
THC D3	318.155	196.100	150	96	31	10

Table 10.3. Mass spectrometer parameters for dried blood spot LC-MS/MS method

Curtain Gas (psi)	Collision Activated Dissociation (psi)	Ionization Spray Voltage (V)	Temperature (°C)	Gas Source 1 (psi)	Gas Source 2 (psi)	Entrance Potential (V)
50	10	5500	600	70	60	10

Sample preparation

10 µL of analyte was added to 190 µL blank human whole blood and vortex mixed for 10 seconds. For double blank and blank samples, 10 µL of LCMS grade methanol was added to 190 µL blank human whole blood. 50 µL of sample was spotted onto the Whatman Protein Saver cards and allowed to fully dry at room temperature in the absence of light (~3 hours). Cards were then collected into plastic bags with desiccant and stored at -20°C until day of analysis.

On the day of analysis, one 6-mm punch was taken from each spot and placed into individual 1.5 mL microcentrifuge tubes. 100 µL of LCMS grade water was added to the tube and vortex mixed for 1 minute. For protein precipitation, 310 µL of cold acetonitrile and internal standard were added to the sample and vortex mixed for an additional 30 seconds. For the double blank, 10 µL LCMS grade methanol was added in place of the internal standard. Samples were centrifuged at 14000 rpm for 10 minutes at 4°C. Samples were then filtered using the Agilent Captiva EMR-Lipid plate. Filtrate was transferred to amber HPLC vials with inserts.

10.1.2 Results

10.1.2.1 Issues with dried blood spot quantitative analysis

With a nine-point standard curve, one or two standard points would fail, with no specific standard point consistently failing. The quantitative method had issues regarding the reproducibility of results. One issue with quantitative analysis for dried blood spots derived from determining the exact volume in the 6-mm discs. With dried blood spots, chromatographic effects, sample heterogeneity, and hematocrit effects would affect the accuracy and precision of measuring the cannabinoid concentrations.[124,125] Chromatographic effects relate to the diffusion of the sample throughout the card. The spot would form a gradient, with the area being spotted having the greatest concentration of analyte and the outer edge having the least concentration. In conjunction to chromatographic effect, sample heterogeneity would cause error to quantitative analysis. The analyte in the sample was not homogenously spread, therefore making some areas of the spot more concentrated than others. These two factors would make it difficult produce consistent, fully saturated discs.

In the future, it would be difficult to determine the exact concentration of cannabinoids in the newborn dried blood spots. When directly dropped onto the card, exact volumes of blood in each DBS disc is unknown. Additionally, cannabinoid concentrations quantified in the DBS sample cannot determine the time of exposure during pregnancy. In conclusion, developing a quantitative method for dried blood spots is not necessary for this study. A qualitative method would be a more suitable approach,

with the intent to detect for the presence of THC-COOH, CBD, and THC in the neonate DBS samples.

10.2 Method Development for Qualitative/Drug Detection Analysis

With all the problems with DBS testing, quantitative analysis was an unsuitable approach to determine prevalence of *Cannabis* exposure in DBS samples. It was also not necessary to measure the accurate concentration of the DBS sample. Time and duration of prenatal *Cannabis* exposure were needed to determine the possible effects to the fetus. However, since samples analyzed were randomized and deidentified, we could not determine these factors.

For qualitative analysis, a standard curve was not required. Instead, the focus would be on development of a limit of detection (LOD), which could not be used for quantification. To determine the LOD, the analyte response needs to be three times greater than the noise response adjacent to the peak of the analyte.

10.2.1 Method

A final concentration of 1.47 ng/mL was used as the LOD for the dried blood spot assay. The LOD working standard (29.42 ng/mL) was prepared following dilution of the previous LLOQ (292.40 ng/mL) working standard. Internal working standard (1.67 µg/mL) was prepared by dilution of 100 µg/mL CBD-d3, THC-d3, and THC-COOH-d3 in methanol.

To prepare the samples, 10 µL of 29.40 ng/mL working standard was added to 190 µL blank pooled human whole blood. 50 µL of the sample was spotted onto each sample disc on the Whatman 903 Protein Saver card. Samples were then dried at room temperature for 3 hours in the absence of light and then stored at -20°C in plastic bags with desiccant until day of analysis.

To extract LOD samples from DBS cards, five 6-mm punches (equivalent to one full disc on the Whatman card) were collected into a 2 mL Eppendorf Lo-bind microcentrifuge tube. 1 mL of LCMS grade methanol and 5 µL of 1.67 µg/mL internal standard were added, and vortex mixed for 1 minute. Samples were then centrifuged at

1500 x g at 4°C for 5 minutes. 800 μL was transferred to new Eppendorf microcentrifuge tubes and subsequently dried using the LabConco SpeedVac for 1 hour. Samples were then reconstituted using 100 μL of mobile phase (25% A:75% B) and transferred to amber HPLC vials with inserts for LC-MS/MS analysis.

10.2.2 Method Validation

No current standard method validation guideline exists for dried blood spots. A fit for purpose method validation protocol had been established for the DBS assay. Method validation would follow the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology guidelines.[154] Based on the SWGTOX method validation guidelines, carryover, interference, matrix effect, limit of detection, and stability would provide sufficient determination of a reliable qualitative assay. Details of the criteria was described as followed.

10.2.2.1 Carryover

Carryover analysis must be conducted to determine if a high concentration may interfere with the subsequent injection of a low concentration. Carryover could lead to inaccurate quantification of samples. To conduct carryover for a qualitative method, a high concentration of analyte was injected into the mass spectrometer, followed by an injection of a blank sample and a low concentration sample. A sequence of high concentrations must be analyzed until presence of carryover. The highest concentration with no detectable peak would be considered the acceptable concentration for carryover absence.

A final concentration of 14.70 ng/mL will be prepared by adding 10 μL of 294.20 ng/mL standard to 190 μL blank pooled whole blood. A volume of 50 μL is spotted onto the Whatman Protein Saver card and allowed to dry for 3 hours. The LOD (final concentration of 1.47 ng/mL) is prepared as the low concentration by adding 10 μL of 29.42 ng/mL standard to 190 μL blank pooled whole blood. A volume of 50 μL is spotted

onto the Whatman Protein Saver card, allowed to dry for 3 hours and stored at -20°C in plastic bags with desiccant until day of analysis.

10.2.2.2 Interference

Interference would assess for any potential endogenous interferences that may be present in the human blood source. Evaluation of any interferences in the matrix required 10 different sources of blood. Blank human whole blood sources were obtained from volunteers, with ethics approval. A volume of 50 µL of blank individual blood was spotted onto the Whatman Protein Saver card, subsequently allowed to dry for three hours in the absence of light and stored in plastic bags with desiccant at -20°C until day of analysis. Samples were extracted following the extraction procedure.

10.2.2.3 Matrix Effects

Matrix factor analysis will be conducted to evaluate for any occurrence of ion suppression or enhancement. A post-column infusion is required to assess for any matrix effect. This requires the use of a tee-connection. Two concentrations, the LOD and a high concentration, are necessary to assess the matrix factor. To conduct a test, the pure standard is directly infused into the mass spectrometer. Baseline of infusion should be constant. Next, a blank dried blood spot sample is injected through the UPLC. SWGTOX recommends evaluating the 10 different sources of blank human whole blood. A matrix factor effect is present if changes in the baseline, increase or decrease in total ion intensity, were present.

10.2.2.4 Limit of Detection

Replicates of 6 LOD samples would be analyzed each day to determine reliability of the concentration based on the signal-to-noise ratio. Both MRM transitions of each cannabinoid must have a signal-to-noise ratio ≥ 3. Additionally, retention time must be monitored. The LOD for all cannabinoids used in the assay was 1.47 ng/mL.

10.2.3 Detection of cannabinoids in neonate dried blood spot samples in Saskatchewan pre-legalization of recreational *Cannabis*

Five hundred residual neonate DBS samples from June 2018 were collected from the Roy Romanow Provincial Lab in Regina, Saskatchewan. The randomly selected DBS cards required fully saturated discs to ensure consistency in each 6-mm punch. Each sample consisted of 5 6-mm punches, collected in 2 mL Eppendorf Lo-bind microcentrifuge tubes, and stored in a large plastic bag with desiccant in a -20°C freezer until day of analysis. 220 samples were extracted following the sample extraction procedure and analyzed subsequently after the LOD assessment from the second day of method validation.

10.3 Results

We have developed a sensitive and selective qualitative LC-MS/MS assay for the detection of CBD, THC, and THC-COOH in dried blood spots. We obtained a LOD of 1.47 ng/mL for all three cannabinoids, with signal-to-noise ratios greater than 3. THC-COOH, CBD, and THC eluted at retention times of 1.82, 1.89, and 2.82 minutes, respectively (Figure 10.1).

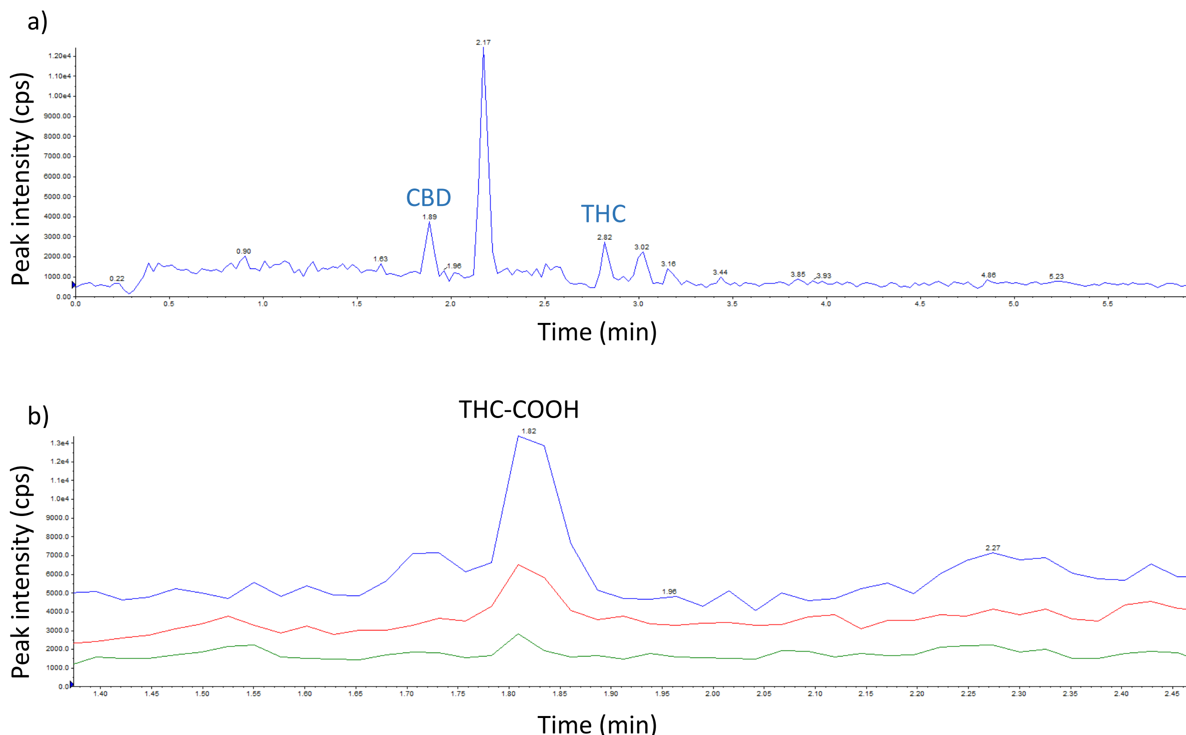

Figure 10.1. Chromatogram displaying the LOD (1.47 ng/mL) of CBD, THC (a) and THC-COOH (b), achieving signal-to-noise (s/n) ratio greater than 3 in positive ionization mode.

10.3.1 Limit of Detection

Evaluation of LOD depended on detection of signal-to-noise ratios greater than 3. Two days of assessment, 6 replicates of LOD concentrations (1.47 ng/mL) each day, achieved signal-to-noise ratios greater than 3. Third day of LOD assessment could not be evaluated due to the COVID-19 pandemic. Method validation will continue once university laboratories open.

10.3.2 Prenatal *Cannabis* exposure detection in Saskatchewan June 2018 neonate DBS samples

220 neonate DBS samples were analyzed, following the second day of method validation. A total of 87 DBS samples displayed peaks for the THC-COOH 1 transition. However, only 11 of samples had detectable peaks for THC-COOH 1 (signal-to-noise ratio greater than 3), displayed in Table 10.4. Additionally, the same 11 samples displayed peaks for all three THC-COOH transitions. Based on Equation 6, prenatal

Cannabis exposure pre-legalization of recreational *Cannabis* displayed a prevalence of 5%. The LC-MS/MS assay determined no CBD or THC detected in all 220 neonate DBS samples. Analysis of the remaining 280 samples will be conducted in the future.

Table 10.4. Detection of THC-COOH, CBD, and THC following analysis of 220 Saskatchewan neonate DBS samples (June 2018) obtained from the Roy Romanow Provincial Lab

Location	Date	Number of Samples Analyzed	Number of Samples with THC-COOH Detected	Number of Samples with CBD Detected	Number of Samples with THC Detected
Saskatchewan	June 2018	220	11	0	0

11 Discussion

11.1 Context

Cannabis has recently gained the interest of researchers and pediatricians as a potential alternative treatment of refractory epileptic encephalopathy. Yet, lack of thorough science-based evidence on the efficacy of *Cannabis* therapy creates uncertainty in the safety of pediatric *Cannabis* use. Furthermore, no standardized dosage regimen exists for the pediatric population. The few clinical studies examining *Cannabis* based medicine (CBM) for pediatric epilepsy have differed in the strain of *Cannabis* plants and administered dose, varying in efficacy and adverse effects.[58-64] A standardized dosage regimen can ensure *Cannabis* therapy is safe and effective for children with refractory epilepsy.

Currently, *Cannabis* dosage regimens for pediatric use are extrapolated from adult dosages, based on body weight.[90] A University of Saskatchewan led national open label dose-escalation study of CBM use, the CARE-E trial, was designed to help identify an appropriate dose of CBM for pediatric refractory encephalopathy by monitoring seizure frequency reduction and adverse effects with increasing doses.[2] The CARE-E study also intended to define the relationship between the bioactive cannabinoid $C_{ss,min}$ levels and dose, evaluating for linear or nonlinear pharmacokinetics and to provide the dose to conduct a single oral pharmacokinetic (PK) study in children. A single oral pharmacokinetic study can determine pharmacokinetic parameters, such as C_{max}, T_{max}, k_e, $t_{1/2}$, and AUC, essential for developing a standard dosage regimen. Since it is widely known that age-dependent pharmacokinetics exist in the pediatric population, characterization of age-dependent pharmacokinetics is required to develop appropriate age-stratified dosage regimens, ensuring the child receives optimal therapeutic responses to the *Cannabis* oil therapy while avoiding or minimizing adverse side effects associated with *Cannabis* consumption.[94]

Before conducting a PK study, a highly sensitive bioanalytical method is required for accurate measurement and to adequately capture the drug plasma concentration versus time profile. Liquid chromatography-tandem mass spectrometry is a bioanalytical tool widely used in pharmaceutical and toxicological analysis. Its high sensitivity and

selectivity are valuable for the analysis of multiple analytes requiring accurate measurements of low concentrations in biological fluids, such as whole blood, plasma, urine, and saliva. This is beneficial to capture an appropriate terminal phase in PK analysis to define k_e, necessary for determining $t_{1/2}$, as well as residual cannabinoids to determine *Cannabis* exposure.

An important factor that may alter pharmacokinetic parameters is diet. CBMs are often used as an adjuvant therapy, administered alongside AEDs and ketogenic diets. Ketogenic diets, consisting of high fat and low carbohydrates, can increase lipoprotein levels in the blood. Cannabinoids appreciably bind to lipoproteins; therefore, increase of lipoproteins may ultimately alter the plasma protein binding profile of cannabinoids.[80] It is imperative to investigate the impact the ketogenic diet will have on the plasma protein binding of cannabinoids and determine if dosage adjustments are necessary. Plasma protein binding assays are challenged by the extensive non-specific binding of cannabinoids to materials conventionally used in plasma protein binding techniques, such as ultrafiltration and equilibrium dialysis.[82] Adsorption to the surfaces of the polymer plastic and membrane filters will result in low recovery of cannabinoids, underestimating both the total and unbound cannabinoid concentration.

While *Cannabis* therapy is proposed for treating many pediatric medical conditions, children may be unintentionally or accidentally exposed. A major concern is the potential adverse effects associated with prenatal *Cannabis* exposure. More reports have emerged of pregnant women using *Cannabis* to help alleviate pregnancy symptoms, such as morning sickness.[50] Legalization of recreational *Cannabis* has increased accessibility of *Cannabis* products, which in turn may have increased *Cannabis* consumption in pregnant women, whether use is intentional or not. Cannabinoids can diffuse through the placental-blood barrier and enter the fetal systemic circulation, producing effects observed immediately after birth or delayed until adolescence/adulthood.[47] It is important to understand the prevalence of prenatal *Cannabis* exposure and the effects associated.

To investigate the knowledge gaps in pediatric *Cannabis* research, specifically for the age-dependent pharmacokinetics of cannabinoids, I measured $C_{ss,min}$ levels of 11-OH-THC, CBD, THC, and CBC in pediatric patients by developing a quantitative LC-

MS/MS method with adequate sensitivity and selectivity. A comparative analysis of plasma protein binding assays, using the same LC-MS/MS method, also determined which technique captured the cannabinoid plasma protein binding profile while avoiding non-specific binding and solubility issues. Additionally, in a separate objective, a qualitative LC-MS/MS method was developed to determine the prevalence of prenatal *Cannabis* exposure by detecting the presence of cannabinoids in neonate DBS samples.

11.2 LC-MS/MS Method Development and Validation

11.2.1 Method Development

LC-MS/MS method development requires identifying appropriate mobile phase conditions (i.e. type of solvents, buffers/pH modifiers, gradient vs isocratic flow, mobile phase ratios, and flow rate) required for achieving sufficient analyte separation and peak shape as well as an appropriate sample clean-up procedure to ensure minimal endogenous interference, reduced matrix effect, and increased sensitivity.

One of the key parameters for method development is determining to use a gradient or isocratic elution for the chromatographic separation. Reverse phase liquid chromatography consists of a non-polar stationary phase column and polar mobile phase. The mobile phase uses a combination of aqueous (mobile phase A) and organic (mobile phase B) solvent. The ratio of organic to aqueous solvent will dictate the elution of the analyte. Initial method development led to LC-MS/MS conditions that provided an isocratic elution with a run time of 5 minutes, held at 90% mobile phase B. 11-OH-THC and CBD peaks overlapped at a retention time of 1.64 minutes. Overlapping analyte peaks may result in matrix effects that can impact the overall sensitivity of the method. To address this issue, LC conditions were modified to a gradient method. Changes to mobile phase ratios, starting with 80% mobile phase B for the first 3.5 minutes, increasing to 90% B for 6.5 minutes, then returning to 80% B for the last 3.5 minutes. Lowering the organic mobile phase during the beginning of the run resulted in the separation of 11-OH-THC and CBD. However, the gradient method increased the total run time to 13.5 minutes. As seen in Figure 7.1b, CBC, the last eluting cannabinoid, has

a retention time of 9.77 minutes. Gradient methods require additional time after the elution of the last analyte to ensure return of mobile phase conditions to initial conditions, with recommendations of 5 column volumes to re-equilibrate the column for the next sample run.

Another LC parameter that contributes to method development optimization is the flow rate. UPLC instrumentation allows for use of increased flow rate, which can aid in shorter total run time. This is beneficial for studies that require high throughput analysis. Using a flow rate of 700 µL/min instead of 250 µL/min allowed for 1) shorter interactions with the stationary phase of the reverse phase column as well as 2) delivery of more cannabinoids to the mass spectrometer. Shorter interaction with the column will lead to shorter retention times and ultimately shorter sample run time. Mobile phase ratios were modified again, to 77.5% mobile phase B to ensure adequate separation 11-OH-THC and CBD while shortening the run time to 10 minutes. Reverting back to an isocratic method, mobile phase conditions remain constant throughout the sample run and do not require any re-equilibration. Delivery of more charged cannabinoid analytes to the mass spectrometer will increase the number of ions transported to the detector, increasing peak intensity and ultimately sensitivity.

Although increased flow rate may deliver more analyte to the mass spectrometer, more solvent is also introduced to the instrument, potentially leading to insufficient desolvation in the ionization chamber. Inadequate evaporation of solvent will decrease the ionization efficiency and subsequently the ions that enter to the quadrupoles. Optimization of gas and temperature in the source is required to ensure adequate evaporation of the solvent. As the final result, an isocratic method with a flow rate of 700 µL/min and run time of 10 minutes, held at 77.5% mobile phase B, provided great separation of 11-OH-THC (1.83 min), CBD (2.19 min), THC (5.55 min), and CBC (8.50 min). Additionally, the LC-MS/MS parameter optimization resulted in narrower and sharper peaks, increasing the peak height of 11-OH-THC, CBD, THC, and CBC, from 2.6×10^3, 6.9×10^3, 3.8×10^3, and 2.1×10^3 to 1.1×10^4, 2.1×10^4, 9.2×10^3, and 6.2×10^3, respectively. Increasing peak height is important when establishing sensitive LLOQ values.[155]

Another way to increase sensitivity is optimizing sample preparation techniques to aid in removing endogenous interferences and increasing analyte concentrations. Many sample preparation approaches were used to increase the sensitivity of the LC-MS/MS method. Sample concentration was achieved by drying off 700 µL supernatant following the protein precipitation step and reconstituting with the mobile phase at a lower volume of 200 µL. Extraction efficiency is calculated by comparing pre-spike samples (samples fortified with analyte prior to extraction) and pure-spike samples (samples fortified with analyte after extraction), where post-spike samples represent 100% recovery of cannabinoids. The increase of cannabinoid concentration will increase peak area and height of the cannabinoids, which is beneficial for increasing sensitivity. The LLOQ in the initial LC-MS/MS method was 0.98 ng/mL for 11-OH-THC, CBD, and THC, and 1.97 ng/mL for CBC. Addition of sample drying and reconstitution with a low mobile phase volume to the sample preparation procedure increased sensitivity by two-fold, resulting in LLOQ concentrations of 0.49 ng/mL for 11-OH-THC, CBD, and THC, and 0.98 ng/mL for CBC.

An additional peak with the same MRM transition for the quantifier ion of CBD, THC, and CBC was present in all samples, eluting at a retention time of 3.05 minutes (Figure 7.3). The peak was also found in the double blank and blank samples, indicating possible endogenous interference. However, the interference did not show the MRM transitions for the qualifier ions. Most importantly, the unknown peak has a different retention time than CBD, THC, and CBC, therefore does not affect accurate quantification of the cannabinoids. Further analysis using NMR is required to determine the compound and ensure no potential interference to future additional cannabinoids.

11.2.2 Method Validation

After development, the method requires validation to ensure accurate quantitative data. Method validation, as per the 2018 FDA Bioanalytical Method Validation guidelines, requires, linearity, accuracy, precision, carryover, stability, and matrix effect testing.[151]

Multiple guidelines for method validation, FDA, EMA, and ICH, consistently emphasize the importance of conducting system suitability tests but do not provide

specific criteria to conduct and measure system suitability.[151,157,158] System suitability is an essential parameter to determine the reproducibility and performance of the instrumentation, ensuring it provides accurate quantification of samples. MDS Pharma Services (MDSPS) has conducted several tests to determine the criteria for system suitability, resulting in a cut-off of 6% CV.[152]

Typically, system suitability is calculated using the peak area ratio between the analyte and internal standard. However, internal standard was not added into the system suitability method validation samples, resulting in the calculation being based solely on the peak area of the analyte. Nevertheless, the system suitability results were within the MDSPS CV criteria, ranging from 0.76 to 3.77%, confirming that the LC-MS system is providing reproducible data (Table 7.3).

Two of the most important criteria for method validation includes testing the accuracy and precision of the LC-MS/MS method, ensuring the method produces measured concentrations comparable to the theoretical concentrations in a reproducible manner. Intra- and inter-day accuracy and precision for all cannabinoids were within the FDA criteria, with observed concentrations of LLOQ and all three QCs within ±20% and 15% of the nominal concentrations, respectively, as displayed in Table 7.4 and 7.5.[151] Greater variation of accuracy and precision was shown with the LLOQ values for all cannabinoids, with accuracy values lower than the nominal LLOQ concentration, due to greater influence of the baseline in the measurement of LLOQ peak area.

Ion ratio, between the quantifier and qualifier ion, is crucial to ensure peak purity and monitor for cannabinoid identification, ensuring no false positive detections due to interfering compounds with the same MRM transitions. Ion ratios can also indicate saturation of the detector, observed with high concentrations. Ion ratio monitoring during method validation, produced CV values ranging from 6.31 to 10.94%, with average ion ratios of 0.33, 0.60, 0.94, and 0.50 for CBD, THC, 11-OH-THC, and CBC, respectively (Table 7.7). Samples used to determine the consistency of ion ratios included the standard curve as well as the accuracy and precision QC samples, ranging in concentrations from 0.49 to 125 ng/mL. Ion ratios calculated during method validation are consistent, regardless of cannabinoid concentration. Ion ratios of clinical samples should be compared to the reference ion ratio, averaged from the standard curve.

Complete stability assessment is essential prior to conducting pharmacokinetic studies to ensure cannabinoid quantification is not compromised by loss of analytes. Stability is important to test under different environments and conditions that may cause loss of cannabinoids, such as during storage, under various temperatures, and after freezing and thawing of sample (i.e., rapid temperature changes).

Short term stability QC samples, both unprocessed and processed, were within 15% of the nominal concentration, with the exception of one LQC unprocessed sample. This is likely due to pipetting error as the remaining 5 LQC unprocessed samples were within the criteria. Short term stability evaluates for loss of cannabinoids due to potential situations where samples may be left at room temperature. These situations can include improper storage conditions, samples accidentally left at room temperature, and malfunction of LC-MS/MS instrumentation during analysis. Results from the short-term stability test indicate if any of the situations occurred; the unprocessed and processed samples will remain stable for 6 hours at room temperature.

Autosampler stability examines if cannabinoids are stable under the autosampler temperature during data analysis. Observed QC concentrations reanalyzed 24 hours after initial analysis, kept in the autosampler at 4°C, remained within 15% of the nominal concentrations, indicating cannabinoid stability. This is important with high throughput analysis, as samples may require long residence time with the autosampler prior to injection and analysis.

Finally, freeze-thaw stability assesses cannabinoid stability through three cycles of freezing and thawing plasma samples. Samples may experience repeated freezing and thawing throughout sample collection, storage, and analysis. Cannabinoid samples from the first and third freeze thaw cycle remained stable, within 15% of the nominal cannabinoid concentration. HQC samples of THC and CBC from the second cycle failed, with plasma cannabinoid concentrations exceeding the nominal values, though CBD and 11-OH-THC were within the acceptable criteria. This is likely due to pipetting error or instability of cannabinoids after the second freeze-thaw cycle. Reanalysis of the complete freeze-thaw stability is required to ensure THC and CBC can endure constant freezing and thawing.

Both CBD and THC displayed 102.33% and 88.25% recovery, respectively. Recovery was calculated by comparing pre-spike and post-spike samples. The high recovery suggests the sample extraction procedure sufficiently removes the majority of endogenous compounds while maintaining integrity of the cannabinoids.

Matrix effects, calculated by comparing post-spike and neat samples, is prominently present in both CBD and THC, expressing 50.37% and 42.35%, respectively (Table 7.9). The high matrix effect indicates substantial ion suppression of the cannabinoids. Sample extraction consists only of protein precipitation, which is an effective process of removing plasma proteins but does not remove phospholipids. Drying and reconstituting will increase the cannabinoid concentrations but will also increase the concentration of phospholipids. Phospholipids exists with diverse characteristics, ranging in polarity and molecular weights, providing difficulty in selectively removing the endogenous compounds during chromatographic separation.[159]

The addition of deuterated internal standards will help to correct for matrix effect, as seen in (Table 7.10). However, phospholipid ion suppression will profoundly affect sensitivity of the LC-MS/MS assay. To overcome ion suppression and achieve greater sensitivity, more extensive sample clean-up is required. Lipid sorbent plates, such as Agilent Captiva EMR-Lipid plates, can sufficiently remove both phospholipids and plasma proteins.[160]

11.3 CARE-E Study

The overall purpose of the CARE-E study was to select an effective dose with minimal adverse effects.[2] This study was the first open-label pediatric study to measure $C_{ss,min}$ CBD, THC, and CBC levels in pediatric patients on *the* CBD rich CBM therapy. The CARE-E dose escalation study started with a dose of 2-3 mg CBD/kg/day, increasing monthly to a maximum dose of 10-12 mg CBD/kg/day. My responsibility in this study was the quantitative analysis of pediatric blood cannabinoid concentrations. This study is the first to quantify cannabinoid plasma concentrations in a pediatric *Cannabis* dose escalation study. Measuring plasma cannabinoid concentrations is

important to assess its relationship to pharmacological effects and establish pharmacokinetic characteristics. Limited information is available on age-dependent cannabinoid pharmacokinetics of the pediatric population. It is necessary to determine the pharmacokinetic differences throughout childhood for the development of an appropriate age-stratified dosage regimen.

11.3.1 Non-specific binding and cannabinoid instability issues

Before conducting any study, it is important to recognize any potential issues related to the physicochemical properties of the cannabinoids and the materials used. Tubes used for blood collection and plasma sample storage as well as the duration of sample storage were two important challenges associated with patient blood cannabinoid analysis. In previous work done in our lab, Barricor tubes were superior to other blood collection tubes commonly used for clinical analysis.[158] Other blood collection tubes displayed lower or higher cannabinoid plasma concentrations compared to the control. Many of these tubes contain additives that can interfere with quantification of cannabinoids through either ion suppression or enhancement. Barricor tubes provided cannabinoid concentrations comparable to the control tube. Additionally, prolonged incubation in the blood collection tubes may subject the cannabinoids to non-specific binding.

Polymer plastics, such as polystyrene, polypropylene, and polyethylene, are highly hydrophobic molecules. Cannabinoids, which are nonpolar and lipophilic, will undergo hydrophobic interaction with the polymer molecules, adsorbing to the sides of the plastic tubes.[161] Adsorption can be reversed by direct addition of a water miscible organic solvent to the tube to release adsorbed cannabinoids from the plastic surface.[162] However, plasma samples may be initially stored in one tube and later aliquoted into specific volumes after. Since cannabinoids adsorb to the plastic surface, reduced cannabinoid levels are transferred to the new container, resulting in underestimated cannabinoid concentrations. To address this issue, plasma samples are immediately aliquoted into low binding plastic tubes upon retrieval of the samples. Furthermore, experience from my thesis work suggests a standardized guideline should exist for all clinical cannabinoid research, comprehensively detailing appropriate

materials and procedures used for blood collection, storage, and analysis. This would ensure cannabinoids maintain stability and can provide an ability for interlaboratory comparisons of clinical cannabinoid concentrations.

Another issue observed during the CARE-E study was a significant decrease in cannabinoid plasma concentrations of the samples reanalyzed 8-10 months after the initial analysis, with approximately 30 to 50% decrease for CBD, THC, and CBC. Participant A-05 experienced a decrease in cannabinoid plasma concentration at dose 10-12 mg CBD/kg/day (Figure 8.1). This was due to stability issues as the sample was analyzed 8 months after blood collection. Long-term stability assessment during method validation only analyzed cannabinoid stability up to 3 months. Instability of cannabinoids at 8 months may be due to a combination of long-term storage of degradation and non-specific binding to polymer plastics.[119,163] After blood collection and plasma separation in Barricor vacutainers, plasma is transferred to polystyrene culture tubes and stored at -80ºC until analysis.

11.3.2 CARE-E sample analysis

All participants showed a wide variation in plasma concentrations, with CBD $C_{ss,min}$ levels ranging from 24.20 - 124.67 ng/mL at the 10 - 12 mg CBD/kg/day dose (Table 8.3). Variation in the bioavailability of cannabinoids as well as differences in systemic clearance likely explains the differences in cannabinoid exposure. Cannabinoids are known to exhibit low and variable bioavailability (THC F= 4-12%; CBD F= 13-19% in animals), which is likely due to interindividual variability of first pass metabolism.[164,165] One important source of variability in the pediatric population is stage of postnatal development. Age plays an important role in the maturation of metabolic enzymes. Participants varied in age, ranging from 1-10 years of age. Participant A-04, who is categorized in the 1-3 year age range, displayed the highest $C_{ss,min}$ value at the 10-12 mg CBD/kg/day dose. CYP3A and CYP2C subfamilies reach maturation by age 2 years, then exceed adult levels until 10 years, and slowly returning to adult levels by puberty.[90] Due to immature CYP3A and CYP2C enzymes, Participant A-04 likely had reduced first pass metabolism, resulting in increased bioavailability and reduced

systemic clearance leading to higher plasma cannabinoid concentrations relative to the size of dose.

The immaturity of CYP3A and 2C enzymes could also explain the non-linear or dose-dependent pharmacokinetics displayed in Participant A-04. With lower expression, metabolic enzymes become saturated at higher cannabinoid concentrations, resulting in higher bioavailability and exposure to cannabinoids. The role of intestinal efflux transporters on cannabinoid bioavailability is uncertain, but CBD is not a substrate of efflux transporters and, therefore, CBD is more likely undergoing saturation of first pass metabolism.[74] A detailed single oral dose-escalation pharmacokinetic study with a larger sample size can investigate the potential non-linear pharmacokinetics of CBD, THC, and CBC.

Systemic clearance (Cl_s) also plays a significant role in steady state plasma concentrations. Systemic clearance, which varies throughout child development, is influenced by organ blood flow, plasma protein binding, and hepatic enzymatic metabolism.[166] Those who experience lower hepatic metabolism may have reduced hepatic clearance and increased oral bioavailability, ultimately leading to increase exposure to CBD, THC, CBC and the metabolites, such as 11-OH-THC, THC-COOH, 6-OH-CBD and 7-OH-CBD.

11.3.3 Factors affecting pharmacokinetic variability

Variability of bioavailability and systemic clearance may be driven by other factors, such as genetic polymorphism in CYP expression, concomitant medications, and diet. CYP3A4, CYP2C9, and CYP2C19 are all major metabolic enzymes responsible for the metabolism of THC and CBD. All three CYP enzymes are known to have high inter-individual variability due to genetic polymorphism. For example, CYP2C9 contains three common variants- CYP2C9.1, CYP2C9.2, and CYP2C9.3.[167] 30% of Caucasians express the CYP2C9.2 and CYP2C9.3 variants.[167] These two variants are uncommon in the Asian and African populations. Approximately 95% of people from the Asian and African populations express the wild-type variant, CYP2C9.1. Variants CYP2C9.2 and CYP2C9.3 have low in vitro THC metabolic activity, whereas THC seems to inhibit the CYP2C9.1 variant.[168]

Drug-drug interactions may also occur with concomitant medications. Many of the AEDs, such as clobazam, topiramate, valproic acid, and phenytoin are metabolized by the same CYP enzymes as phytocannabinoids.[169] As a potent inhibitor of CYP2C9, CYP2C19, and CYP2D6, CBD may alter the metabolic activity of several AEDs, resulting in varying pharmacological response.[170] A common drug-drug interaction often seen is CBD and clobazam. Clobazam, a substrate to CYP3A4, undergoes demethylation to produce active metabolite, N-desmethylclobazam, a CYP2C19 substrate.[171] Co-administration of CBD and clobazam can increase plasma steady state concentrations of clobazam and its active metabolite. Dependent on the therapeutic window and steady-state plasma concentrations, increased clobazam and N-desmethylclobazam levels may lead to better therapeutic outcomes or toxicity, making it imperative to closely monitor each AED plasma concentration.

THC intoxication has been a concern with use of CBM for pediatric patients. As reported in the literature, THC intoxication is exhibited at a plasma concentration of 5 ng/mL.[172] None of the participants reached a THC concentration of 5 ng/mL at the highest dose. While participants A-01 and A-04 reached THC concentrations close to 5 ng/mL at the 10-12 mg CBD/kg/day dose, they did not display any form of intoxication during the CBD rich CBM therapy. The possible attenuation of THC intoxication may be due to CBD acting as a negative allosteric modulator (NAM) of CB_1 receptors, decreasing the efficacy of THC.[173] Orthosteric ligands of CB_1 receptors, such as THC, are linked to the psychoactive effects of *Cannabis*.

During the dose escalation study, three participants, A-03, A-06, and A-07, became seizure free. Participants A-06 and A-07 also had the highest CBC plasma concentrations. Recently, researchers have discovered potential antiseizure properties in CBC.[174] The high CBC plasma concentrations in the two participants may be linked to the improvements in seizure frequency. All three participants had mid-range $C_{ss,min}$ levels for CBD. This disparity is possibly due to inter-individual variability of pharmacodynamic processes, specifically age-dependent maturation of cannabinoid receptors. The endocannabinoid system (ECS) plays an important role in neural development. CB_1 receptors have been found in the fetus brain at 14 weeks of gestation, though the levels are significantly lower compared to adults.[175] CB_1 receptor

expression in the prefrontal cortex peaks around the age of 5 years before slowly decreasing into adulthood.[176] In other regions of the brain, such as the frontal cortex, hippocampus, basal ganglia, and cerebellum, CB_1 receptor expression increases from the gestational period to adulthood.[175,176] Varying receptor expression may influence the pharmacodynamic processes of cannabinoids. More research is required to fully understand cannabinoid receptor ontogeny during child development, such as receptor density and functionality.

Although the CARE-E study provided valuable findings for CBM and refractory epilepsy, the study was limited to the small sample size. It is difficult to conclusively determine the age-dependent PK characteristics from 7 participants. Age groups were disproportional, with two participants within 1-3 years, four participants within 4-6 years, and 1 participant within 7-10 years of age. It is crucial to have an adequate sample size as children in different age groups (i.e. infant, toddler, early child, child, and adolescence) greatly differ in physical size, body composition (body water and fat proportions), and physiological processes (organ maturation).[94] Furthermore, children of the same age group can produce variable pharmacokinetic responses due to factors such as disease progression, genetic polymorphism, and concomitant medications that may lead to drug-drug interactions.[177] Even amongst the same age group, children can develop at different rates. PK studies using larger sample sizes with equal and sufficient numbers of participants in each age category is necessary to establish PK parameters related to age differences. The single oral dose PK study will contain a sample size of n=6/group with 4 groups- infant (<2 - 3 years old), early child (4 - 6 years old), child (7 - 10 years old), and adolescence (11-18 years old).

11.4 Plasma Protein Binding Assay

As previously mentioned, diet can impact the pharmacokinetics of cannabinoids. Many children with pediatric epilepsy are placed on a ketogenic diet, a diet containing high fat content, to help reduce the seizure frequency. The plasma protein binding profile of cannabinoids may alter with increased lipoprotein production due to the high fat diet.[80]

The unbound cannabinoid fraction plays a significant role in understanding the changes in the pharmacokinetics of cannabinoids with modifications in plasma protein levels. Cannabinoids are classified as high extraction ratio drugs.[178,179] The unbound cannabinoid plasma concentration represents the concentration producing a pharmacological effect. Changes in the unbound fraction of high extraction drugs will not affect the unbound steady state plasma concentration following oral administrations, but total steady state plasma concentrations will change due to changes in oral bioavailability.[180] Typically, bioanalytical methods quantify for the total plasma concentration. Hence, any observed alteration in total steady state plasma concentrations with changes in plasma protein binding may compel changes to the dosing regimen if it is not understood that unbound steady state concentrations remain unchanged.

Changes in the unbound fraction will also impact the distributional characteristics of the cannabinoids. Since only the unbound concentration can elicit a pharmacological effect, changes in the unbound fraction may result in pharmacodynamic changes depending upon the relative volume of distribution and the extent in plasma protein binding change.[75] Such changes in pharmacodynamics may compel a dosing adjustment. Therefore, it is important to define the extent of altered lipoprotein levels to calculate the unbound plasma concentrations and monitor if dosage adjustments are required. Plasma protein binding profiles can be achieved using plasma protein binding assays, such as ultrafiltration and equilibrium dialysis.

Common plasma protein techniques typically are not suitable for plasma protein binding analysis of highly lipophilic compounds owing to extensive non-specific binding to hydrophobic plastics, membrane filters, and glassware. Ultrafiltration was conducted at total CBD concentrations of 500, 1500, and 3000 ng/mL. However, the ultracentrifugation filtrate contained no detectable unbound CBD concentration at any of the three levels of total concentrations, indicating non-specific binding. As lipophilic compounds, CBD likely underwent hydrophobic interaction with the polystyrene sample reservoir.[161] Additionally, cannabinoids are possibly sticking to the membrane filter, preventing any free cannabinoid to pass into the filtrate cup with the ultrafiltrate. This

high non-specific binding suggests that ultrafiltration is not a suitable technique for cannabinoid or any lipophilic compound plasma protein binding analysis.[181]

RED also provided challenges with measuring the unbound cannabinoid concentration. THC and CBC unbound concentrations were not detectable with the RED technique. However, the observed total concentrations were comparable to the theoretical total concentrations for both cannabinoids, indicating no loss of cannabinoids due to non-specific binding to the plastic and membrane filters. Additional tests determined cannabinoids have low solubility in buffer, with 65.8 - 76.2% and 78.9 - 87.6% loss of CBD and THC, respectively, after a 2-hour incubation. The loss of cannabinoids correlates with the degree of lipophilicity, CBD being less lipophilic compared to THC. Cannabinoid concentrations in plasma remained constant after 2 hours. Buffer is commonly used in research for its biologically relevance in pH (7.4) and isotonicity, similar to the human physiological system. Additionally, as cannabinoids are highly plasma protein bound, the plasma proteins may protect cannabinoids from adsorption to the surfaces of the apparatus and membrane filter. With the absence of plasma proteins in buffer, cannabinoids were also subjected to surface adsorption.[182] It was also determined that increase of salt concentration increased the polarity of the PBS solution, resulting in loss of cannabinoids due to insolubility in aqueous solutions, as seen when comparing Hyclone PBS to Gibco DPBS.

The 3-solvent extraction procedure was selected as an alternative technique for plasma protein binding analysis.[131] This technique avoids the use of materials susceptible to non-specific binding. Sample extraction was conducted in low-binding microcentrifuge tubes. The first step of the three-solvent procedure involves the addition of isooctane to the plasma sample. Isooctane is a highly nonpolar, water immiscible solvent.[131] This allows only the free cannabinoids to partition into the nonpolar phase without disrupting the plasma protein bound cannabinoids. The second solvent used in the procedure is 2-octanol, an alcohol with an 8-carbon tail. 2-Octanol displays characteristics of an alcohol and lipid solvent. With thorough mixing, 2-octanol may interact with lipoproteins, modifying the fluidity of the hydrophilic layer of the lipoprotein and potentially allowing the cannabinoids to release.[132] Due to its 8-carbon tail, 2-octanol provides an environment for the released cannabinoids to partition into the non-

polar phase. Other plasma proteins would not be affected as they are more polar compared to lipoproteins. Acetonitrile is an organic, water miscible solvent commonly used for protein precipitation and is added in the third step of the three-solvent procedure. Acetonitrile can reduce the hydration layer surrounding plasma proteins, which is important for protein folding.[133] As the hydration layer is reduced, plasma proteins undergo aggregation and subsequent precipitation with each other resulting in the release of the cannabinoids.

The total observed cannabinoid concentration, calculated by summation of cannabinoid concentrations from the three fractions, was comparable to the total theoretical cannabinoid concentration for 11-OH-THC, CBD, THC, and CBC. This indicates that the 3-solvent extraction technique yields high recovery, with no or minimal indications of non-specific binding. This technique also allows the separation of the lipoprotein bound cannabinoids from the other plasma protein bound cannabinoids. Isolation of lipoprotein bound fraction can determine altered plasma protein binding with increased lipoprotein levels due to ketogenic diets.

To accurately quantify the cannabinoids, equal internal standard concentrations were present in isooctane, 2-octanol, and acetonitrile. This ensures consistent internal standard peak area in all three solvents. However, there is concern about the deuterated internal standard undergoing plasma protein binding similar to the analyte, which would inaccurately calculate the unbound, lipoprotein-bound, and remaining plasma protein bound fractions. If that were the case, the internal standard peak area in isooctane would be lower compared to the standard curve and accumulate in the 2-octanol and acetonitrile fractions. However, the internal standard peak area in the isooctane layer was consistent with the internal standard peak areas of the standard curve. The internal standard peak area in the 2-octanol layer was significantly lower. Due to its high viscosity, 2-octanol required longer drying time. It is possible cannabinoids in 2-octanol underwent degradation, but the internal standard would have corrected for the issue. Additionally, viscosity can affect the ionization efficiency of cannabinoids.[183] The higher the viscosity, the lower the rate of evaporation. Incomplete solvent evaporation can lower ionization efficiency. Nonetheless, the internal standard peak area in the acetonitrile layer remained consistent with the internal standard peak

area of the standard curve, indicating no shift of plasma protein binding of the internal standard from 2-octanol.

Based on the 3-solvent extraction technique, the unbound fraction of 11-OH-THC, CBD, THC, and CBC was 17.3%, 17.9%, 13.0%, and 6.6%, respectively (Table 9.3). The results follow a trend where increasing lipophilicity is associated with increase of plasma protein binding. Lipoprotein bound fractions of 11-OH-THC, CBD, THC, and CBC ranged from 12.3 to 20.9% (Table 9.2). Interestingly, 11-OH-THC displayed higher lipoprotein bound fractions compared to CBC. It was assumed increase of lipophilicity would increase lipoprotein binding; however, the inverse relationship observed indicates otherwise. This may be due to the insufficient interaction time between the cannabinoids and 2-octanol. The higher lipophilic compounds, such as THC and CBC, may require longer interaction times with 2-octanol for the complete extraction of lipoprotein bound cannabinoids. Further analysis with multiple different extraction time periods is necessary to accurately quantify the lipoprotein bound fraction of cannabinoids.

The results obtained from the 3-solvent extraction technique deviates from the previous literature, which has reported cannabinoids are 95-99% plasma protein bound, or 1-5% unbound.[72] The assessment of cannabinoid plasma protein binding was conducted in the 1970s and 1980s. Many limitations were discovered in each of the studies as followed in Table 11.1.

Table 11.1. Summary of three cannabinoid plasma protein binding assays, detailing the technique used, plasma protein bound fraction, and limitations to the study.

Study	Technique Used	Plasma Protein Bound Cannabinoids	Limitations
Klausner et al.[129]	Zonal ultracentrifugation	62 - 65% lipoprotein bound; 35 - 38% residue fraction (mainly albumin)	No measurement of the unbound fraction; assumption of 100% plasma protein bound.
Wahlqvist et al.[81]	Disc electrophoresis/ Agar gel electrophoresis	80 - 95% lipoprotein bound; no albumin bound	Inadequate separation of each plasma protein

			fraction; inability to accurately quantify each fraction. No albumin fraction measured which contradicts Klausner et al.
Hunt et al.[130]	Equilibrium dialysis	92 - 99% total plasma protein bound	Use of PBS solution; cannabinoids have solubility issues in buffer.

The 3-solvent extraction technique provides advantages over the previous cannabinoid plasma protein binding assays. Each fraction is isolated from the other two, allowing for distinct separation between the unbound, lipoprotein bound, and remaining plasma protein bound cannabinoids. The 3-solvent extraction technique accounts for the unbound cannabinoid concentration. None of the three previous studies provided information on the unbound THC concentration.

In addition, the previous studies provide semi-quantitative analysis, indicating cannabinoids are highly plasma protein bound. In fact, Wahlqvist and Klausner agreed that THC predominantly binds to lipoproteins.[81,129] Based on our results, cannabinoids were 12-20% lipoprotein bound. It is possible that more time is necessary to thoroughly mix 2-octanol with the plasma sample to allow the solvent to completely interact with the lipoproteins. Analysis of multiple time points will determine an optimal mixing period. Additional assessment, using other analytes with well-established plasma protein binding characteristics, is critical to confirm accuracy and precision of the 3-solvent extraction method.

11.5 Dried Blood Spot Assay

With the legalization of recreational *Cannabis*, there may be an increase in prenatal *Cannabis* exposure. To evaluate the prevalence of prenatal *Cannabis* exposure, neonate dried blood spots were utilized. Neonate DBS samples are readily available to retroactively detect prenatal *Cannabis* exposure. Newborn DBS screening is mandatory for all newborns in Canada. Additionally, all provinces in Canada store the neonate DBS samples for approximately 10-20 years, becoming readily available to retroactively detect for prenatal *Cannabis* exposure in a large population at random. Cannabinoids can be extracted from the DBS samples and analyzed using LC-MS/MS.

Quantitative LC-MS/MS analysis was deemed unfit for DBS cannabinoid analysis, with the standard curve consistently failing linearity. Major factors affecting the accurate quantification of cannabinoids in DBS include hematocrit effect, paper chromatographic or "volcano" effect, sample heterogeneity, and unknown blood spot volumes.[124,125] Hematocrit effect, the main factor responsible for inaccuracy, is based on the interindividual variability of red blood cell levels in whole blood. Higher RBC levels tend to have high viscosity, which limits the homogeneous spread of blood in the delimited blood spot area of the card.[124] This can be problematic as all samples will vary in hematocrit level and viscosity; therefore, neonate DBS samples may over- or underestimate measured blood cannabinoid concentration.

As blood is spotted onto the disc, the blood will diffuse in a chromatographic manner, with the center being the most concentrated and the outer edges being less concentrated.[125] This effect will make it difficult to obtain dried blood spot consistency when punching spots from DBS sample. Paper chromatographic effect can be avoided by extracting the whole DBS sample; however, this can only be done if blood spot volumes are constant in all samples. Exact blood spot volumes are required for accurate quantification. This may be problematic with neonate DBS collection, as the newborn's heel is pricked with a lancet, allowing blood to drop directly onto the card.

Lastly, sample heterogeneity plays a significant role in accuracy. Typically, samples require thorough mixing to ensure homogeneously distributed analytes. However, after drying, analyte remains stationary with randomized distribution. Parts of

the blood spot may have higher amounts of cannabinoids than other areas, which can produce inaccurate quantification by either overestimating or underestimating of the actual concentrations.[125]

Exact quantification of cannabinoids is not necessary for determining prenatal *Cannabis* exposure. Assessment of randomized and deidentified neonate DBS samples cannot provide information about the time and duration of prenatal *Cannabis* exposure, making it impossible to define a concentration-effect relationship. A qualitative analysis was developed instead to detect for the presence of cannabinoids, specifically THC-COOH, CBD, and THC, in neonate DBS samples.

Initially for qualitative method development, blank human whole blood samples were collected anonymously from the Royal University Hospital in Saskatoon, Saskatchewan. Upon assessing for interferences/selectivity, which requires analyzing 10 different sources of matrix, many blank samples tested positive for THC-COOH. As recreational *Cannabis* is now legal and widely used in adults, anonymous whole blood samples cannot be used as blank matrices due to potential cannabinoid contamination. To overcome this situation, ethics approval was obtained to collect whole blood samples from consented participants who do not use *Cannabis* or have not used within 3 months. All 10 sources of blank human whole blood did not detect any endogenous interferences or cannabinoids.

Sample preparation includes methanol extraction, followed by centrifugation, drying and reconstituting. A centrifugal concentrator was used instead of a dry bath with filtered air to increase sensitivity. With the dry bath, THC-COOH underwent degradation, extensively decreasing concentration. Use of the centrifugal concentrator maintains stability of THC-COOH while increasing the concentration.

For qualitative LC-MS/MS analysis, only a LOD (1.47 ng/mL) is required to detect the presence of THC-COOH, CBD, and THC, with the signal-to-noise ratios greater than or equal to 3. Three MRM transitions are used to confirm the presence of each cannabinoid. To ensure the accuracy of the LOD value, the experiment was conducted in two days with 6 replicates, showing that the S/N ratio remained greater than 3. The LC-MS/MS qualitative method provided enough sensitivity to detect THC-COOH, CBD, and THC in DBS samples, at a concentration of 1.47 ng/mL.

Following LOD determination, we initiated the analysis of neonate DBS samples, starting with the analysis of pre-legalization DBS samples from Saskatchewan. Up to now, 220 Saskatchewan neonate DBS samples from June 2018 have been analyzed. Of the 220 samples, 11 tested positive for THC-COOH, so far indicating a 5% exposure rate pre-legalization of recreational *Cannabis*. This exposure rate is comparable with Spain, reporting a prevalence of *Cannabis* exposure of 5.3%.[47]

CBD and THC were not detected in the samples. Both CBD and THC have shorter half-lives than THC-COOH, making them more suitable as drug biomarkers for recent drug exposure.[23,69] Additionally, CBD and THC stability in DBS samples has not been conducted, but CBD and THC in whole blood samples at -80°C have observed instability after 52 weeks.[119] DBS samples are considered more stable than whole blood samples; however, the Saskatchewan samples were stored at 4°C. Therefore, with higher storage temperature, it is possible cannabinoids are unstable after 52 weeks. Stability assessment is required using the qualitative LC-MS/MS method for confirmation.

12 Future Work

12.1 LC-MS/MS optimization

LC-MS/MS assays typically undergo modifications to optimize and improve sensitivity and sample run times. More cannabinoids will be added to the assay in the future, including 6-OH-CBD, 7-OH-CBD, 7-COOH-CBD, CBN, THCV, CBDV, and CBG. The addition of analytes requires sufficient separation; therefore, an isocratic method may not be suitable. A gradient method will probably be needed to ensure cannabinoids are well separated in a timely manner.

The use of the UPLC instrument can further be advantageous with the use of a UHPLC column. These columns contain sub-2-μm particles, which allow for shorter sample run times while maintaining peak separation. The columns can also provide better peak shapes by increasing peak height and decreasing peak width.[184] This would be beneficial for THC and CBC as both still display short, wide peaks with the currently used LC-MS/MS assay. Shorter sample run times while maintaining adequate separation is ideal for high-throughput analysis.

Column choice is another potential analytical optimization step. The phenyl-hexyl column provides great selectivity to aromatic compounds. This column contains a hexyl or 6-carbon alkyl chain and phenyl group attached. The hexyl hydrocarbon chain provides greater separation for aromatic analytes. The nonpolar stationary column interacts by π-π stacking electron rich bonds between the analyte and stationary phase, which will minimize any potential interferences and also reduce background noise, leading to greater sensitivity.[185,186]

Removal of phospholipids can minimize matrix effect and ion suppression while increasing sensitivity. Agilent Captiva Enhanced Matrix Removal (EMR) Lipid plates combines the efficiency of protein precipitation and effectiveness of solid phase extraction into one technique.[160] This plate uses a proprietary material to selectively interact with long chain lipids, trapping them within the filter. Addition of an organic solvent, such as acetonitrile, would precipitate plasma proteins. The removal of the two major endogenous interferences should provide a much cleaner sample and lower

chromatographic baseline. Additionally, the cannabinoids will have less competition for ionization, increasing ionization efficiency as phospholipids compete with the analyte for charge and surface area on the charged droplet.

12.2 CARE-E Dose Escalation Study

The CARE-E study is a nationwide study lead by the University of Saskatchewan, with study sites at the Universities of British Columbia, Alberta, Manitoba, and Montreal. Seven participants from Site A (Saskatchewan) have completed the dose escalation study, with more participants recruited and undergoing completion of study. Most universities have completed recruitment and the study, with 7 participants completing the study in British Columbia. We are currently anticipating the receival of all patient samples for analysis, necessary for the completion of the CARE-E study.

12.3 Pharmacokinetic studies

It is important to understand the physicochemical properties of the analyte(s) prior to conducting clinical studies. Potential issues throughout the whole process of the clinical study, including blood collection, blood storage, sample preparation, and LC-MS/MS analysis must be addressed to ensure no potential loss of analyte due to non-specific binding or unsuitable storage processes.

Once approved by Health Canada, the CRIS research group will conduct human clinical trials, including single oral dose pharmacokinetic studies in children and adults. The pediatric PK study will categorize participants into three different experimental groups, separated by age- infant (2-3 years old), child (4-6 years old), older child (7-10 years old), and adolescent (11-18 years old). Conducting the PK study of pediatric patients in the diseased state will allow for a better understanding of the pharmacokinetics of cannabinoids in patients to identify more optimal dosing requirements. Therefore, the PK study will recruit pediatric patients undergoing palliative care.

The validated human plasma LC-MS/MS assay will be used to quantify the cannabinoid plasma concentrations that are necessary for obtaining pharmacokinetic parameters, such as C_{max}, T_{max}, elimination rate constant (k), $t_{1/2}$, and AUC. These parameters are essential to determine age-dependent pharmacokinetic differences of cannabinoids, allowing the establishment of suitable dosage regimens in the heterogenous pediatric population.

In conjunction to the age-dependent pediatric pharmacokinetic study, the plasma protein binding assay will aid the understanding of the influence of the ketogenic diet on the level of lipoproteins. Dietary lipids can increase lipoprotein levels, which may alter the plasma protein binding profiles of cannabinoids. The 3-solvent extraction technique can determine the possible impact and determine whether or not a dosage adjustment is required to ensure adequate therapeutic response to the CBM.

12.4 Dried Blood Spot- Prevalence of Prenatal *Cannabis* Exposure

The DBS assay will aid in the assessment of the prevalence of prenatal *Cannabis* exposure pre-and post-legalization of recreational *Cannabis*. Included in this study are British Columbia, Manitoba, and Saskatchewan. The assay will also compare differences in prenatal *Cannabis* exposure in the three provinces. The assay still requires completion of method validation, including carry-over, matrix effect, and the last day of LOD determination.

Once method validation is complete 1750 newborn dried blood spot samples will be taken from April, May, and June 2018 (pre-legalization) and another 1750 samples taken from April, May, and June 2019 (post-legalization) from BC, Manitoba, and Saskatchewan. Sample size was calculated to have enough statistical power to conclude a 2% change or no change in prevalence. Dates were chosen to avoid bias in seasonal changes of *Cannabis* consumption.

Many retail stores in Canada could not open immediately following legalization of recreational *Cannabis* or faced shortage issues of *Cannabis* products. April, May, and June 2020 will also be assessed to determine any changes of prenatal *Cannabis* exposure due to greater accessibility of *Cannabis* products. It is likely that greater

accessibility of *Cannabis* products in 2020 will provide a more accurate evaluation of prenatal *Cannabis* exposure pre- and post-legalization of recreational *Cannabis*.

References

1. Specchio, N., Trivisano, M., Scott, R. C., & Ferrie, C. (2012). Epileptic Encephalopathy. *Epilepsy Research and Treatment, 2012*(1), 1-2. doi:10.1155/2012/460256
2. Huntsman, R.J., Tang-Wai, R., Alcorn, J., Vuong, S., Acton, B., Corley, S., Laprairie, R., Lyon, A.W., Meier, S., Mousseau, D.D., Newmeyer, D., Prosser-Loose, E., Seifert, B., Tellez-Zenteno, J., Huh, L., Leung, E., & Major, P. (2019) Dosage Related Efficacy and Tolerability of Cannabidiol in Children with Treatment-Resistant Epileptic Encephalopathy: Preliminary Results of the CARE-E Study. *Frontiers Neurology, 10*. doi: 10.3389/fneur.2019.00716
3. Reddy, D.S. & Golub, V.M. (2016). The Pharmacological Basis of *Cannabis* Therapy for Epilepsy. *The Journal of Pharmacology and Experimental Therapeutics, 357*, 45-55. doi:10.1124/jpet.115.230151
4. Camfield, P.R. (2011). Definition and natural history of Lennox-Gastaut syndrome. *Epilepsia, 52 (5)*, 3-9. doi: 10.1111/j.1528-1167.2011.03177.x
5. Archer, J.S., Warren, A.E.L., Stagnitti, M.R., Masterton, R.A.J., Abbott, D.F., & Jackson, G.D. (2014). Lennox-Gastaut syndrome and phenotyping; Secondary network epilepsies. *Epilepsia, 55 (8)*, 1245-1254. doi:10.1111/epi.12682
6. Villas, N., Meskis, M.E., & Goodliffe, S. (2017). Dravet syndrome: Characteristics, comorbidities, and caregiver concerns. *Epilepsy & Behavior, 74*, 81-86. http://dx.doi.org/10.1016/j.yebeh.2017.06.031
7. Guerrini, R., Striano, P., Catarino, C., & Sisodiya, S.M. (2011). Neuroimaging and neuropathology of Dravet syndrome. *Epilepsia, 52 (2)*, 30-34. doi:10.1111/j.1528-1167.2011.02998.x
8. Bureau, M. & Bernardina, B.D. (2011). Electroencephalographic characteristics of Dravet syndrome. *Epilepsia, 52 (2)*, 13-23. doi:10.1111/j.1528-1167.2011.02996.x
9. Mert, G.G., Herguner, M.O., Incecik, F., Altunbasak, S., Sahan, D., & Unal, I. (2017). Risk factors affecting prognosis in infantile spasm. *International Journal of Neuroscience, 127 (11)*, 1012-1018. doi:10.1080/00207454.2017.1289379

10. Duloc, O., Soufflet, C., Chiron, C., & Kaminska, A. (2002). What is west syndrome? In P.A. Schwartzkroin & J.M. Rho (Eds.) *Epilepsy, Infantile Spasms, and Development Encephalopathy* (1-22). https://doi.org/10.1016/S0074-7742(02)49003-4
11. Singhal, N.S. & Sullivan, J.E. (2014). Continuous Spike-Wave during Slow Wave Sleep and Related Conditions. *ISRN Neurology, 2014*, 1-6. http://dx.doi.org/10.1155/2014/619079
12. Varadkar, S. (2017). Continuous spike-wave during sleep: treating an epilepsy without seizures. *Developmental Medicine & Child Neurology,* 1. doi: 10.1111/dmcn.13650
13. Alyautdin, R., Romanov, B., Pasternak, E., Bunyatyan, N., & Merkulov, V. (2016). Analysis of Factors Influencing the Interchangeability of Antiepileptic Drugs. *Pharmaceutical Chemistry Journal, 50 (3)*, 181-184. doi:10.1007/s11094-016-1418-x
14. Charuta, J., Kolbe, D. L., Mansilla, A., Manson, S., Smith, R. J., & Campbell, C. A. (2016). Ketogenic diet: A novel treatment for early epileptic encephalopathy due to PIGA deficiency. *Brain and Development, 38*, 848-851. doi:10.1016/j.braindev.2016.04.004
15. Yu, J.Y. & Pearl, P.L. (2013). Metabolic Causes of Epileptic Encephalopathy. *Epilepsy Research and Treatment, 2013*, 1-20. http://dx.doi.org/10.1155/2013/124934
16. Lutas, A. & Yellen, G. (2012). The ketogenic diet: metabolic influences on brain excitability and epilepsy. *Trends in Neurosciences, 36 (1)*, 32-40. https://doi.org/10.1016/j.tins.2012.11.005
17. Zgair, A., Wong, J.C.M., Lee, J.B., Mistry, J., Sivak, O., Wasan, K.M., Hennig, I.M., Barret, D.A., Constantinescu, C.S., Fischer, P.M., Gershkovich, P. (2016). Dietary Fats and Pharmaceutical Lipid Excipients Increase Systemic Exposure to Orally Administered *Cannabis* and *Cannabis*-based Medicine. *American Journal of Translational Research, 8*(8), 3448-3459.

18. Dhamija, R., Eckert, S., &Wirrell, E. (2013). Ketogenic Diet. *The Canadian Journal of Neurological Science, 40*, 158-167. https://doi.org/10.1017/S0317167100013676
19. Sills, M.A., Forsythe, W.I., Haidukewych, D., Macdonald, A., &The Medium Chain Robinson, M. (1986). Triglyceride diet and intractable epilepsy. *Archives of Diseases in Childhood, 61*, 1168-1172. http://dx.doi.org/10.1136/adc.61.12.1168
20. Mitchell, J.W., Seri, S., & Cavanna, A.E. (2012). Pharmacotherapeutics and Non-Pharmacological Options for Refractory and Difficult to Treat Seizures. *Journal of Central Nervous System Disease, 4*, 105-115. doi:10.4137/JCNSD.S8315
21. Wheless, J.W. (2001). The ketogenic diet: an effective medical therapy with side effects. *Journal of Child Neurology, 16 (9)*, 633-635.
22. Mackie, K. (2006). Mechanisms of CB1 receptor signalling: endocannabinoid modulation of synaptic strength. *International Journal of Obesity, 30,* 19-23. doi:10.1038/sj.ijo.0803273
23. Ashton, C.H. (2001). Pharmacology and effects of *cannabis*: a brief review. *British Journal of Psychiatry, 178*, 101-106. https://doi.org/10.1192/bjp.178.2.101
24. Ibsen. M.S., Connor, M. & Glass M. (2017). Cannabinoid CB_1 and CB_2 receptor signalling and bias. *Cannabis And Cannabinoid Research, 2*, 48-60. doi: 10.1089/can.2016.0037
25. Turnham, R.E. & Scott, J.D. (2016). Protein kinase A catalytic subunit isoform PRKACA; History, function and physiology. *Gene, 577(2)*, 101-108. https://doi.org/10.1016/j.gene.2015.11.052
26. Elphick, M.R. & Egertová, M. (2001). The Neurobiology and Evolution of Cannabinoid Signalling. *Philisophical Transactions: Biological Sciences, 356*, 381-408. doi: 10.1098/rstb.2000.0787
27. Leboffe, L., Masi, A. D., Trezza, V., Polticelli, F., & Ascenzi, P. (2017). Human serum albumin: A modulator of cannabinoid drugs. *IUBMB Life, 69*(11), 834-840. doi:10.1002/iub.1682
28. Mechoulam, R., Fride, E., & Di Marzo, V. (1998). Endocannabinoids. *European Journal of Pharmacology, 359*, 1-18. https://doi.org/10.1016/S0014-2999(98)00649-9

29. Vandevoorde, S., Saha, B., Mahadevan, A., Razdan, R.K., Pertwee, R.G., Martin, B.R., Fowler, C.J. (2005). Influence of the degree of unsaturation of the acyl side chain upon the interaction of analogues of 1-arachidonoylglycerol with monoacylglycerol lipase and fatty acid amide hydrolase. *Biochemical and Biophysical Research Communication, 337 (1)*, 104-109. https://doi.org/10.1016/j.bbrc.2005.09.015
30. Fowler, C.J. (2013). Transport of Endocannabinoids Across the Plasma Membrane and Within the Cell. *The Federation of European Biochemical Societies Journal, 280*(90), 1895-1904. https://doi.org/10.1111/febs.12212
31. Elmes, M.W., Kaczocha, M., Berger, W.T., Leung, K.N., Ralph, B.P., Wang, L., Sweeney, J.M., Miyauchi, J.T., Tsirka, S.E., Ojima, I., Deustch D.G. (2015). Fatty Acid-Binding Proteins (FABPs) are Intracellular Carriers for Δ^9-Tetrahydrocannabinol (THC) and Cannabidiol (CBD). *The Journal of Biological Chemistry, 290 (14)*, 8711-8721. doi:10.1074/jbc.M114.618447
32. Pertwee, R.G. (2005). Pharmacological Actions of Cannabinoids, *Handbook of Experimental Pharmacology, 168*, 1-51.
33. Grotenhermen, F. & Müller-Vahn, K. (2016). Medicinal Uses of Marijuana and Cannabinoids. *Critical Reviews of Plant Sciences, 35(5-6)*, 378-405. doi:10.1080/07352689.2016.1265360
34. Grotenhermen, F. (2003). Pharmacokinetics and Pharmacodynamics of Cannabinoids. *Clinical Pharmacokinetics, 42*(4), 327-360. doi:10.2165/00003088-200342040-00003
35. Macoveiciuc, A. (2017, November 03). Known and potential side effects of cannabidiol. Retrieved February 05, 2018, from https://elixinol.com/blog/known-and-potential-side-effects-of-cannabidiol-cbd/
36. Devinsky, O., Cilio, M.R., Cross, H., Fernandez-Ruiz, J., French, J., Hill, C., Katz, R., Di Marzo V., Jutras-Aswad, D., Notcutt, W.G., Martinez-Orgado, J., Robson, P.J., Rorhback, B.G., Thiele, E., Whalley, B., & Friedman, D. (2014). Cannabidiol: Pharmacological and Potential Therapeutic Role in Epilepsy and Other Neuropsychiatric Disorders. *Epilepsia, 55*(6), 791-802. doi: 10.1111/epi/12631

37. Laun, A. S., & Song, Z. (2017). GPR3 and GPR6, novel molecular targets for cannabidiol. *Biochemical and Biophysical Research Communications, 490*(1), 17-21. doi:10.1016/j.bbrc.2017.05.165
38. Console-Bram, L., Marcu, J., & Abood, M. E. (2012). Cannabinoid receptors: nomenclature and pharmacological principles. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 38*(1), 4-15. doi:10.1016/j.pnpbp.2012.02.009
39. Zuardi, A.W. (2008). Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action. *Revista Brasileira de Psiquiatria, 30(3)*, 271-280. http://dx.doi.org/10.1590/S1516-44462008000300015
40. Adamowicz, P., Gieron, J., Gil, D., Lechowicz, W., Skulska, A., & Tokarczyk, B. (2017). The effects of synthetic cannabinoid UR-144 on the human body- A review of 39 cases. *Forensic Science International, 273*, 18-21. https://doi.org/10.1016/j.forsciint.2017.02.031
41. Camp, N.E. (2011). Synthetic Cannabinoids. *Journal of Emergency Nursing, 37 (3)*, 292-293. https://doi.org/10.1016/j.jen.2011.01.006
42. Cao, M.H., Li, Y.Y., Xu, J., Feng, Y.J., Lin, X.H., Li, K., Han, T., & Chen, C.J. (2012). Cannabinoid HU210 protects isolated rat stomach against impairment caused by serum of rats with experimental acute pancreatitis. *PLoS One, 7 (12)*, 1-9. doi:10.1371/journal.pone.0052921
43. Ossato, A., Vigolo, A., Trapella, C., Seri, C., Rimondo, C., Serpelloni, G., & Marti, M. (2015). JWH-018 impairs sensorimotor functions in mice. *Neuroscience, 300*, 174-188. https://doi.org/10.1016/j.neuroscience.2015.05.021
44. Lauritsen, K.J. & Rosenberg, H. (2016). Comparison of outcome expectanciesfor synthetic cannabinoids and botanical marijuana. *The American Journal of Drug and Alcohol Abuse, 42 (4)*, 377-384. https://doi-org.cyber.usask.ca/10.3109/00952990.2015.1135158
45. Strouse, T. B. (2016). Cannabinoids in Medical Practice. *Cannabis and Cannabinoid Research,1*(1), 38-43. doi:10.1089/can.2015.0010

46. Barrus, D.G., Capogrossi, K.L., Cates, S.C., Gourdet C.K., Peiper N.C., Novak, S.P., Lefever, T.W., & Wiley, J.L. (2016). Tasty THC: Promises and Challenges of *Cannabis* Edibles. *Methods Report,* 1-22.
47. Roncero, C., Valriberas-Herrero, I., Mezzatesta-Gava, M., Villegas, J.L., Aguilar, L., & Grau-Lopez, L. (2020). *Cannabis* use during pregnancy and its relationship with fetal developmental outcomes and psychiatric disorders. A systematic review. *Reproductive Health, 17*(25), https://doi.org/10.1186/s12978-020-0880-9.
48. Dong, C., Chen, J., Harrington, A., Yaragydri Vinoid, K., Hedge, M.L., & Hedge, V.L. (2019). Cannabinoid exposure during pregnancy and its impact on immune function. *Cellular and Molecular Life Sciences, 76*, 729-743. https://doi.org/10.1007/s00018-018-2955-0.
49. Corsi, D.J., Hsu, H., Weiss, D., Fell, D.B., & Walker, M. (2019). Trends and correlates of *cannabis* use in pregnancy: a population-based study in Ontario, Canada from 2012-2017. *Canadian Journal of Public Health,* 110, 76-84. https://doi.org/10.17269/s41997-018-0148-0.
50. Dickson, B., Mansfield, C., Guiahi, M., Allshouse, A.A., Borgelt, L.M., Sheeder, J., Silver, R.M., & Metz, T.D. (2018). Recommendations from *Cannabis* Dispensaries About First-Trimester *Cannabis* Use. *Obstetrics and Gynecology*, 131(6), 1031-1038. doi:10.1097/AOG.0000000000002619.
51. Walker, O.S., Hooloway, A.C., & Raha, S. (2019). The role of the endocannabinoid system in female reproductive tissues. *Journal of Ovarian Research, 12*(3). https://doi.org/10.1186/s13048-018-0478-9.
52. Correa, F., Wolfson, M.L., Valchi, P., Aisemberg, J., & Franchi, A.M. (2016). Endocannabinoid system and pregnancy. *Reproduction, 152*, 191-200. DOI: 10.1530/REP-16-0167.
53. Feinshtein, V., Erez, O., Ben-Zvi, Z., Eshkoli, T., Sheizaf, B., Sheiner, E., & Holcberg, G. (2013). Cannabidiol enhances xenobiotic permeability through the human placental barrier by direct inhibition of breast cancer resistance protein: an ex vivo study. *American Journal of Obstetrics and Gynecology, 209*(573), 1-15. http://dx.doi.org/10.1016/j.ajog.2013.08.005.

54. Jacques S.C., Kingsbury, A., Henshcke, P., Chomchai, C., Clews, S., Falconer, J., Abdel-Latif, M.E., Feller, J.M., & Oei, J.L. (2014). *Cannabis*, the pregnany woman and her child: weeding out the myths. *Journal of Perinatology, 34*, 417-424.
55. Campbell, C.T., Phillips, M.S., & Manasco, K. (2017). Cannabinoids in Pediatrics. *Journal of Pediatric Pharmacology and Therapeutics, 22*(3), 176-185. DOI: 10.583/1551-6776-22.3.176
56. Wang, G.S. (2017). Pediatric Concerns Due to Expanded *Cannabis* Use: Unintended Consequences of Legalization. *Journal of Medical Toxicology, 13*, 99-105. DOI 10.1007/s13181-016-0552-x
57. Soltesz, I., Alger, B.E., Kano, M., Lee, S., Lovinger, D.M., Ohno-Shosaku T., & Watanabe, M. (2015). Weeding out bad waves: towards selective cannabinoid circuit control in epilepsy. *Nature Reviews Neuroscience, 16*(5), 264-277. doi: 10.1038/nrn3937.
58. Devinsky, O., Marsh, E., Friedman, D., Thiele, E., Laux, L., Sullivan, J., Miller, I., Flamini, R., Wilfong, A., Filloux, F., Wong, M., Tilton, N., Bruno, P., Bluvstein, J., Hedlund, J., Kamens, R., Maclean, J., Nangia, S., Singhal, N.S., Wilson, C.A., Patel, A., & Cilio, M.R. (2016). Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *Lancet Neurology, 15*(3), 270-278. doi: 10.1016/S1474-4422(15)00379-8
59. Devinsky, O., Cross, H., Laux, L., Marsh, E., Miller, I., Nabbout, R., Scheffer, I.E., Thiele, E.A., & Wright, S. (2017). Trial of Cannabidiol for Drug-Resistant Seizures in Dravet Syndrome. *The New England Journal of Medicine, 376*(21), 2011-2020.
60. Thiele, E.A., Marsh, E.D., French, J.A., Mazurkiewicz-Beldzinska, M., Benbadis, S.R., Joshi, C., Lyons, P.D., Taylor, A., Roberts, C., & Sommerville, K. (2018). Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. *The Lancet, 391*(10125), 1085-1096. http://dx.doi.org/10.1016/S0140-6736(18)30136-3

61. Russo, E.B. (2011). Taming THC: potential *cannabis* synergy and phytocannabinoid-terpenoid entourage effects. *British Journal of Pharmacology, 163*(7), 1344-1364. doi: 10.1111/j.1476-5381.2011.01238.x.

62. McCoy, B., Wang, L., Zak, M., Al-Mehmadi, S., Kabir, N., Alhadid, K., McDonald, K., Zhang, G., Sharma, R., Whitney, R., Sinopoli, K., & Snead, O.C. (2018). A prospective open-label trial of a CBD/THC *cannabis* oil in dravet syndrome. *Annals of Clinical and Translational Neurology, 5*(9), 1077-1088. doi: 10.1002/acn3.621

63. Tzadok, M., Uliel-Siboni, S., Linder, I., Kramer, U., Epstein, O., Menascu, S., Nissenkorn, A., Yosef, O.B., Hyman, E., Granot, D., Dor, M., Lerman-Sagie, T., & Ben-Zeev, B. (2016). CBD-enriched medical *cannabis* for intractable pediatric epilepsy: The current Israeli experience. *Seizure, 35*, 41-44. http://dx.doi.org/10.1016/j.seizure.2016.01.004

64. Hausman-Kedem, M., Menascu, S., & Kramer, U. (2018). Efficacy of CBD-enriched medical *cannabis* for treatment of refractory epilepsy in children and adolescents- An observational, longitudinal study. *Brain & Development,* 4, 544-551. https://doi.org/10.1016/j.braindev.2018.03.013

65. Huntsman, R.J., Tang-Wai, R., & Shackelford, A.E. (2020). *Cannabis* for Pediatric Epilepsy. *Journal of Clinical Neurophysiology, 37*(1), 1-8. DOI 10.1097/WNP.0000000000000641

66. Elliot, J., DeJean, D., Clifford, T., Coyle, D., Potter, B.K., Skidmore, B., Alexander, C., Repetski, A.E., Shukla, V., McCoy, B., & Wells, G.A. (2018). *Cannabis*-based products for pediatric epilepsy; A systematic review. *Epilepsia, 2019*(60), 6-19. DOI: 10.1111/epi.14608

67. Reithmeier, D., Tang-Wai, R., Seifrt, B., Lyon, A.W., Alcorn, J., Acton, B., Corley, S., Prosser-Loose, E., Mousseau, D.D., Lim, H.J., Tellez-Zenteno, J., Huh, L., Leung, E., Carmant, L., and Huntsman, R.J. (2018). The protocol for the Cannabidiol in children with refractory epileptic encephalopathy (CARE-E) study: a phase 1 dosage escalation study. *BMC Pediatrics, 18*(221). https://doi.org/10.1186/s12887-018-1191-y.

68. Sadhir, M. (2016). Pharmacology of *Cannabis*. *Journal of Pain Management, 9(4)*, 375-379.

69. Bridgeman, M.B. & Abazia, D.T. (2017). Medicinal *cannabis*: history, pharmacology, and implications for the acute care setting. *Pharmacy and Therapeutics, 42(3)*, 180-188.

70. Millar, S., Stone, N.L., Yates, A.S., & O'Sullivan, S.E. (2018). A Systematic Review on the Pharmacokinetics of Cannabidiol in Humans. *Frontiers in Pharmacology, 9*(1365). doi: 10.3389/fphar.2018.01365.

71. Lucas, C.J., Galettis, P., & Schneider, J. (2018). The pharmacokinetics and the pharmacodynamics of cannabinoids. *British Journal of Clinical Pharmacology, 84*(11), 2477-2482. DOI:10.1111/bcp.13710.

72. Huestis, M.A. (2007). Human Cannabinoid Pharmacokinetics. *Chemistry & Biodiversity, 4*(8), 1170-1804. doi:10.1002/cbdv.200790152.

73. Nagao,M., Nakano, Y., Tajima, M., Sugiyama, E., Sato, V.H., Inada, M., & Sato, H. (2020). Nonlinear Disposition and Metabolic Interactions of Cannabidiol Through CYP3A Inhibition *In Vivo* in Rats. *Cannabis and Cannabinoid Research.* DOI: 10.1089/can.2019.0098.

74. Calapai, F., Cardia, L., Sorbara, E.E., Navarra, M., Gangemi, S., Calapai, G., & Mannucci, C. (2020). Cannabinoids, Blood-Brain Barrier, and Brain Disposition. *Pharmaceutics, 12*(265). doi:10.3390/pharmaceutics12030265.

75. Bohnert, T. & Gan, L.S. (2013). Plasma Protein Binding: From Discovery to Development. *Journal of Pharmaceutical Sciences, 102 (9)*, 2953-2994. doi: 10.1002/jps.23614

76. Singh, S.S. & Mehta, J. (2016). Measurement of drug-protein binding by immobilized human serum albumin-HPLC and comparison with ultrafiltration. *Journal of Chromatography B, 837 (1-2)*, 108-116. https://doi.org/10.1016/j.jchromb.2006.02.053

77. Skopp, G., Potsch, L., Mauden, M., & Richter, B. (2002). Partition coefficient, blood to plasma ratio, protein binding and short-term stability of 11-nor-Δ^9-caroxy tetrahydrocannabinol glucuronide. *Forensic Science International, 126*, 17-22.

78. Goto, K., Shiihara, H., Eguchi, H., Utsumi, Y., Kuromori, S., & Ariizumi, M. (1980). Immunoglobulin G (IgG) in Cerebrospinal Fluid in Children with Febrile Convulsion and Epilepsy. *Psychiatry and Clinical Neurosciences, 34 (3)*, 382. doi:10.1111/j.1440-1819.1980.tb01590.x

79. Greineisen, W.E. & Turner, H. (2010). Immunactive effects of cannabinoid: Considerations for the therapeutic use of cannabinoid receptor agonists and antagonists. *International Immunopharmacology, 10*, 547-555. doi:10.1016/j.intimp.2010.02.012

80. Kwiterovich, P.O., Vining, E.P.G., Pyzik, P., Skolasky, R., Freeman, J.M. (2003). Effect of a High-Fat Ketogenic Diet on Plasma Levels of Lipids, Lipoproteins, and Apolipoproteins in Children. *Journal of American Medical Association, 290*(7), 912-920. doi:10.1001/jama.290.7.912

81. Wahlqvist, M., Nilsson, I.M., Sandberg, F., & Agurell, S. (1970). Binding of ∆Tetrahydrocannabinol to Human Plasma Proteins. *Biochemical Pharmacology, 19*, 2579-2584.

82. de Boer, T. and Meijering, H. (2019). Equilibrium Dialysis, Ultracentrifugation and Ultrafiltration in LC-MS Bioanalysis. *Sample Preparation in LC-MS Bioanalysis*, W. Li, W. Jian, and Y. Fu (Eds), 45-51. doi:10.1002/9781119274315.ch3

83. Karschner, E.L, Darwin, D.W., Goodwin, R.S., Wright, S. & Huestis, M.A. (2010). Plasma Cannabinoid Pharmacokinetics Following Controlled Oral Δ^9-Tetrahydrocannabinol and Oromucosal *Cannabis* Extract Administration. *Clinical Chemistry, 57*, 66-75. doi: 10.1373/clinchem.2010.152439

84. Mazur, A., Lichti, C.F., Prather, P.L., Zielinska, A.K., Bratton, S.M., Gallus-Zawada, A., Finel, M., Miller, G.P., Radominska-Pandya, A., & Moran, J.H. (2009). Characterization of Human Hepatic and Extrahepatic UDP-Glucuronosyltransferase Enzymes Involved in the Metabolism of Classic Cannabinoids. *Drug Metabolism and Disposition, 37*(10), 1496-1504. doi:10.1124/dmd.109.026898.

85. Sharma, P., Murthy, P., & Bharanth, S. (2012). Chemistry, Metabolism and Toxicology of Cannabis: Clinical Implications. *Iranian Journal of Psychiatry, 7*(4), 149-156.

86. Ujvary I., & Hanus, L. (2016). Human Metabolites of Cannabidiol: A Review on Their Formation, Biological Activity, and Relevance in Therapy. *Cannabis and Cannabinoid Research, 1*(1), 90-101. DOI: 10.1089/can.2015.0012

87. Yamaori, S., Ebisawa, J., Okushima, Y., Yamamoto, I., & Watanabe, K. (2011). Potent inhibition of human cytochrome P450 3A isoforms by cannabidiol: Role of phenolic hydroxyl groups in the resorcinol moiety. *Life Sciences, 88*, 730-736. doi:10.1016/j.lfs.2011.02.017.

88. Bergamaschi, M., Barnes, A., Queiroz, R.H.C., Hurd, Y.L., & Huestis, M.A. (2013). Impact of enzymatic and alkaline hydrolysis on CBD concentration in urine. *Analytical and Bioanalytical Chemistry,* 405, 4679-4689. DOI 10.1007/s00216-013-6837-x

89. Scuderi, C., De Filippis, D., Iuvone, T., Blasio, A., Steardo, A., & Esposito, G. (2008). Cannabidiol in Medicine: A Review of its Therapeutic Potential in CNS Disorders. *Phytotherapy Research, 23(5)*, 597-602. doi: 10.1002/ptr.2625

90. Alcorn, J. & McNamara, P.J. (2003). Pharmacokinetics in the newborn. *Advanced Drug Delivery Reviews, 55*, 667-686. doi:10.1016/S0169-409X(03)00030-9

91. Matalova, P., Urbanek, K., & Anzenbacher, P. (2016). Special features of pharmacokinetics in children. *Drug Metabolism Reviews, 48*(1), 70-79. doi:10.3109/03602532.2015.1135941

92. Fernandez, E., Perez, R., Hernandez, A., Tejada, P., Arteta, M., & Ramos, J.T. (2011). Factors and mechanisms for Pharmacokinetic Differences between Pediatric Population and Adults. *Pharmaceutics, 3(1)*, 53-72. doi:10.3390/pharmaceutics3010053

93. Bruckner, J.V. (1999). Differences in Sensitivity of Children and Adults to Chemical Toxicity: The NAS Panel Report. *Regulatory Toxicology and Pharmacology, 31*, 280-285. doi:10.1006/rtph.2000.1393

94. Lu, H. & Rosenbaum, S. (2014). Developmental Pharmacokinetics in Pediatric Populations. *Journal of Pediatric Pharmacology and Therapeutics, 19*(4), 262-276.

95. Arain, M., Haque M., Johal, L., Mathur P., Nel, W., Rais, A., Sandhu, R., & Sharma, S. (2013). Maturation of the adolescent brain. *Neuropsychiatric Disease and Treatment, 9*, 449-461. http://dx.doi.org/10.2147/NDT.S39776

96. Yao, L.P. & Jose P.A. (1995). Developmental renal hemodynamics. *Pediatric Nephrology, 9*, 632-637.

97. Citti, C., Ciccarella, G., Braghiroli, D., Parenti C., Vandrello, M.A., & Cannazza (2016). Medicinal *cannabis*: Principal cannabinoids concentration and their stability evaluated by a high performance liquid chromatography coupled to diode array and quadrupole time of flight mass spectrometry method. *Journal of Pharmaceutical and Biomedical Analysis, 128*(5), 201-209. https://doi.org/10.1016/j.jpba.2016.05.033

98. Zivovinovic, S., Alder, R., Allenspach, M.D., & Steuer, C. (2018). Determination of cannabinoids in *Cannabis* sativa L. samples for recreational, medical, and forensic purposes by reversed-phase liquid chromatography-ultraviolet detection. *Journal of Analytical Science and Technology, 9*(27). https://doi.org/10.1186/s40543-018-0159-8

99. Ciolini, L.A., Ranieri, T.L., & Taylor, A.M. (2018). Commercial *cannabis* consumer products part 1: GC-MS qualitative analysis of *cannabis* cannabinoids. *Forensic Science International, 289*, 429-437. https://doi.org/10.1016/j.forsciint.2018.05.032

100. Booth, J.K. & Bohlmann, J. (2019). Terpenes in *Cannabis* sativa- From plant genome to humans. *Plant Science, 284*, 67-72. https://doi.org/10.1016/j.plantsci.2019.03.022.

101. Shapira, A., Berman, P., Futoran, K., Gubrman, O., & Meiri, D. (2019). Tandem Mass Spectrometric Quantification of 93 Terpenoids in *Cannabis* Using Static Headspace Injections. *Analytical Chemistry, 91*, 11425-11432. DOI: 10.1021/acs.analchem.9b02844

102. Dryburgh, L.M., Bolan, N.S., Grof, C.P.L., Galettis, P., Schneider, J., Lucas, C.J., & Martin, J.H. (2018). *Cannabis* contaminants: sources, distribution, human toxicity, and pharmacologic effects. *British Journal of Clinical Pharmacology, 84*(11), 2468-2476. doi: 10.1111/bcp.13695

103. Brown A.K., Xia, Z., Bulloch, P., Idowu, I., Francisco, O., Stetfeld, J., Stout, J., Zimmer, J., Marvin, C., Letcher, R.J., & Tomy, G. (2019). Validated quantitative *cannabis* profiling for Canadian regulatory compliance- Cannabinoids, aflatoxins, and terpenes. *Analytica Chimica Acta, 1088*, 79-88. https://doi.org/10.1016/j.aca.2019.08.042

104. Craven, C.B., Wawryk, N., Jiang, P., Liu, Z., & Li, X.F. (2019). Pesticides and trace elements in *cannabis*: Analytical and environmental challenges and opportunities. *Journal of Environmental Sciences, 85*, 82-93. https://doi.org/10.1016/j.jes.2019.04.028

105. Atapattu, S.N. & Johnson, K.R.D. (2020). Pesticide analysis in *cannabis* products. *Journal of Chromatography A, 1612*. https://doi.org/10.1016/j.chroma.2019.460656

106. Schwope, D.M., Karschner, E.L., Gorelick, D.A., & Huestis, M.A. (2011). Identification of Recent *Cannabis* Use: Whole-Blood and Plasma Free and Glucuronidated Cannabinoid Pharmacokinetics following Controlled Smoked *Cannabis* Administration. *Clinical Chemistry, 57*(10), 1406-1414. DOI: 10.1373/clinchem.2011.171777

107. Desrosier, N.A., Lee, D., Concheiro-Guisan, M., Scheidweiler, K.B., Gorelick, D.A., & Huestis, M.A. (2014). Urinary Cannabinoid Disposition in Occasional and Frequent Smokers: Is THC-Glucuronide in Sequential Urine Samples a Marker of Recent Use in Frequent Smokers? *Clinical Chemistry, 60*(2), 361-372.

108. Scheidweiler, K.B., Desrosier, N.A., & Huestis, M.A. (2012). Simultaneous quantification of free and glucuronidated cannabinoids in human urine by liquid chromatography tandem mass spectrometry. *Clinica Chimica Acta, 413*, 1839-1847. doi:10.1016/j.cca.2012.06.034

109. Huestis, M.A. & Cone, E.J. (2004). Relationship of Δ^9-Tetrahydrocannabinol Concentrations in Oral Fluid and Plasma after Controlled Administration of Smoked *Cannabis*. *Journal of Analytical Toxicology, 28*, 394-399.

110. Lee, D., Milman, G., Barnes, A.J., Goodwin, R.S., Hirvonen, J., & Huestis M.A. (2011). Oral Fluid Cannabinoids in Chronic, Daily *Cannabis* Smokers during

Sustained, Monitored Abstinence. *Clinical Chemistry, 57*(8), 1127-1136. https://doi.org/10.1373/clinchem.2011.164822

111. Lee, D. & Huestis, M.A. (2013). Current knowledge on cannabinoids in oral fluid. *Drug Testing and Analysis, 6*(1-2), 88-111. https://doi.org/10.1002/dta.1514
112. Milman, G., Schwope, D.M., Gorelick, D.A., & Huestis, M.A. (2012). Cannabinoids and metabolites in expectorated oral fluid following controlled smoked *cannabis*. *Clinica Chemica Acta, 413*(7-8), 765-770. https://doi.org/10.1016/j.cca.2012.01.011
113. Vandrey, R., Herrmann, E.S., Mitchell, J.M., Bigelow, G.E., Flegel, R., LoDico, C., & Cone, E.J. (2017). Pharmacokinetic Profile of Oral *Cannabis* in Humans: Blood and Oral Fluid Disposition and Relation to Pharmacodynamic Outcomes. *Journal of Analytical Toxicology, 2017*(41), 83-99. doi: 10.1093/jat/bkx012
114. Cirimele, V., Kintz, P., & Mangin, P. (1995). Testing human hair for *cannabis*. *Forensic Science International, 70*(3), 175-182. https://doi.org/10.1016/0379-0738(94)01615-C
115. Auwarter, V., Wahlfarth, A., Traver, J., Thieme, D., & Weinmann, W. (2010). Hair analysis for ∆9-tetrahydrocannabinolic acid A—New insights into the mechanism of drug incorporation of cannabinoids into hair. *Forensic Science International, 196*(3), 10-13. https://doi.org/10.1016/j.forsciint.2009.12.023
116. Moosmann, B., Roth, N., & Auwarter, V. (2015). Finding cannabinoids in hair does not prove *cannabis* consumption. *Scientific Reports.* DOI: 10.1038/srep14906
117. Schwilke, E.W., Schwope, D.M., Karschner, E.L., Lowe, R.H., Darwin, W.D., Kelly, D.L., Goodwin, R.S., Gorelick, D.A., & Huestis, M.A. (2009). ∆9-Tetrahydrocannabinol (THC), 11-Hydroxy-THC, and 11-Nor-9-carboxy-THC Plasma Pharmacokinetics during and after Continuous High-Dose Oral THC. *Clinical Chemistry, 55*(12), 2180-2189. https://doi.org/10.1373/clinchem.2008.122119
118. Hughes, N.C., Bajaj, N., Fan, J., & Wong, E.Y.K. (2009). Assessing the matric effects of hemolyzed samples in bioanalysis. *Bioanalysis, 1*(6), 1057-1066. doi: 10.4155/bio.09.91.

119. Scheidweiler, K.B., Schwope, D.M., Karschner, E.L., Desrosier, N.A., Gorelick, D.A., & Huestis, M.A. (2013). In Vitro Stability of Free and Glucuronidated Cannabinoids in Blood and Plasma Following Controlled Smoked *Cannabis*. *Clinical Chemistry, 59*(7), 1108-1117. doi:10.1373/clinchem.2012.201467.

120. Mercolini, L., Mandrioli, R., Sorella, V., Somaini, L., Giocondi, D., Serpelloni, G., & Raggi, M.A. (2013). Dried blood spots: liquid chromatography-mass spectrometry analysis of ∆9-tetrahydrocannabinol and its main metabolites. *Journal of Chromatography A, 1271*(1), 33-40. doi: 10.1016/j.chroma.2012.11.030.

121. Protti, M., Rudge, J., Sberna, A.E., Gerra, G., & Mercolini, L. (2017). Dried haematic microsamples and LC-MS/MS for the analysis of natural and synthetic cannabinoids. *Journal of Chromatography B, 1044-1045*, 77-86. http://dx.doi.org/10.1016/j.jchromb.2016.12.038

122. Kyriakou, C., Marchei, E., Scaravelli, G., Garcia-Algar, O., Supervia, A., & Graziano, S.(2016). Identification and quantification of psychoactive drugs in *whole blood using dried blood spot (DBS) by ultra-performance liquid* chromatography tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 128*, 53-60. http://dx.doi.org/10.1016/j.jpba.2016.05.011

123. Nie, B., Henion, J., & Ryona, I. (2019). The Role of Mass Spectrometry in the *Cannabis* Industry. *Journal of the American Society for Mass Spectrometry, 30*, 719-730. DOI: 10.1007/s13361-019-02164-z

124. De Kesel, P.M.M., Capiau, S., Lambert, W.E., & Stove, C.P. (2014). Current strategies for coping with the hematocrit problem in dried blood spot analysis. *Bioanalysis 6*(14), 1871-1874. doi:10.4155/BIO.14.151

125. Zakaria, R., Allen, K.J., Koplin, J.J., Roche, P., & Greaves, R.F. (2016). Advantages and Challenger of Dried Blood Spot Analysis by Mass Spctrometry Across the Total Testing Process. *Electronic Journal of the International Federation of Clinical Chemistry and Laboratory Medicine, 27*(4), 288-317.

126. Stove, C.P., Ingels, A.S., De Kesel, P.M., & Lambert, W.E. (2012). Dried blood spots in toxicology: from cradle to the grave? *Critical Reviews in Toxicology, 42*(3), 230-243. doi: 10.3109/10408444.2011.650790.
127. Avard, D., Vallance, H., Greenberg, C., Laberge, C., Kharaboyan, L., & Plant, M. (2006). Variability in the Storage and Use of Newborn Dried Bloodspots in *Canada: Is it Time for National Standard? Genomics, Society, and Policy, 2(3),* 80-95.
128. Buscher, B., Laasko, S., Mascher, H., Pusecker, K., Doig, M., Dillen, L., Wagner-Redeker, W., Pfeifer, T., Delrat, P., & Timmerman, P. (2014). Bioanalysis for plasma protein binding studies in drug discovery and drug development: views and recommendations of the European Bioanalysis Forum. *Bioanalysis, 6*(5), 673-682. doi: 10.4155/bio.13.338.
129. Klausner, H.A., Wilcox, H.G., & Dingell, J.V. (1975). The use of zonal ultracentrifugation in the investigation of the binding of Δ^9-tetrahydrocannabinol by plasma lipoproteins. *Drug Metabolism and Disposition, 3*(4), 314-319.
130. Hunt, C.A. & Jones, R.T. (1980). Tolerance and Disposition of Tetrahydrocannabinol in Man. *Journal of Pharmacology and Experimental Therapeutics, 215*(1), 35-44.
131. Sethi, P.K., Muralidhara, S., Bruckner, J.V., & White, C.A (2014). Measurement of plasma protein and lipoprotein binding of pyrethroids. *Journal of Pharmacology and Toxicology Methods, 70*(1), 106-111. doi: 10.1016/j.vascn.2014.06.002.
132. Waters, L.L. (1964). The Extraction of Lipids from Human Plasma or Serum by 2-Octanol. *Yale Journal of Biology and Medicine, 37*(3), 204-210.
133. Polson, C., Sarkar, P., Incledon, B., Raguvaran, V., & Grant, R. (2003). Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B, 785*, 263-275.
134. Peschel, W. & Politi, M. (2015). ^{1}H NMR and HPLC/DAD for *Cannabis* sativa L. chemotype distinction, extract profiling and specification. *Talanta, 140*(1), 150-165. doi: 10.1016/j.talanta.2015.02.040

135. Koskela, H., Hakala, U., Loiske, L., Vanninen, P., & Szilvay, I. (2011). Separation and structural characterization of a synthetic cannabinoid found in herbal product using off-line LC-DAD-NMR. *Analytical Methods, 10*(3), 2307-2312. DOI: 10.1039/c1ay05287j

136. Hewavitharana, A.K., Golding, G., Tempany, G., King, G., & Holling, N. (2005). Quantitative GC-MS Analysis of Δ^9-Tetrahydrocannabinol in Fiber Hemp Varieties. *Journal of Analytical Toxiology, 29*(4), 258-261. DOI: 10.1093/jat/29.4.258

137. Cardenia, V., Toschi, T.G., Scappini, S., Rubino, R.C., & Rodriguez-Estrada, M.T. (2018). Development and validation of a Fast gas chromatography method for the determination of cannabinoids in *Cannabis sativa* L. *Journal of Food and Drug Analysis, 26*(4), 1283-1292. https://doi.org/10.1016/j.jfda.2018.06.001

138. Wang, M., Wang, Y.H., Avula, B., Radwan, M.M., Wanas, A.S., van Antwerp, J., Parcher, J.F., ElSohly, M.A.., & Khan I.A. (2016). Decarboxylation Study of Acidic Cannabinoids: A Novel Approach Using Ultra-High-Performance Supercritical Fluid Chromatography/Photodiode Array-Mass Spectrometry. *Cannabis and Cannabinoid Research, 1*(1), 262-271. doi: 10.1089/can.2016.0020

139. Choi, H., Heo, S., Choe, S., Yang, W., Park, Y., Kim, E., Chung, H., & Lee, J. (2013). Simultaneous analysis of synthetic cannabinoids in the materials seized during drug trafficking using GC-MS. *Analytical and Bioanalytical Chemistry, 405*, 3937-3944. DOI 10.1007/s00216-012-6560-z

140. Fialkov, A.B., Gordin, A., & Amirav, A. (2003). Extending the range of compounds amenable for gas chromatography-mass spectrometric analysis. *Journal of Chromatography A, 991*, 217-240. doi:10.1016 /S0021-9673(03)00247-4

141. Citti, C., Bragiroli, D., Vandelli, M.A., & Cannazza, G. (2018). Pharmaceutical and biomedical analysis of cannabinoids: A critical review. *Journal of Pharmaceutical and Biomedical Analysis, 147*, 565-579. http://dx.doi.org/10.1016/j.jpba.2017.06.003

142. De Backer, B., Debrus, B., Lebrun, P., Theunis, L., Dubois, N., Decock, L., Verstraete, A., Hubert, P., & Charlier, C. (2009). Innovative development and

validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in *cannabis* plant material. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Lfe Sciences, 877*(32), 4115-4124. doi: 10.1016/j.jchromb.2009.11.004

143. Patel, B., Wene, D., & Fan, Z. (2017). Qualitative and quantitative measurement of cannabinoids in *cannabis* using modified HPLC/DAD method. *Journal of Pharmaceutical and Biomedical Analysis, 146*(30), 15-23. https://doi.org/10.1016/j.jpba.2017.07.021

144. Aizpurua-Olaizola, O., Zarandona, I., Ortiz, L., Navarro, P., Etxebarria, N., & Usobiaga, A. (2017). Simultaneous quantification of major cannabinoids and metabolites in human urine and plasma by HPLC-MS/MS and enzyme-alkaline hydrolysis. *Drug Testing and Analysis, 9*(4), 626-633. doi: 10.1002/dta.1998

145. Jamey, C., Szwarc, E., Tracqui, A., & Ludes, B. (2008). Determination of Cannabinoids in Whole Blood by UPLC-MS-MS. *Journal of Analytical Toxicology, 32*(5), 349-354. https://doi.org/10.1093/jat/32.5.349

146. Hutter, S., Broecker, S., Kneisel, S., & Auwarter, V. (2012). Identification of the major urinary metabolites in man of seven synthetic cannabinoids of the aminoalkylindole type present as adulterants in ‘herbal mixtures’ using LC-MS/MS techniques. *Journal of Mass Spectrometry, 47*(1), 54-65. https://doi.org/10.1002/jms.2026

147. Snyder, L.R., Kirkland, J.J., and Dolan, J.W. (2010). *Introduction to modern liquid chromatography*. Oxford: Wiley-Blackwell.

148. Kazakevich, Y.V. and LoBrutto, R. (2007). *HPLC for Pharmaceutical Scientists*.Wiley-Interscience.

149. Honours, J.W. (2011). Development and Validation of Quantitative Assay Based on Tandem Mass Spectrometry. *Annals of Clinical Biochemistry,* 48, 97-111. doi:10.1258/acb.2010.010176

150. Ho, C.S., Lam, C.W.K., Chan, M.H.M., Cheung, R.C.K., Law, L.K., Lit, L.C.K., Ng, K.F., Suen, M.W.M., and Tai, H.L. (2003). Electrospray Ionization Mass Spectrometry: Principles and Clinical Applications. *Clinical Biochemistry Reviews,* 24, 3-12.

151. Food and Drug Administration (FDA): Guidance for Industry: Bioanalytical Method Validation. (2018). https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf

152. Briscoe, C., Stiles, M.R., & Hage, D.S. (2007). System suitability in bioanalytical LC/MS/MS. *Journal of Pharmaceutical and Biomedical Analysis, 44*(2), 484-491.

153. European Commission. (2002). https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF

154. Scientific Working Group for Forensic Toxicology (SWGTOX) (2013). Standard Practices for Method Validation in Forensic Toxicology. *Journal of Analytical Toxicology, 37*, 452-474.

155. Rainville, P.D., Smith, N.W., Cowan, D., & Plumb, R.S. (2012). Comprehensive investigation of the influence of acidic, basic, and organic mobile phase compositions on bioanalytical assay sensitivity in positive ESI mode LC/MS/MS. *Journal of Pharmaceutical and Bioanalytical Analysis, 59*, 138-150.

156. European Medicines Agency (EMA): Guidelines on bioanalytical method validation. (2011). https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf

157. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH): ICH guideline M10 on bioanalytical method validation. (2019). https://www.ema.europa.eu/en/documents/scientific-guideline/draft-ich-guideline-m10-bioanalytical-method-validation-step-2b_en.pdf

158. Vuong, S., Michel, D., Alcorn, J., Huntsman, R., Tang-Wai, R., & Lyon, A.W. (2018). BD Vacutainer Barricor Blood Collection Tube is the Tube of Choice for LC-MS/MS Analysis of Bioactive Cannabinoids in Plasma.

159. Guo, X. & Lankmayr, E. (2011). Phospholipid-based matrix effects in LC-MS bioanalysis. *Bioanalysis, 3*(4), 349-352. https://doi.org/10.4155/bio.10.213

160. Vozella, V., Zibardi, C., Ahmed, F., & Piomelli, D. (2019). Fast and Sensitive Quantification of Δ^9-Tetrahydrocannabinol and its Main Oxidative Metabolites by Liquid Chromatography/Tandem Mass Spectrometry. *Cannabis and Cannabinoid Research, 4*(2), 110-123. doi: 10.1089/can.2018.0075.

161. Palmgren, J.J., Monkkonen, J., Korjamo, T., Hassinen, A., & Auriola, S. (2006). Drug adsorption to plastic containers and retention of drugs in cultured cells under in vitro conditions. *European Journal of Pharmaceutics and Biopharmaceutics, 64*, 369-378. doi:10.1016/j.ejpb.2006.06.005
162. Fukazawa, T., Yamazaki, Y., & Miyamoto, Y. (2010). Reduction of non-specific adsorption of drugs to plastic containers used in bioassays or analyses. *Journal of Pharmacological and Toxicological Methods, 61*(3), 329-333.
163. Christophersen, A.S. (1986). Tetrahydrocannabinol stability in whole blood: plastic versus glass containers. *Journal of Analytical Toxicology, 10*(4), 129-131.
164. McGilveray, I.J. (2005). Pharmacokinetics of cannabinoids. *Pain Research and Management, 10*, 15-22.
165. Mechoulam, R., Parker, L.A., & Gallily, R. (2014). Cannabidiol: An Overview of Some Pharmacological Aspects. *The Journal of Clinical Pharmacology, 41*(1), 11-19.
166. Wilkinson, G.R. & Shand, D.G. (1975). A physiological approach to hepatic drug clearance. *Clinical Pharmacology & Therapeutics, 18*(4), 377-390.
167. Sanderson, S., Emery, J., & Higgins, J. (2005). CYP2C9 gene variants, drug dose, and bleeding risk in warfarin-treated patients: A HuGEnet systematic review and meta-analysis. *Genetics in Medicine, 7*(2), 97-104.
168. Yamaori, S., Kushihara, M., Koeda, K., Yamamoto, I., & Watanabe, K. (2013). Significance of CYP2C9 genetic polymorphism in inhibitory effect of Δ^9-tetrahydrocannabinol on CYP2C9 activity. *Forensic Toxicology, 31*, 70-75.
169. Johannessen, S. & Johannessen Landmark, C. (2010). Antiepileptic Drug Interactions- Principles and Clinical Implications. *Current Neuropharmacology, 8*(3), 254-267.
170. Gaston, T.E., Bebin, E.M., Cutter, G.R., Liu, Y., & Szaflarski, J.P. (2017). Interactions between cannabidiol and commonly used antiepileptic drugs. *Epilepsia, 58*(9), 1586-1592.
171. Anderson, L.L., Absalom, N.L., Abelev, S.V., Low, I.K., Doohan, P.T., Martin, L.J., Chebib, M., McGregor, I.S., & Arnold, J.C. (2019). Coadministered

cannabidiol and clobazam: Preclinical evidence for both pharmacodynamic and pharmacokinetic interactions. *Epilepsia, 60*, 2224-2234.

172. Kulig, K. (2017). Interpretation of Workplace Tests for Cannabinoids. *Journal of Medical Toxicology, 13*(1), 106-110.
173. Laprairie, R.B., Bagher, A.M., Kelly, M.E.M., & Denovan-Wright, E.M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB_1 receptor. *British Journal of Pharmacology*, *172*(20), 4790-4805. doi: 10.1111/bph.13250
174. Anderson, C.L., Evans, V.F., DeMarse, T.B., Febo, M., Johnson, C.R., Carney P.R. (2017). Cannabidiol for the Treatment of Drug-Resistant Epilepsy in Children: Current State of Research. *Journal of Pediatric Neurology, 15*(4), 143-150. doi: 10.1055/s-0037-1598109
175. Fride, E. (2008). Multiple Roles for the Endocannabinoid System During the Earliest Stages of Life: Pre- and Postnatal Development. *Journal of Neuroendocrinology, 20*(1), 75-81.
176. Meyer, H.C., Lee F.S., & Gee D.G. (2018). The Role of the Endocannabinoid System and Genetic Variation in Adolescent Brain Development. *Neuropsychopharmacology, 43*(1), 21-33.
177. Marscot, A. (2018). Pharmacokinetic Variability in Pediatrics and Intensive Care: Toward a Personalized Dosing Approach. *Journal of Pharmacy and Pharmaceutical Sciences, 21*, 354-362.
178. Ohlsson, A., Lindgren, J.E., Andersson, S., Agurell, S., Gillespie, H., & Hollister, L.E. (1986). Single-dose kinetics of deuterium-labelled cannabidiol in man after smoking and intravenous administration. *Biomedical & Environmental Mass Spectrometry, 13*(2), 77-83. doi: 10.1002/bms.1200130206
179. Ohlsson, A., Lindgren, J.E., Wahlen, A., Agurell, S., Hollister, L.E., & Gillespie, H.K. (1982). Single-dose of deuterium-labelled delta-1-tetrahydrocannabinol in heavy and light cannabis users. Biomedical Mass Spectrometry, 9(1), 6-10. DOI: 10.1002/bms.1200090103
180. Rowland M. (1984). Protein Binding and Drug Clearance. *Clinical Pharmacokinetics, 9*(1), 10-17.

181. Taylor, S. & Harker, A. (2006). Modifications of the ultrafiltration technique to overcome solubility and non-specific binding challenges associated with the measurement of plasma protein binding of corticosteroids. *Journal of Pharmaceutical and Biomedical Analysis, 41*(1), 299-303.

182. Katneni, K., Pham, T., Saunders, J., Chen, G., Patil, R., White, K.L., Abla, N., Chiu, F.C.K., Shackleford, D.M., & Charman, S.A. (2018). Using Human Plasma as an Assay Medium in Caco-2 Studies Improves Mass Balance for Lipophilic Compounds. *Pharmaceutical Research, 35*(201), 1-13. https://doi.org/10.1007/s11095-018-2493-3

183. Leito, I., Herodes, K., Huopolainen, M., Virro, K., Künnapas, A., Kruve, A., & Tanner, R. (2007). Towards the electrospray ionization mass spectrometry ionization efficiency scale of organic compounds. *Rapid Communications in Mass Spectrometry, 22*, 379-384. DOI: 10.1002/rcm.3371.

184. Zhao, L. & Li, F. (2014). UHPLC-MS strategies and applications for bioanalyses related to pharmacokinetics and drug metabolism. *Trends in Analytical Chemistry, 63*, 170-179. https://doi.org/10.1016/j.trac.2014.08.006.

185. Reubsaet, J.L.E. & Vieskar, R. (1999). Characterisation of π-π interactions which determine retention of aromatic compounds in reversed-phase liquid chromatography. *Journal of Chromatography A, 841*(2), 147-154.

186. Croes, K., Steffens, A., Marchand, D.H., & Snyder, L.R. (2005). Relevance of π-π and Dipole-Dipole Interactions for Retention on Cyano and Phenyl Columns in Reversed-Phased Liquid Chromatography. *Journal of Chromatography A, 1098*(1-2), 123-130.

Appendix

Ethics Certificates of Approval, Re-approval, and Amendment

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)

SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
PRAIRIE PLANT SYSTEMS INC.
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)

UNIVERSITY OF SASKATCHEWAN

TITLE

Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
15-Jul-2015	30-Jan-2017	Study Protocol CARE-E-01, Version 3 (22 December 2016) Information And Consent Form For Parents And Caregivers (Including Assent), v.3 (22 December 2016) Medication Card, v.2 (28 October 2016) Daily Seizure Record, v.1 (05 December 2016) Modified Quality of Life in Childhood Epilepsy Questionnaire (QOLCE), v.4 (28 October 2016) Side Effect Rating Scale, v.1 (06 December 2016) Parental Survey, v.1 (06 December 2016) Acknowledgement of: Health Canada No Objection Letter RE: Protocol # CARE-E-01 (12 January 2017) Investigator's Brochure for CanniMed® 20:1 CBD:THC Oil Suspension, Edition 1 (15 December 2016) Saskatchewan Health Research Foundation (SHRF) Application Review Letter Curriculum Vitae for Dr. Richard Huntsman Application for Biomedical Research Ethics Review, v.3 (18 January 2017) Response to Notice of Ethical Review (17 February 2016	29-Jan-2018

Delegated Review ☐ Full Board Meeting ☒ Date of Full Board Meeting: 15-Jul-2015 (REB-2)

IRB 1 FWA Registration #00001471 ☐ IRB 2 FWA Registration #00008358 ☒ Not Applicable ☐

CERTIFICATION

The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. If a protocol has been reviewed at a full board meeting, a subsequent study of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific

Please send all correspondence to: Research Services and Ethics Office
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been approved by the Minister of Health, Province of Saskatchewan, to serve as a Research Ethics Board (REB) for research studies involving human participants under section 29 of The Health Information Protection Act (HIPA).

Ildiko Badea, PhD., Vice-Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Services and Ethics Office
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
PRAIRIE PLANT SYSTEMS INC.
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN

TITLE
Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Protocol Clarification Letter, dated 02 February 2017 (signed 10 February 2017) Information and Consent Form For Parents And Caregivers (Including Assent), v.4 (02 February 2017) Modified Quality of Life in Childhood Epilepsy Questionnaire (QOLCE), v.5 (02 February 2017) Side Effect Rating Scale, v.2 (02 February 2017)	22-Feb-2017	29-Jan-2018

Acknowledgement of:
Application for Biomedical Research Ethics Review, v.4 (13 February 2017)

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION
The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This

Please send all correspondence to:
Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon SK S7N 4J8

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Ildiko Badea, Vice-Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607-110 Gymnasium Place
Saskatoon SK S7N 4J8

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)

SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
PRAIRIE PLANT SYSTEMS INC.
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)

UNIVERSITY OF SASKATCHEWAN

TITLE

Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Information And Consent Form For Parents And Caregivers (Including Assent), v.5 (02 March 2017) Parental Survey, v.2 (28 February 2017)	09-Mar-2017	29-Jan-2018

Acknowledgement of:
Addition of Sub-Investigator: Dr. Simona Hasal (Pediatric Neurology)

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon SK S7N 4J8

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Gordon McKay, PhD., Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607-110 Gymnasium Place
Saskatoon SK S7N 4J8

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
PRAIRIE PLANT SYSTEMS INC.
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN

TITLE
Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Daily Seizure Record, v.2 (07 April 2017)	19-Apr-2017	29-Jan-2018

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Ildiko Badea, Vice-Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon SK S7N 4J8

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR
Richard Huntsman

DEPARTMENT
Pediatrics

Bio #
15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
PRAIRIE PLANT SYSTEMS INC.
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN

TITLE
Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

APPROVAL OF
Study Protocol CARE-E-01, Version 4 (09 May 2017)
Information And Consent Form For Parents And Caregivers (Including Assent), v.6 (02 May 2017)

APPROVED ON
17-May-2017

CURRENT EXPIRY DATE
29-Jan-2018

Acknowledgement of
Health Canada Leaflet: Consumer Information – Cannabis (July 2016)

Delegated Review ☐ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☐

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Ildiko Badea, Vice-Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon SK S7N 4J8

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)

SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
PRAIRIE PLANT SYSTEMS INC.
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)

UNIVERSITY OF SASKATCHEWAN

TITLE

Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Protocol Clarification Letter (07 June 2017)	14-Jun-2017	29-Jan-2018

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Ildiko Badea, Vice-Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon SK S7N 4J8

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive

Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
PRAIRIE PLANT SYSTEMS INC.
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN

TITLE
Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Study Protocol CARE-E-01, Version 5 (09 August 2017) Information And Consent Form For Parents And Caregivers (Including Assent), v.7 (27 July 2017) Modified Quality of Life in Childhood Epilepsy Questionnaire (QOLCE), v.6 (20 July 2017) Daily Seizure Record, v.3 (25 July 2017) Acknowledge Receipt of: Study Protocol CARE-E-01, Version 5, Summary of Changes (09 August 2017)	23-Aug-2017	29-Jan-2018

Delegated Review ☐ Full Board Meeting ☒ Date of Full Board Meeting: 23-August-2017 (REB 1&2)

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are

Please send all correspondence to:
Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon SK Canada S7N 4J8

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Gordon McKay, PhD., Chair
University of Saskatchewan
Biomedical Research Ethics Board

Biomedical Research Ethics Board (Bio-REB)

Certificate of Re-Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN

TITLE
Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

RE-APPROVED ON
03-Jan-2018

EXPIRY DATE
02-Jan-2019

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION
The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Gordon McKay, PhD., Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 – Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN

TITLE
Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Information And Consent Form For Parents And Caregivers (Including Assent), v.8 (01 May 2018) Protocol Clarification Letter (02 May 2018) Daily Seizure Record, v.4 (23 November 2017) Medication Card, v.3 (16 November 2017) Modified QOLCE Questionnaire, v.7 (16 November 2017) Participant Diary, v.1 (21 February 2018) Side Effect Rating Scale, v.3 (16 November 2017)	09-May-2018	02-Jan-2019

Acknowledgement of
Biomedical Amendment Form (Completed 02 May 2018)

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION
The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB.

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon SK Canada S7N 4J8

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Ildiko Badea, Vice-Chair
Biomedical Research Ethics Board
University of Saskatchewan

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon SK Canada S7N 4J8

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

College of Pharmacy and Nutrition
University of Saskatchewan

FUNDER(S)
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)
CHILDREN'S HOSPITAL FOUNDATION
SAVOY FOUNDATION

SPONSOR(S)
UNIVERSITY OF SASKATCHEWAN

TITLE
Protocol CARE-E-01: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Study Protocol CARE-E-01, Version 6 (29 August 2018) Information and Consent Form for Parents and Caregivers (Including Assent), v.9 (29 August 2018)	10-Oct-2018	02-Jan-2019

Acknowledgement of
Health Canada No Objection Letter RE: Amendment to Protocol # CARE-E-01 (Version 6) (10 October 2018)
Study Protocol CARE-E-01, Version 6 Summary of Changes (06 September 2018)
Biomedical Amendment Form (completion date of 07 September 2018)

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION
The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB.

This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Richard Huntsman	Pediatrics	15-192

Digitally Approved by Ildiko Badea
Vice-Chair, Biomedical Research Ethics Board
University of Saskatchewan

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Re-Approval

Application ID:

Ethics Number: 15-192

Principal Investigator: Richard Huntsman

Department: Department of Pediatrics

Locations Where Research Activities are Conducted: Royal University Hospital, Canada
College of Pharmacy and Nutrition, Canada

Student(s):

Funder(s): Jim Pattison Childrens Hospital Foundation
Saskatchewan Health Research Foundation
Savoy Foundation
University of Alberta

Sponsor: University of Saskatchewan

Title: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

Protocol Number: CARE-E-01

Approved On: 17/12/2018

Expiry Date: 16/12/2019

Acknowledgment Of: Biomedical Prospective Renewal Form

Review Type: Delegated Review

IRB Registration Number: Not Applicable

* This study, inclusive of all previously approved documents, has been re-approved until the expiry date noted above

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2014).

Digitally Approved by Gordon McKay, Ph.D.
Chair, Biomedical Research Ethics Board
University of Saskatchewan

1 / 1

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 25-Feb-2019

Certificate of Approval Amendment

Ethics Number: 15-192

Principal Investigator: Richard Huntsman Department: Department of Pediatrics

Locations Where Research Activities are Conducted: Royal University Hospital, Canada
College of Pharmacy and Nutrition, Canada

Student(s):

Funder(s): Jim Pattison Childrens Hospital Foundation
Saskatchewan Health Research Foundation
Savoy Foundation

Sponsor: University of Saskatchewan

Title: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

Protocol Number: CARE-E-01

Approved On: 20/02/2019

Expiry Date: 16/12/2019
Approval Of: Information and Consent Form for Parents and Caregivers (Including Assent) – Manitoba Patients, version 1, 09-Jan-2019

Acknowledgment Of: Biomedical Amendment Form; Submission Memo

Review Type: Delegated Review

IRB Registration Number: Not Applicable

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2014).

Digitally Approved by Gordon McKay, Ph.D.
Chair, Biomedical Research Ethics Board
University of Saskatchewan

Biomedical Research Ethics Board (Bio-REB) 04-Oct-2019

Certificate of Approval Amendment

Ethics Number: 15-192

Principal Investigator: Richard Huntsman

Department: Department of Pediatrics

Locations Where Research Activities are Conducted: Royal University Hospital, Canada
College of Pharmacy and Nutrition, Canada
Jim Pattison Children's Hospital, Canada

Student(s):

Funder(s): Jim Pattison Childrens Hospital Foundation
Saskatchewan Health Research Foundation
Savoy Foundation

Sponsor: University of Saskatchewan

Title: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

Protocol Number: CARE-E-01

Approved On: 02/10/2019

Expiry Date: 16/12/2019

Approval Of:

* Study Protocol CARE-E-01, Version 7 (24 September 2019)
* Information and Consent Form for Parents and Caregivers (Including Assent), v.10 (24 September 2019)
* Information and Consent Form for Parents and Caregivers (Including Assent) Manitoba Patients, v.2 (24 September 2019)
* Addition of Study Site: Jim Pattison Children's Hospital (JPCH)

Acknowledgment Of:

* Study Protocol CARE-E-01, Version 7 Summary of Changes (25 September 2019)
* Biomedical Amendment Form (completion date of 01 October 2019)
* Addition of Sub-Investigator: Dr. Michael Szafron

Review Type: Delegated Review

IRB Registration Number: Not Applicable

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

Digitally Approved by Gordon McKay, Ph.D.
Chair, Biomedical Research Ethics Board
University of Saskatchewan

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 05-Dec-2019

Certificate of Re-Approval

Ethics Number: 15-192

Principal Investigator: Richard Huntsman

Department: Department of Pediatrics

Locations Where Research Activities are Conducted: Royal University Hospital, Canada
College of Pharmacy and Nutrition, Canada
Jim Pattison Children's Hospital, Canada

Student(s):

Funder(s): Jim Pattison Childrens Hospital Foundation
Saskatchewan Health Research Foundation
Savoy Foundation

Sponsor: University of Saskatchewan

Title: Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)

Protocol Number: CARE-E-01

Approved On: 05/12/2019

Expiry Date: 04/12/2020

Acknowledgment Of:

Review Type: Delegated Review

IRB Registration Number: Not Applicable

* This study, inclusive of all previously approved documents, has been re-approved until the expiry date noted above

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

Digitally Approved by Gordon McKay, Ph.D.
Chair, Biomedical Research Ethics Board
University of Saskatchewan

Biomedical Research Ethics Board (Bio-REB) 05-Feb-2020

Certificate of Approval Amendment

Ethics Number:	15-192
Principal Investigator:	Richard Huntsman
Department:	Department of Pediatrics
Locations Where Research Activities are Conducted:	Royal University Hospital, Canada College of Pharmacy and Nutrition, Canada Jim Pattison Children's Hospital, Canada
Student(s):	
Funder(s):	Jim Pattison Childrens Hospital Foundation Saskatchewan Health Research Foundation Savoy Foundation
Sponsor:	University of Saskatchewan
Title:	Cannabidiol in Children with Refractory Epileptic Encephalopathy: A Phase 1 Open Label Dose Escalation Study (CARE-E)
Protocol Number:	CARE-E-01
Approved On:	05/02/2020
Expiry Date:	04/12/2020
Approval Of:	* Study Protocol Administrative Clarification Letter (04 December 2019)
Acknowledgment Of:	* Response to Research Ethics Office Email of 21 November 2019 (06 December 2019)
Review Type:	Delegated Review
IRB Registration Number:	Not Applicable

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Jane Alcorn	Pharmacy	17-319

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
Health Sciences Building
University of Saskatchewan
Saskatoon Sk

STUDENT RESEARCHER(S)
Stephanie Vuong

FUNDER(S)
UNIVERSITY OF SASKATCHEWAN - COLLEGE OF MEDICINE

TITLE
Protocol: Prevalence of Prenatal Exposure to Cannabis: A proof of principle study

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
10-Jan-2018	10-Jan-2018	Application for Use of Existing Biological Materials in Research	09-Jan-2019

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. If a protocol has been reviewed at a full board meeting, a subsequent study of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Gordon McKay, PhD., Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Re-Approval

Application ID:

Ethics Number: 17-319

Principal Investigator: Jane Alcorn

Department: College of Pharmacy and Nutrition

Locations Where Research Activities are Conducted: Health Sciences Building, Canada

Student(s): Stephanie Vuong

Funder(s): College of Medicine

Sponsor:

Title: Prevalence of Prenatal Exposure to Cannabis: A proof of principle study

Protocol Number:

Approved On: 06/12/2018

Expiry Date: 05/12/2019

Acknowledgment Of: none

Review Type: Delegated Review

IRB Registration Number: Not Applicable

* This study, inclusive of all previously approved documents, has been re-approved until the expiry date noted above

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2014).

Digitally Approved by Gordon McKay, Ph.D.
Chair, Biomedical Research Ethics Board
University of Saskatchewan

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 23-Aug-2019

Certificate of Approval Amendment

Ethics Number: 17-319

Principal Investigator: Jane Alcorn

Department: College of Pharmacy and Nutrition

Locations Where Research Activities are Conducted: Health Sciences Building, Canada

Student(s): Amelie Cyr
Stephanie Vuong

Funder(s): College of Medicine

Sponsor:

Title: Prevalence of Prenatal Exposure to Cannabis: A proof of principle study

Protocol Number:

Approved On: 09/08/2019

Expiry Date: 05/12/2019

Approval Of:

* Biomedical Amendment Form
* Email Correspondence with REB rec'd 17-Jul-2019

Acknowledgment Of:

Review Type: Delegated Review

IRB Registration Number:

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2014).

Digitally Approved by Beth Davis, Ph.D.,
Vice-Chair, Biomedical Research Ethics Board
University of Saskatchewan

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 05-Dec-2019

Certificate of Re-Approval

Ethics Number: 17-319

Principal Investigator: Jane Alcorn

Department: College of Pharmacy and Nutrition

Locations Where Research Activities are Conducted: Health Sciences Building, Canada

Student(s): Amelie Cyr
Stephanie Vuong

Funder(s): College of Medicine

Sponsor:

Title: Prevalence of Prenatal Exposure to Cannabis: A proof of principle study

Protocol Number:

Approved On: 04/12/2019

Expiry Date: 03/12/2020

Acknowledgment Of:

Review Type: Delegated Review

IRB Registration Number: Not Applicable

* This study, inclusive of all previously approved documents, has been re-approved until the expiry date noted above

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

Digitally Approved by Gordon McKay, Ph.D.
Chair, Biomedical Research Ethics Board
University of Saskatchewan

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 30-Jan-2020

Certificate of Approval Amendment

Ethics Number: 17-319

Principal Investigator: Jane Alcorn

Department: College of Pharmacy and Nutrition

Locations Where Research Activities are Conducted: Health Sciences Building, Canada

Student(s): Amelie Cyr
Stephanie Vuong

Funder(s): College of Medicine

Sponsor: University of Saskatchewan

Title: Prevalence of Prenatal Exposure to Cannabis: A proof of principle study

Protocol Number:

Approved On: 24/01/2020

Expiry Date: 03/12/2020

Approval Of:

* Biomedical Amendment Form, rec'd 15-Jan-2020
* Subject Information and Consent Form, January 2020

Acknowledgment Of:

Review Type: Delegated Review

IRB Registration Number:

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

Digitally Approved by Beth Davis, Ph.D.
Vice-Chair, Biomedical Research Ethics Board
University of Saskatchewan