Curriculum Vitae

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EDUCATION

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August 2007 – August 2011
PhD Toxicology
Advisor: Marilyn A. Huestis, PhD
Dissertation: *Free and Glucuronidated Cannabinoids, Cognitive, Subjective and Physiological Effects and In Vitro Cannabinoid Stability Following Controlled Smoked Cannabis Administration*

University of Illinois, Chicago, IL
August 2003 – May 2005
MS Forensic Science
Advisor: William J. Mills, PhD
Thesis: *Improvements in Sampling and Analytical Methods for Polychlorinated Biphenyls in Environmental Samples*

Miami University, Oxford, OH
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PROFESSIONAL POSITIONS

National Institute on Drug Abuse (NIH/NIDA/IRP)
Chemistry and Drug Metabolism Section
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Intramural Research Training Award Fellow (Pre-Doctoral)

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Animal Forensic Toxicology Laboratory
March 2006 – June 2007
Senior Analytical Forensic Toxicology Specialist

University of Illinois at Chicago
Animal Forensic Toxicology Laboratory
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Analytical Forensic Toxicology Specialist

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Associate Scientist

SPECIFIC EXPERIENCE

AB Sciex 3200 Q Trap LC-MS/MS System, Agilent 6890 GC-ECD/FID (including two-dimensional GC) Agilent 5973&5975 MSD, Agilent 1100 Series Ion Trap LC-MS/MS System, Porter Lee BEAST LIMS, SPSS for Windows, Pharsight WinNonlin, Microsoft Office 2007(Windows), 2011 (Macintosh)

MEMBERSHIPS

American Chemical Society (ACS)
Student Member, 2002 – Present

Society of Forensic Toxicologists (SOFT)
Student Member, 2009 – Present
Young Forensic Toxicologists (YFT) Committee, 2009 – Present

HONORS/AWARDS

Society of Forensic Toxicologists
Educational Research Award (ERA) Recipient, October, 2010

National Institute on Drug Abuse, National Institutes of Health
Pre-doctoral Intramural Research Training Award (IRTA), September 2009-Present

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MANUSCRIPTS

Schwope DM, Karschner EL, Gorelick DA, Huestis MA. Identification of recent cannabis use: Whole blood and plasma free and glucuronidated cannabinoid pharmacokinetics following controlled smoked cannabis administration. *Clinical Chemistry*. 2011. *In Press*.

Schwope DM, Scheidweiler KB, Huestis MA. Direct quantification of cannabinoids and cannabinoid glucuronides in whole blood by liquid chromatography tandem mass

spectrometry. *Analytical and Bioanalytical Chemistry*. 2011. *In press*, DOI 10.1007/s00216-011-5197-7.

Holland MG, **Schwope DM**, Stoppacher R, Gillen SB, Huestis MA. Postmortem redistribution of Delta-9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (THCCOOH). *Forensic Science International*. 2011. *In Press*, DOI 10.1016/j.forsciint.2011.06.028.

Goodwin RA, Baumann MH, Gorelick DA, Schwilke E, **Schwope DM**, Darwin WD, Kelly DL, Schroeder JR, Ortemann-Renon C, Bonnet D, Huestis MA. CB_1-cannabinoid receptor antagonist effects on cortisol in cannabis-dependent men. *The American Journal of Drug and Alcohol Abuse*. 2011. *In press*, DOI 10.3109/00952990.2011.600398

Milman G, Barnes AJ, **Schwope DM**, Schwilke EW, Goodwin RS, Kelly DL, Gorelick DA, Huestis MA. Cannabinoids and metabolites in expectorated oral fluid after eight days of controlled around-the-clock oral THC administration. *Analytical and Bioanalytical Chemistry*. 2011. 401 (2) 599-607.

Milman G, Barnes AJ, **Schwope DM**, Schwilke EW, Darwin WD, Goodwin RS, Kelly DL, Gorelick DA, Huestis MA. Disposition of cannabinoids in oral fluid after controlled around-the-clock oral THC administration. *Clinical Chemistry*. 2010. 56 (7) 1261-1269.

Schwope DM, Milman G, Huestis MA. Validation of an enzyme immunoassay for detection and semi-quantification of cannabinoids in oral fluid. *Clinical Chemistry*. 2010. 56 (6) 1007-1014.

Schwilke EW, **Schwope DM**, Karschner EL, Lowe RH, Darwin WD, Kelly DL, Goodwin RS, Gorelick DA, Huestis MA. Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC & 11-nor-9-carboxy-THC plasma pharmacokinetics during and after continuous high-dose oral THC. *Clinical Chemistry*. 2009. 55 (12) 2180-2189.

Luckie C, Whitney C, Benoit M, Taddei L, Sukta A, Peterson J, **Schwope D**, Gaensslen RE, Negrusz A. Detection and quantification of low levels of benzoylecgonine in equine urine. *Forensic Science International*. 2008. 177 (1) 21-24.

GIVEN WORKSHOPS & SEMINARS

Schwope DM. Cannabinoids in oral fluid. American College of Medical Toxicology Seminar in Forensic Toxicology: Ethanol and Marijuana, San Francisco, Dec 13-14, 2010.

Schwope DM. Cannabinoids in oral fluid – background and screening. Marijuana Pharmacology – Practical Applications for the Forensic Toxicologist Workshop. Society of Forensic Toxicologists Annual Meeting, Richmond, Oct 18, 2010.

Schwope DM. Cannabinoids in oral fluid. American College of Medical Toxicology Seminar in Forensic Toxicology: Ethanol and Marijuana, Baltimore, Nov 18-19, 2009.

SELECTED ABSTRACTS

Schwope DM, Karschner EL, Scheidweiler KB, Gorelick DA, Huestis, MA. *In vitro* stability of cannabinoids and cannabinoid glucuronides in authentic whole blood and plasma specimens following controlled smoked cannabis. Joint SOFT-TIAFT International Conference and Exposition, San Francisco, September 25-30, 2011. *Accepted*

Schwope DM, Scheidweiler KB, Huestis, MA. Direct LC-MS/MS quantification of plasma cannabinoids and their glucuronides. American Academy of Forensic Sciences Annual Meeting, Chicago, Feb 21-26, 2011.

Schwope DM, Holland MG, Gillen S, Stoppacher R, Huestis MA. Postmortem redistribution of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (THCCOOH). Society of Forensic Toxicologists Annual Meeting, Richmond, Oct 18-22, 2010.

Schwope DM, Milman G, Huestis MA. Validation of enzyme immunoassay for semi-quantification of cannabinoids in oral fluid. Society of Forensic Toxicologists Annual Meeting, Oklahoma City, Oct 19-23, 2009.

Schwope DM, Karschner EL, Schwilke EW, Goodwin RS, Kelly DL, Gorelick DA, Huestis MA. Total whole blood cannabinoids following multiple daily oral THC dosing. IATDMCT Congress, Montreal, Oct 4-8, 2009.

Schwope DM, Schwilke EW, Karschner EL, Lowe RH, Kelly DL, Goodwin RS, Gorelick DA, Huestis MA. Whole blood cannabinoids in daily cannabis users during eight days of controlled multiple oral THC doses. Society of Forensic Toxicologists Annual Meeting, Phoenix, October 26-31, 2008.

Schwope DM, Luckie C, Whitney C, Benoit M, Taddei L, Sukta A, Peterson J, Gaensslen RE, Negrusz A. Determination of verapamil and its metabolites in equine samples. Society of Forensic Toxicologists Annual Meeting, Austin, October 3-7, 2006.

Schwope DM, Li A, Nienow C, Bloomgren C, Mills WJ. Improved methods for PCB air sampling and analysis. 27th Midwest Environmental Chemistry Workshop, Madison, October 15-17, 2004.

Schwope DM, Malcolm R, Mills WJ. Characterization of air emissions from PCB transformers. 26th Midwest Environmental Chemistry Workshop, Iowa City, October 11-12, 2003.

Schwope DM, Minto RE. Progress toward the synthesis of fistulina hepatica natural products. 225th American Chemical Society National Meeting, New Orleans, March 24-29, 2003.

Abstract

Title of Dissertation: Free and Glucuronidated Cannabinoids, Subjective, Physiological and Psychomotor Effects and *In Vitro* Cannabinoid Stability Following Controlled Smoked Cannabis Administration

David M. Schwope, Doctor of Philosophy, 2011

Dissertation Directed by: Marilyn A. Huestis, PhD, Chief, Chemistry and Drug Metabolism, National Institute on Drug Abuse, National Institutes of Health; Adjunct Professor, Department of Epidemiology and Preventive Medicine, University of Maryland School of Medicine, Baltimore, MD

Δ^9-tetrahydrocannabinol (THC) is the illicit drug most frequently observed in accident and driving under the influence of drugs (DUID) investigations. Whole blood is often the only available specimen collected during such investigations. However, whole blood cannabinoid concentrations (including phase II cannabinoid glucuronide metabolites) and relationships between subjective and psychomotor effects and cannabinoid blood concentrations following cannabis smoking have not been examined. Furthermore, there was no analytical method to investigate whole blood cannabinoid glucuronides directly.

Nine male and one female chronic, heavy cannabis smokers resided on a closed research unit and smoked *ad libitum* one 6.8% THC cannabis cigarette. Whole blood and plasma specimens were collected up to 22 h after smoking. THC, 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), cannabidiol (CBD), cannabinol (CBN), THC-glucuronide and THCCOOH-glucuronide were directly quantified by a new, validated liquid chromatography-tandem mass spectrometry method. Specimens were analyzed within 24 h of collection to obviate stability issues. Assessments were

performed before and up to 6 h after smoking, including subjective (visual analog scales [VAS] and Likert scales), physiological (heart rate, blood pressure, respirations) and psychomotor (critical tracking and divided attention tasks) measures.

Analytical method linearity ($R^2 \geq 0.997$) ranged from 0.5-50 μg/L (THC-glucuronide), 1.0-100 μg/L (THC, 11-OH-THC, THCCOOH, CBD and CBN) and 5.0-250 μg/L (THCCOOH-glucuronide). Median whole blood (plasma) observed C_{max} were 50 (76), 6.4 (10), 41 (67), 1.3 (2.0), 2.4 (3.6), 89 (190), and 0.7 (1.4) μg/L 0.25 h after starting smoking for THC, 11-OH-THC, THCCOOH, CBD, CBN, and THCCOOH-glucuronide, respectively, and 0.5 h for THC-glucuronide. CBD and CBN were not detectable after 1 h. THC significantly increased VAS responses and heart rate, with concentration-effect curves demonstrating counter-clockwise hysteresis. No differences were observed for critical tracking or divided attention task performance in this cohort of heavy, chronic cannabis smokers.

These are the first authentic human whole blood cannabinoid metabolite data following controlled cannabis smoking. Minor cannabinoids and cannabinoid glucuronide data were evaluated as markers of recent cannabis use. These findings will improve interpretation of whole blood and plasma cannabinoid results, further identification of recent cannabis intake, and inform our understanding of impairment and subjective effects following acute smoked cannabis.

Free and Glucuronidated Cannabinoids, Subjective, Physiological and Psychomotor Effects and *In Vitro* Cannabinoid Stability Following Controlled Smoked Cannabis Administration

by
David M. Schwope

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2011

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Finally, a huge thanks to my family. Mom, Dad and Steve, you guys are great. Thanks Rach for listening to my complaints and keeping it real about grad school. Becky, you deserve much more than a single line, or even a single page. I can't thank you enough for all you've done and the time you've given up over the last four years. I'm excited to begin the next chapter of our lives, especially as we add to our family in December.

DMS

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List of Abbreviations

11-OH-THC – 11-hydroxy-Δ^9-tetrahydrocannabinol
2-AG – 2-arachidonoyl glycerol
2D-GC-MS – two-dimensional gas chromatography-mass spectrometry
ACN – acetonitrile
AEA – anandamide
ANOVA – analysis of variance
AUC – area under the curve
BART – balloon analogue risk task
BIS-11 – Barratt Impulsiveness Scale Version 11
BMI – body mass index (kg/m^2)
BP – blood pressure
BPRU – Johns Hopkins Behavioral Pharmacology Research Unit
BRC – National Institute on Drug Abuse Biomedical Research Center
CB_1 – cannabinoid receptor subtype 1
CB_2 – cannabinoid receptor subtype 2
CBC – complete blood count
CBD – cannabidiol
CBN – cannabinol
CDM – Chemistry and Drug Metabolism Section
CE – collision energy
CI – chemical ionization
CIF – cannabis influence factor
C_{max} – maximum observed concentration
CNS – central nervous system
CTT – critical tracking task
CV – coefficients of variance
CYP – cytochrome P450
DAG – diacylglycerol
DAT – divided attention task
DBP – diastolic blood pressure
DP – declustering potential
DUID – driving under the influence of drugs
ECG – electrocardiogram
EI – electron-impact ionization
ELISA – enzyme-linked immunosorbent assay
EMIT – enzyme multiplied immunoassay technique
ESI – electrospray ionization
FDA – Food and Drug Administration
FID – flame ionization detector
FN – false negative
FP – false positive

GC – gas chromatography
GC-MS – gas chromatography-mass spectrometry
GLM – general linear model
HPLC – high performance liquid chromatography
HR – heart rate
IQR – interquartile range
IRB – institutional review board
IRP – Intramural Research Program
IV – intravenous
λ_c – lambda-c
LC-MS – liquid chromatography-mass spectrometry
LOD – limits of detection
LOQ – limits of quantification
MJ – marijuana, cannabis
MRM – multiple reaction monitoring
MS/MS – tandem mass spectrometry
NAPE – *N*-arachidonoyl-phosphatidylethanolamine
NHTSA – National Highway Traffic Safety Administration
NIDA – National Institute on Drug Abuse
NIDA IRP CDM – Chemistry and Drug Metabolism Section, National Institute on Drug Abuse Intramural Research Program, National Institutes of Health
NIH – National Institutes of Health
OF – oral fluid
PD-PK – pharmacodynamic-pharmacokinetic
POCT – point-of-collection testing device
QC – quality control
RT – room temperature
SAMHSA – Substance and Mental Health Services Administration
SBP – systolic blood pressure
SD – standard deviation
SEM – standard error of the mean
SES – subjective effects scales
SIM – selected ion monitoring
SPE – solid phase extraction
THC – Δ^9-tetrahydrocannabinol
THCA-A – Δ^9-tetrahydrocannabinolic acid A
THCCOOH – 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol
T_{max} – time to maximum concentration
TN – true negative
TP – true positive
UGT – uridine 5'-diphospho-glucuronosyltransferase
VAS – visual analogue scale
V_d – volume of distribution
WRAT – Wide Range Achievement Test
ZKPQ – Zuckerman-Kuhlman Personality Questionnaire

Chapter 1 – Introduction

Cannabis sativa (cannabis, marijuana, MJ) has been smoked for its medicinal and psychoactive properties for thousands of years and is the most widely used illicit drug in the world (1-2). The primary psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), was identified and synthesized in 1964 (3). Potency of confiscated cannabis in the United States has increased over time. In the 1980's and early 1990's the average THC content was approximately 3–4%; currently, cannabis potency is approximately 9% (4).

Extensive cannabis use in the United States began in the early 1900's, peaked in the 1970's and is most prevalent among adolescents, teens and young adults. In 2009, an estimated 16.7 million (6.6% of the population) Americans 12 years or older had smoked cannabis in the past month, more than in 2008 (15.2 million or 6.1%) and 2007 (14.4 million or 5.8%) (5). Additionally, 36.7% (6.1 million) of these individuals smoked cannabis almost daily (≥ 20 times) in the past month. Experimental evidence indicates increased likelihood of more dangerous drug use (e.g. cocaine and heroin) when cannabis ingestion begins at an early age; animal studies suggest this may be due to drug-induced alterations in brain function (6).

Cannabis Pharmacology

THC's pharmacological action is mediated by G-protein-coupled cannabinoid receptors (7). Presently, two subtypes (CB_1 and CB_2) are cloned (8-9). Additional subtypes GPR55 and GPR119 are suggested novel cannabinoid receptors, but biological relevance is still unclear (10). *In vivo*, THC psychoactive effects are primarily mediated by the CB_1 subtype that is widely distributed in the brain, including hippocampus, cerebral cortex, basal ganglia, and cerebellum (11-14), although CB_1 receptors also are found in peripheral tissues and cells. CB_2 receptors are largely found peripherally, functioning as mediators of immune system cell signaling (15), although recent studies also identified CB_2 receptors in rat brain glial cells (16-18). Primary endogenous cannabinoid receptor ligands are 2-arachidonyl glycerol (2-AG) (19), and arachidonylethanolamide (anandamide, AEA) (20). 2-arachidonylglyceryl ether (21), *N*-arachidonyl-dopamine (22), and *O*-arachidonyl-ethanolamine (23) also were identified as receptor ligands; research continues to identify other potential candidates.

The primary function of endogenous cannabinoids (specifically AEA and 2-AG) is regulation of pre-synaptic neurotransmitter release (e.g., GABA, glutamate) (24). Endogenous cannabinoids are released on demand (in contrast to other typical neurotransmitters stored in vesicles) from post-synaptic cells and bind to CB_1 receptors located on pre-synaptic nerve terminals (25). The most likely pathway for AEA synthesis begins with formation of *N*-arachidonoyl-phosphatidylethanolamine (NAPE) from phoshatidylcholine and phosphatidylethanolamine via *N*-acyltransferase (26). NAPE is

cleaved by phospholipase D to form AEA and phosphatidic acid. 2-AG is synthesized from diacylglycerol (DAG) via DAG-lipase. Enzymes responsible for endocannabinoid deactivation are intracellular, necessitating transport across the plasma membrane. While active transport was observed in multiple cell types (27-28), the protein responsible has yet to be identified. Following uptake, AEA and 2-AG are deactivated by membrane bound fatty acid amide hydrolase (29) and monoacylglycerol lipase (19), respectively.

Cannabis Toxicity

Serious acute toxicity from cannabis is rare, with no reported deaths due solely to cannabinoid use (30). Panic attacks, psychosis, and CNS depression may occur (31). The FDA-approved package insert for dronabinol (synthetic THC) estimates the lethal human dose of intravenous THC as 30 mg/kg, based on extrapolation from rodent studies (32). Toxicity studies in dogs and monkeys (33) indicate that the human LD_{50} for oral THC must be more than three orders of magnitude greater (3,000 mg/kg). The lowest oral THC dose causing any deaths in dogs or monkeys was about 1000 mg/kg.

Cannabinoid Blood Pharmacokinetics

THC is strongly lipophilic and rapidly distributes into highly perfused tissues such as the lungs, heart, brain, and liver (34). Steady-state volume of distribution (V_d) is approximately 3.4 L/kg (35-36), with 95-99% of THC in plasma bound to lipoproteins and a lesser amount to albumin and red blood cells (34, 37). Adipose tissue accumulates THC following chronic cannabis use, and slowly releases THC back into the blood over time (38-41). The rate-limiting step in THC excretion is the redistribution of THC from fat deposits into the systemic circulation (34). Over 100 metabolites of THC have been

identified (34, 37, 42). Phase I hydroxylation of THC at C_9 by hepatic cytochrome P450 enzymes forms the pharmacologically active monohydroxy compound, 11-OH-THC (34). Alternative products of oxidation include minimal amounts of 8α- and substantial amounts of 8β-hydroxy-Δ^9-THC. CYP2C9 is the main P450 enzyme oxidizing THC to 11-OH-THC, although CYP2C19, and CYP3A4 also contribute to overall THC hepatic oxidation (34). Although liver is the major site of THC metabolism, other tissues, such as lung, also are capable of metabolizing cannabinoids (34, 37, 42). Subsequent 11-OH-THC oxidation yields the non-psychoactive THCCOOH metabolite. Phase II conjugation of glucuronic acid to THCCOOH increases water solubility facilitating urinary excretion (Figure 1). THC and 11-OH-THC undergo direct conjugation as well; conjugation rates of 10% and 60%, respectively, were reported following oral dosing (43). Alternative conjugation with glutathione, amino acids, and fatty acids is less common; it is unknown to what extent sulfate conjugation occurs (34).

Wall et al. (44) reported no significant differences in THC metabolism between men and women. Although Lemberger et al. (45-46) reported more rapid metabolism in frequent as compared to infrequent cannabis smokers, these results were not substantiated by other investigators (47-49). After five days, 85-90% of a THC dose is excreted, primarily as acid metabolites, with 20-35% excreted in urine and 65-80% in feces (44, 50-51). Cannabinoid terminal plasma elimination half-lives are difficult to measure due to low analyte concentrations, the need for a long monitoring period and potential loss of measurable drug due to its adsorptivity to multiple surfaces (37).

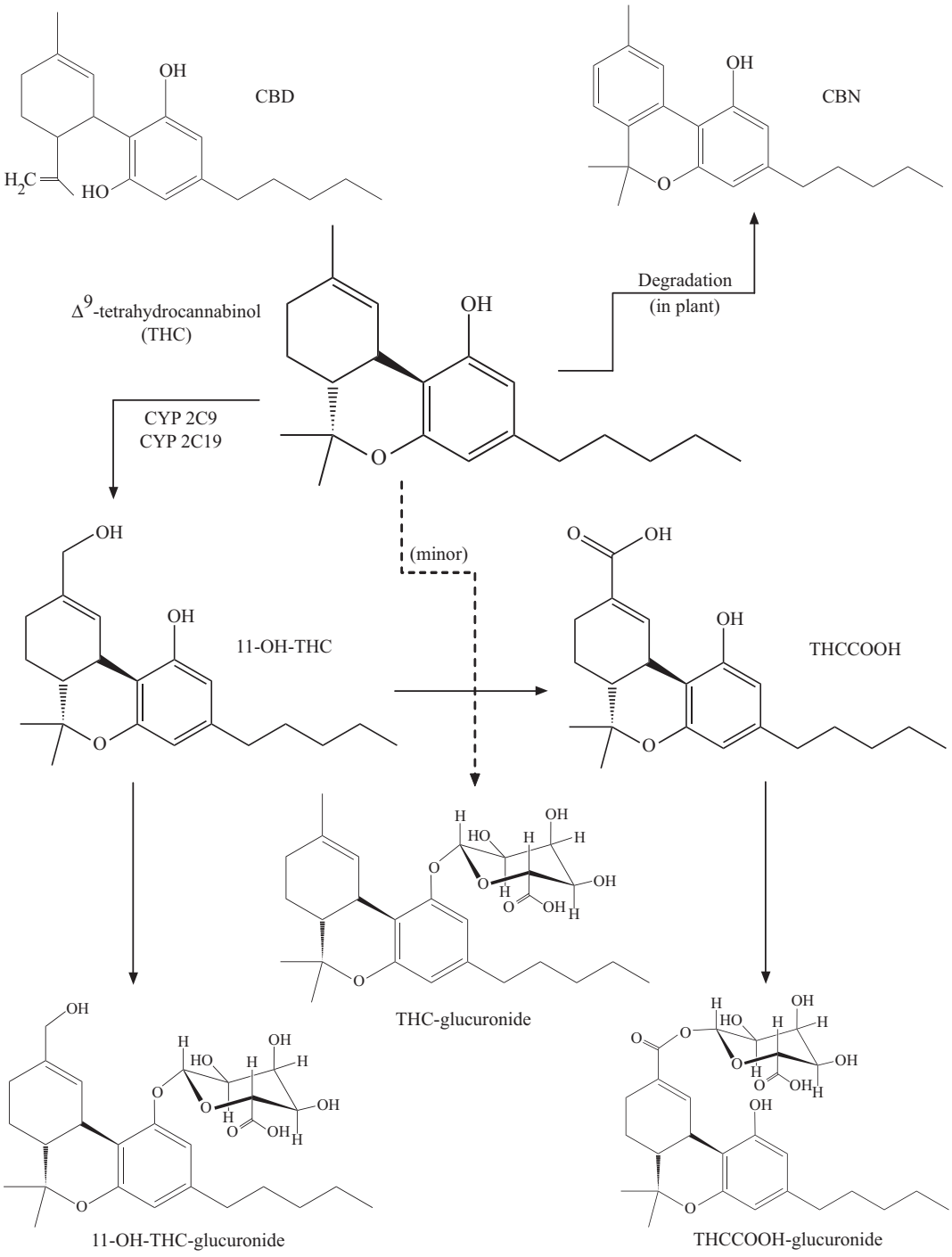


Figure 1. Metabolic pathway for Δ^9-tetrahydrocannabinol.

Solid arrows indicate major metabolic pathways; dashed arrows indicate minor pathways. CBD: cannabidiol; CBN: cannabinol; THC: Δ^9-tetraydrocannabinol; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; CYP: cytochrome P450

Smoking is the primary route of cannabis intake. Drug delivery during cannabis smoking is characterized by rapid absorption of THC with slightly lower peak concentrations than those found after intravenous administration (52). Due to uncertainty in dose delivery and variation in smoking topography, bioavailability following cannabis smoking was reported as 18–50% (47, 53). The primary variables in determining actual drug exposure include the number, duration, and spacing of puffs, hold time, and inhalation volume (54-56). THC can be measured in plasma within seconds after the first inhalation of cannabis smoke (57). In a study where participants smoked either a 1.75% or 3.55% THC cigarette, cannabinoid concentrations increased rapidly, with peak concentrations at 9.0 min. Mean (n = 6) peak THC concentrations were 84.3 and 162.2 μg/L for the low and high doses, respectively (57). Within 2 h, plasma THC concentrations were at or below 5 μg/L. Times of last THC detection (limit of quantification (LOQ) 0.5 μg/L) varied, but were generally less than 12 and 24 h after the low- and high-dose cannabis cigarettes. In other studies, peak THC concentrations ranged between 45.6 to 187.8 μg/L following smoking of an approximately 1% THC cigarette (58), 33–118 μg/L 3 min after *ad libitum* smoking of an approximately 2% THC cigarette (52), and 24-462 μg/L 12 min after smoking a cigarette containing approximately 69 mg THC (59).

Pharmacodynamics

The subjective "high" from cannabis is characterized by euphoria, relaxation and distorted perceptions (37). These effects depend largely on dose that varies depending on how an individual smokes, also known as smoking topography. Experienced cannabis smokers often titrate their dose by varying depth of inhalation and hold time (47).

Subjective/Physiological Effects

Acute psychological effects of cannabinoids include euphoria, dysphoria, sedation, and altered perception (60-62). Intensity of euphoria/dysphoria was associated with dose, mode of administration, vehicle in which drug was administered (60, 63), users' expectations of effects, and environment and personality of the user (60).

Cannabis acutely increases heart rate and peripheral vasodilatation (52, 64-66); supine hypertension and hypotension upon standing also may occur (67). Conjunctival reddening (37, 52) peaks approximately 1 hour following smoking (68) and skin temperature decreases were observed after IV (64) and smoked (66) THC administration. Tolerance to cardiovascular effects was demonstrated after two to three weeks of chronic oral THC administration, possibly due to a compensatory increase in blood plasma volume (69).

Psychomotor Effects

Acute cannabis intoxication produces dose-related impairments in cognitive and psychomotor functioning, as well as risk-taking behavior (70-74). Additionally, reaction time, perception, short-term memory and attention, motor skills, tracking, and skilled activities are altered (75-77).

While several observational studies evaluated the relationship between past cannabis use and risk-taking, e.g., criminal (78-80) and risky sexual behavior (81-83), and driving under the influence of cannabis (84-85), few studies examined whether acute cannabis exposure directly affects risk-taking. Lane et al (72) observed significant effects on risk-taking following smoking of 3.58% THC cigarettes, but not those with

lower (1.77%) THC concentrations. McDonald et al (73) found that 15 mg oral THC yielded mixed results across four different impulsivity tasks. This difference may reflect decreased bioavailability and lower peak blood THC concentrations following oral dronabinol (THC) administration compared to smoked cannabis.

From a public health perspective, driving and work-related accidents are a major concern following acute cannabis intoxication (86). Cannabis-induced driving impairment was demonstrated in on-the-road driving tests (87-88), where impairment increased with dose and was more persistent in driving skills requiring sustained attention. Additionally, performance impairment on a critical tracking task in the 2 h following a smoked cannabis dose (250 μg/kg) and also 2-6 h after smoking was similar to impairment observed in participants performing the same task with blood alcohol concentrations of approximately 0.11 g/dL and 0.07 g/dL, respectively (88-89). However, the relationship between cannabis intake and impairment is complex and may be related to prior use history, setting, and task familiarity. Ramaekers et al studied effects of cannabis smoking (13% THC, 500 μg/kg) in heavy (> 4 days/week) and occasional (≤ 1x/week) smokers and found significant impairment in tracking performance, divided attention, and inhibitory control in occasional, but not heavy smokers (90). Papafotiou examined cannabis-induced driving simulator impairment in cannabis smokers after one week of abstinence; significant impairment was observed 80 min after smoking a 1.74% or 2.93% THC cannabis cigarette (91). Further research is required to elucidate impairment differences between occasional and heavy cannabis smokers; however, these data indicate that cannabis intake can yield impairment similar

to alcohol concentrations at or above the legal limit (0.08 g/dL), contributing to increased accident rates.

Pharmacodynamic-Pharmacokinetic Relationships

Law enforcement and triers of fact are often interested in relating concentrations of THC and its metabolites to drug-induced effects. Typically the question "What concentration of blood THC produces psychoactive effects similar to the current legal limit of alcohol?" arises during investigations and criminal/civil trials. Unfortunately, this answer is not as forthcoming as with alcohol, due to the lipophilicity of THC and its pharmacokinetics following smoked or oral administration.

When VAS subjective effects are compared to simultaneously collected whole blood THC concentrations and plotted by time, a counter-clockwise hysteresis is obtained (Figure 2). Cocchetto et al (92) first detailed hysteresis following cannabis smoking, indicating a delay in the onset of subjective effects as compared to plasma cannabinoid concentrations. Barnett et al (93) also observed a counter-clockwise hysteresis for heart rate and THC plasma concentrations, and Chiang et al (94) described differences in areas within the hysteresis for oral, smoked and intravenous THC. Cone and Huestis described a similar phenomenon for "How much drug effect do you feel" VAS (66, 95). Counter-clockwise hysteresis is typically observed for drugs with a larger volume of distribution, drugs with active metabolites, or an indirect mechanism of drug action (96). THC, with 2- or 3-compartment pharmacokinetics and an equipotent metabolite (11-OH-THC) is a likely candidate for a counter-clockwise hysteresis. These hystereses can result in misinterpretation of cannabinoid blood concentrations or effect, as observed

pharmacodynamic effect (i.e. subjective "high") can be similar over a large THC concentration range. Vice versa, a similar concentration of THC can yield quite different responses for subjective "High," depending on what time the specimens were collected after intake. Additionally, caution is warranted as inter-individual variability is high for hysteresis areas (96), and could vary substantially depending on dose, setting or prior use, yielding further variability in determining concentration-effect relationships.

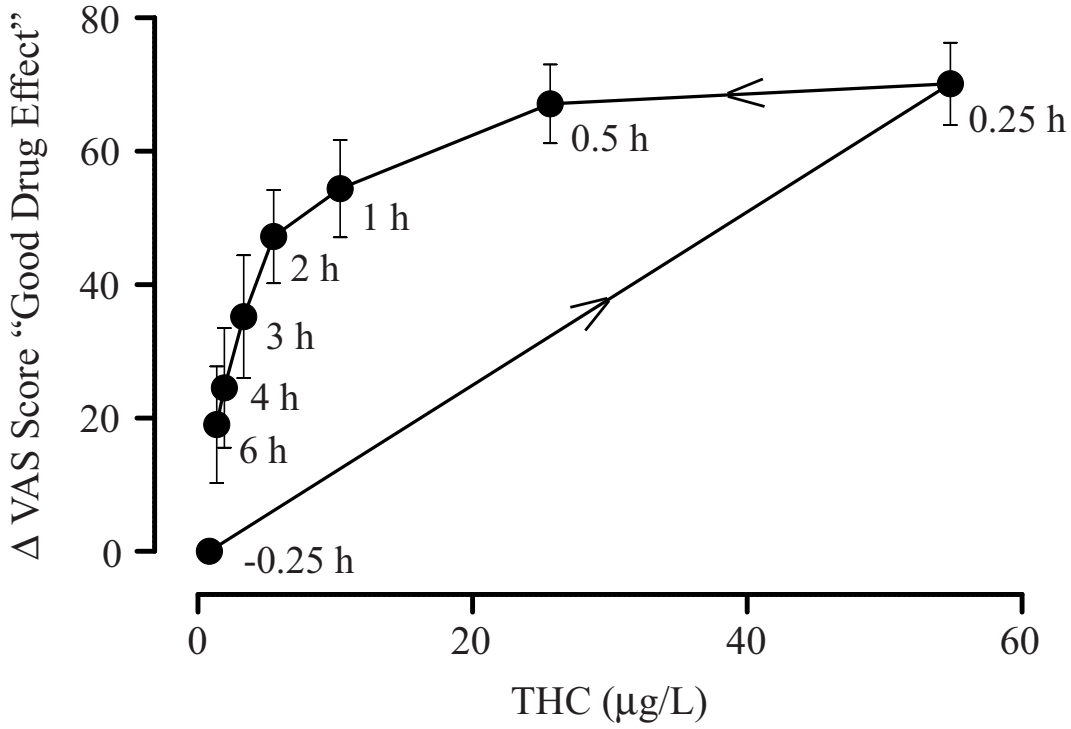


Figure 2. Counter-clockwise hysteresis example.

Median whole-blood THC concentrations and mean (SEM) VAS "Good Drug Effect" following smoking of a 6.8% THC cannabis cigarette (n = 10). VAS scores are calculated as change from baseline (-0.5 h). Samples collected at -0.5, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 after starting smoking. Arrows in hysteresis plot indicate samples collected over increasing time.

Cannabinoid Detection and Quantification

Numerous confirmation methodologies have been developed for cannabinoids, including gas chromatography (GC) (97-99), high performance liquid chromatography (HPLC) (100-108), liquid chromatography tandem mass spectrometry (LC-MS/MS) (109-112), gas-chromatography/mass spectrometry (GC-MS) (113-121) and GC-MS/MS (122-124). Confirmation of forensic specimens usually includes mass spectrometric or tandem mass spectrometric identification due to the improved specificity of these techniques. Selected ion monitoring (SIM), full-scan ion monitoring, chemical ionization methods, direct probe insertion GC-MS, and neutral loss and product ion methods have been utilized for the confirmation of cannabinoids in body fluids and tissues.

Compared to other drugs of abuse, cannabinoid analysis presents difficult challenges. THC and 11-OH-THC are highly lipophilic and present in low concentrations in body fluids. Complex specimen matrices (e.g. blood, plasma and hair) may require multi-step extractions to separate cannabinoids from lipids and proteins. Cannabinoid extraction techniques include liquid-liquid extractions, as well as solid-phase extraction with bonded silica, anion exchange and mixed-mode chemistry (115, 125-129).

Two-dimensional GC-MS (2D-GC-MS) is an analytical tool that improves analyte separation from co-eluting matrix, increase signal-to-noise ratio and improve sensitivity. For forensic purposes, 2D-GC-MS was first employed to quantify cannabinoids in whole blood (130), and shortly thereafter, applied to plasma (131), oral fluid (132-134), hair (135), and meconium (136-137).

Typically, methods are limited to parent THC, phase I metabolites and other minor cannabinoids, and fail to consider implications of phase II metabolites. Specifically, factors such as poor hydrolysis efficiency (43, 138) and glucuronide instability (139) can introduce unnecessary (and potentially substantial) error into quantitative determinations. Direct identification and quantification of glucuronides negates hydrolysis efficiency concerns and can yield novel insight into glucuronide pharmacokinetics and glucuronide *in vitro* stability while offering the possibility of utilizing cannabinoid glucuronides as markers of recent cannabis intake.

In Vitro Whole Blood and Plasma Cannabinoid Stability

In clinical and forensic toxicology, drug stability during storage is an important consideration for interpretation of drug concentrations and metabolite patterns (139). In order to investigate pharmacokinetic parameters such as elimination half lives and detection windows (49), and to utilize prediction models of last cannabis use (140-142), it is essential to establish stability for cannabinoid biomarkers. To our knowledge, the stability of major cannabinoids in authentic whole blood and/or plasma specimens was not systematically investigated. Cannabinoid stability in fortified drug-free whole blood and plasma specimens was reported (143-148), sometimes with highly variable stability (145-147). THC was stable in blood stored at 5, -5, and -20 °C for up to 17 weeks (147), with THC degradation after this time until none was detected by 23 weeks. Temperature conditions did not influence drug recovery, nor did repetitive freeze-thaw cycles. The authors hypothesized that decreasing THC concentrations may have been due to unsuccessful extractions due to irretrievable binding to degraded proteins. In another study, Johnson found no significant changes in concentrations for the first month of

storage at room temperature, 4 °C, or -10 °C for THC, 11-OH-THC, and THCCOOH in blood and plasma (146). However, THC and 11-OH-THC concentrations decreased significantly after storing for 2 months at room temperature. Blood stored at 4 °C showed no significant changes for 4 months, but extractions after 6 months demonstrated poor precision and inefficient extraction.

Skopp et al conducted several studies documenting free and glucuronidated serum cannabinoid stability (139, 149), concluding that *in vitro* stability of samples fortified with THCCOOH-glucuronide and stored at greater than -20 °C was compromised by formation of free THCCOOH. This called into question free THCCOOH as a reliable marker for recent cannabis consumption, as previously reported (139, 148). Studies of fortified samples could differ significantly from authentic whole blood and/or plasma specimens (145, 150-152). Therefore, characterization of cannabinoid stability in authentic specimens after cannabis smoking represents a major knowledge gap. Extensive protein binding in authentic specimens may limit loss via adsorption to container surfaces, but as blood ages, precipitant material forms. THC binds to surfaces and precipitant. To complicate the issue further, over time THC binding may change, releasing the drug back into the liquid phase. Pilot studies in our laboratory indicated that plasma protein binding in authentic specimens could be a critical determinant for *in vitro* cannabinoid stability. Indeed, we found that storage of fortified whole blood samples for 2 weeks at -20 °C produced cannabinoid concentration decreases of up to 20% (153).

Summary

Whole blood cannabinoid pharmacokinetics, phase II cannabinoid metabolite time course and cannabinoid stability in whole blood and plasma have not been characterized in authentic specimens collected after controlled cannabis administration. Furthermore, the relationship between cannabinoid pharmacodynamics and pharmacokinetics requires additional investigation. I developed a controlled smoked cannabis protocol to specifically address these knowledge gaps. The protocol was approved by the NIDA Institutional Review Board, an Investigational New Drug Application was obtained from the Food and Drug Administration and participants provided written, informed consent. For the first time ever, cannabinoids were quantified in whole blood and plasma within 24 h of collection by a novel LC-MS/MS method, minimizing *in vitro* stability concerns. This protocol was a major laboratory effort requiring resource dedication of two LC-MS/MS instruments to analyze plasma and whole blood specimens and four 2D-GC-MS instruments for oral fluid specimen analysis. Previous pharmacokinetic data (from our laboratory and others) were reported for authentic specimens frozen for months to years, leading to questions regarding stability of these concentrations over time. Therefore, we believe data from this protocol will be instrumental for interpreting blood cannabinoid concentrations in forensic DUID accident investigations and clinical monitoring scenarios, while furthering our understanding of potential markers of recent cannabis intake and *in vitro* cannabinoid stability.

Chapter 2 – Oral fluid, Plasma and Whole Blood Pharmacokinetics and Stability Following Smoked Cannabis (NIDA IRP Clinical Protocol 10-DA-N458)

Data presented in this dissertation were generated through an Institutional Review Board (IRB)-approved clinical research protocol conducted at the Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health (NIH/NIDA/IRP), in Baltimore MD from January 2011 through March 2011. The procedures and policies detailed are as submitted November 24, 2010.

Study Objectives

The primary objectives of the protocol were to (1) characterize the disposition of THC and its metabolites in oral fluid following smoked administration and determine whole blood and plasma/oral fluid ratios, (2) document stability of cannabinoids in authentic whole blood, plasma and oral fluid specimens over time and under different storage conditions, (3) assess the association between cannabis-induced performance impairment and/or risk-taking behavior with THC and metabolite concentrations in whole blood, plasma, and oral fluid, and (4) assess whether THC-glucuronide or other cannabinoid biomarkers are reliable indicators of recent smoked cannabis use.

Participants

A total of 10 cannabis users were needed to complete the study.

Inclusion Criteria

1. 18 to 45 years of age;
2. Cannabis use with a minimum frequency of at least twice per month during the three months prior to study entry;
3. A positive urine cannabinoid screen;
4. Peripheral veins suitable for repeated venipuncture and/or placement of an intravenous catheter;
5. Blood pressure (BP) and heart rate (HR) at or below the following values while sitting after five min rest: Systolic BP (SBP) 140 mm Hg, diastolic BP (DBP) 90 mm Hg, heart rate (HR) 100 bpm;
6. ECG and three-minute rhythm strip without clinically relevant abnormalities;

Exclusion criteria

1. History or presence of any clinically significant illness, as detected by history, physical examination, and/or laboratory tests, that might put the subject at increased risk of adverse events;
2. History of a clinically significant adverse event associated with cannabis intoxication;
3. Donation of more than 450 mL of blood within 30 days of study drug administration;

4. If female, pregnant or nursing;
5. Currently interested in or participating in drug abuse treatment, or participated in drug abuse treatment within 60 days preceding study enrollment.

Study Designs and Methods

Overview

This was a single session protocol in which participants smoked one cannabis cigarette, provided blood, oral fluid and urine specimens, performed neurocognitive tasks and completed subjective questionnaires.

Recruitment

Cannabis users were recruited from the community using print, radio, television and web-based advertisements previously approved by the NIDA IRB.

Screening Methods

Participants were first enrolled in a screening protocol (Protocol 10-DA-N415, Evaluation of Potential Research Subjects) and screened for study entry. Specific screening tests are detailed in Appendix A.

Study Design

Participants could complete the study session as outpatients or they could spend the night prior to and/or following drug administration at the Behavioral Pharmacology Research Unit (BPRU). Participants provided a negative urine drug screen if they did not

spend the evening prior to testing at BPRU. If the drug screen was positive for any illicit substances at the time of the study session, these participants were rescheduled in order to ensure lack of intoxication on neurocognitive and subjective measures. If participants resided on the BPRU prior to testing, urine test results were recorded but negative results were not required to proceed with the session. Participants had baseline measures collected on all parameters and whole blood, plasma, oral fluid and urine collected prior to *ad libitum* smoking of a single 6.8% THC cigarette. Whole blood, plasma, and oral fluid specimens were collected frequently after smoking until discharge. All urine specimens were collected *ad libitum*. No more than 450 mL of blood (for whole blood and plasma specimens) was collected from each participant throughout the study. Participants were discharged after passing a neuromotor exam no earlier than 6 h after cannabis smoking if vital signs were normal (SBP < 140, DBP < 90, HR < 100). Participants could be requested to stay overnight on the BPRU in the unlikely event that there were concerns for the subjects' safety due to prolonged intoxication. Whole blood, plasma and oral fluid specimens were utilized to characterize the disposition of cannabinoid biomarkers and to determine stability under different storage conditions (Appendix B). Biological fluid cannabinoid concentrations were correlated to neurocognitive, physiological, and subjective effects.

Cannabis Cigarettes

Standardized cannabis cigarettes were supplied by the NIDA Research Technology Branch. Based on gas chromatographic analysis, cigarettes contained approximately 6.8% or approximately 54 mg THC.

Study Procedures

Prior to the smoking session, participants completed risk-behavior trait assessments and neurocognitive task training to become acquainted with the tasks and normalize performance. Upon arrival for the smoking session, the neuromotor exam (Appendix C) was performed, baseline performance on three neurocognitive tasks and subjective parameters was assessed, and vital signs recorded. A urine pregnancy test was administered to females. Participants had an intravenous catheter placed to facilitate repeated blood draws. Participants had whole blood, plasma and oral fluid collected prior to smoking. One cannabis cigarette was smoked *ad libitum* within 10 min. Blood was collected at 8 times after the start of smoking (Appendix D). No more than 450 mL total blood was collected during the study session, and no more than 70 mL in any single collection. Oral fluid specimens were collected by expectoration and with the Quantisal collection device at the same times as blood. Oral fluid was collected by the Draeger DrugTest 5000 test cassette at 0.5 h before dosing and 1 h, 2 h, 3 h, 4 h, 6 h and 22 h after dosing. Urine was collected *ad libitum* throughout the study. Neurocognitive task performance was assessed at three time points after smoking. If participants spent the night after cannabis smoking, a final neurocognitive task assessment as well as blood, oral fluid and urine collection occurred approximately 22 h after dosing (prior to discharge). Participants were discharged if vital signs were normal (SBP < 140, DBP < 90, HR < 100) and they had successfully completed the post-session neuromotor exam.

The time commitment for each participant was approximately 10 h, unless they chose to reside on the BPRU prior to or after cannabis smoking. Participants were

required to have a negative urine drug screen and breathalyzer test on the morning of the study unless they stayed overnight at the BPRU the evening prior to the session.

Assessment of Risk Perception, Risk Taking, and Impulsivity Traits

Currently, relationships among risk perception, impulsivity, risk-taking (or risk propensity), and risk-reward decision-making (e.g., as measured by gambling tasks) are not well known. These constructs may consist of overlapping mixtures of more fundamental constructs (brain functions) which have a neurobiological basis (154), and may be somewhat differentially affected by various licit and illicit drugs, including THC. We used a variety of validated self-report questionnaire instruments to assess multiple aspects of these constructs, for correlation with BART performance and with acute cannabis effects.

Risk Perception Questionnaire

The risk perception questionnaire (155) consists of 40 different events, each accompanied by a 7-point response-option scale (Appendix E). For each event, the participants answered the question, "Compared to other adults of your age and gender, how likely is it that you will <fill in event here> at some point in your lifetime?" The response options are numbers between –3 and 3, in which 0 represents "equally likely"; –1, –2, and –3 represents "a little less likely," "less likely," and "much less likely," respectively; and 1, 2, and 3 represent "a little more likely," "more likely," and "much more likely," respectively. The numbers and operational phrases are provided on the scale. The 40 events differ with regard to controllability and are of three types: controllable, uncontrollable, and neutral. The degree to which participants in each group

judge themselves to be "less likely" than others to experience the individual events reflects the groups' level of optimism. Differences in levels of optimism across item types reflect the influence of controllability on the groups' optimistic biases.

During a brief introduction and practice phase, participants were instructed on how to complete the questionnaire. Two practice events were completed with the help of the interviewer before the participants began to answer the questionnaire. When the practice trials were completed, the interviewer read each event on the questionnaire aloud to each participant to control for differences in reading ability and to provide opportunities for the participants to ask questions about the events. The participants were assured they did not have to report their responses aloud and they did not have to answer any questions that made them feel uncomfortable.

Two versions of the questionnaire were created, differing only in the order in which the 40 items are presented, to control for the potential effect of item-order on participants' responses. Each participant was assigned randomly to complete one of the two versions. Test duration was approximately 10 min.

Self-Assessment of Risk Perception

The 6-item self-assessment of risk perception (156) is one of three short-form assessments (the others are in the Risk-taking and Impulsivity Assessments section) designed by Cherpitel and colleagues through factor analyses of data from a larger national alcohol survey (Appendix F). Subjects responded to the question "How likely is it that something bad will happen to you if you…" for 6 items (e.g., "…had sex with

someone you just met") using on a 5-point scale (1=Very Unlikely, 5=Very Likely). Test duration was approximately 2 min.

Impulsive Sensation-Seeking subscale from the Zuckerman-Kuhlman Personality Questionnaire

The Impulsive Sensation-Seeking subscale of the Zuckerman-Kuhlman Personality Questionnaire (ZKPQ) (157) has published normative data known to predict a variety of risky behaviors (Appendix G). Subjects answered 19 True/False questions (e.g., "I like doing things just for the thrill of it."; "Before I begin a complicated job, I make careful plans." Test duration was approximately 5 min.

Self-Assessment of Risk-Taking/Impulsivity

The 5-item risk-taking/impulsivity scale (156) is one of three short-form assessments designed by Cherpitel and colleagues through factor analyses of data from a larger national alcohol survey (Appendix F). Subjects responded to the question "How much does each of the following statements describe you?" for 5 items (e.g., "I often act on the spur of the moment without stopping to think.") using on a 4-point scale (1 = Not at all, 5 = Quite a lot). Test duration was 2 min.

Self-Assessment of Sensation-Seeking

The 4-item sensation-seeking scale (156) is one of three short-form assessments designed by Cherpitel and colleagues through factor analyses of data from a larger national alcohol survey (Appendix F). Subjects responded to the question "How much does each of the following statements describe you?" for 4 items (e.g., "I like to try new

things for the excitement.") using on a 4-point scale (1=Not at all, 5=Quite a lot). Test duration was approximately 2 min.

Barratt Impulsiveness Scale Version 11

The Barratt Impulsiveness Scale Version 11 (BIS-11) (158) is a widely-used 30-question self-report questionnaire designed to assess one's impulsiveness (Appendix H). All 30 questions are assessed on a 4-point scale: Never/Rarely (1 point), Occasionally (2 points), Often (3 points), or Almost Always/Always (4 points). Higher scores suggest greater impulsivity. Item scores were summed to give an overall impulsivity score. To minimize response bias, selected items were worded to so that higher scores suggest less impulsivity. Test duration was approximately 5 min.

Melbourne Decision Making Questionnaire

The Melbourne Decision Making Questionnaire is a 22-item self-report questionnaire (159) that assesses decision making style in terms of preferred coping patterns or strategies for dealing with decisional conflict (Appendix I). The questionnaire evaluates four factors in decision-making: vigilance (e.g., When making decisions, I like to collects lots of information), hyper-vigilance (e.g., I feel as if I'm under tremendous pressure when making decisions), buck-passing (e.g., I prefer to leave decisions to others), and procrastination (e.g., I put off making decisions). Scores were counted on a 3-point scale, including the items 'true for me' (2 points), 'sometimes true' (1 point), and 'not true for me' (0 points). Test duration was approximately 5 min.

Assessment of Cannabinoid Intoxication

Symptoms of cannabinoid intoxication were assessed prior to and following smoking the cannabis cigarette.

Subjective Effects

Subjective effects were assessed with seven 160-mm visual-analogue scales, taking about 1.5 min to complete, and thirteen five-point Likert scale items, taking about two min to complete (Appendix J). Both parts measured psychological and physical symptoms associated with cannabinoid intoxication likely to appear or change over several min to several h.

Neurocognitive Performance

Four neurocognitive tasks were used to assess tracking, divided attention, working memory and behavioral risk taking (70, 75-77, 160).

Critical Tracking Task

The critical tracking task (161) measures the subject's ability to control a displayed error signal in a 1st-order compensatory tracking task. Error appears as a horizontal deviation of a cursor from the midpoint on a horizontal, linear scale. Subjects have to null the error by returning the cursor to the midpoint by means of compensatory joy-stick movements. The frequency of cursor deviations, and therefore, its velocity, increase as a stochastic, linear function of time. The subject is required to make compensatory movements with a progressively higher frequency. Eventually his/her

response frequency lags the error signal by 180° and control is lost. The frequency at which control loss occurs is commonly called the critical frequency or lambda-c (λ_c). The test was repeated five times and the median lambda-c over the five trials was used as the dependent variable. Total test duration was 5 min.

Participants were trained accordingly before beginning the first test session (baseline measurement). The objective of the training was to obtain the participant's consistent optimal performance (λ_c).

1. When 5 trials are completed, a mean lambda score appeared on the screen for the set of 5 trials. This mean was derived from the 5 trials after removing the lowest and highest scores (i.e. the mean of the middle 3 scores was presented). This score for the set was recorded. There were 3 sets of 5 trials (15 total trials providing 3 mean lambda scores) in the training series.

2. When the training series was completed, the participant's performance should have become increasingly more consistent. At this point, the variability of each lambda obtained during the 3 sets was determined. The objective was for the lambda scores of the 3 sets to display < 15% variability relative to the mean.

3. In the event that three consecutive sets failed to obtain lambda variability < 15%, an additional set of trials was performed, and the lambda variability was re-calculated for the final three consecutive sets. This was repeated until variability < 15% was obtained.

4. The mean lambda score for the final set was then divided by two and this value was used during the divided attention task (see below).

Divided Attention Task

The divided attention task (162) assesses the ability to divide attention between two tasks performed simultaneously. The primary task is the same as the critical tracking task described above with the exception that the velocity of the error signal is kept constant at 50% of the subject's optimal performance ($\lambda_c/2$). Tracking error is measured by the absolute distance (mm) between the cursor's position and the center of the screen. The secondary task involves monitoring 24 single-digit numbers (0-9) that are displayed in the four corners of the screen. The numbers change asynchronously every 5 seconds. The subject is required to remove his/her foot from a pedal-switch as quickly as possible any time the target numeral "2" appears. Main dependent variables are tracking error (primary task), reaction time and number of correct target detections (secondary task). Test duration was 12 min.

Training on the divided attention task (DAT) portion of the test was done once after the critical tracking portion of training. After $\lambda_c/2$ was obtained during the CTT training, the participant practiced the DAT for approximately 12 minutes (one testing series), utilizing the $\lambda_c/2$ value obtained during the CTT training series. The practice time for this task was recorded.

Working Memory (n-Back Task)

The n-back task is a parametric working memory task to assess the effects of manipulating cognitive load on working memory performance. The variation for this study involved a delayed match-to-sample task, in which participants were asked to determine whether a given stimulus matched a stimulus presented in either the previous

trial (1-back), two trials previously (2-back), or three trials previously (3-back). The task also demanded that participants encode novel stimulus items at the same time as retrieval during 1-, 2-, and 3-back trials. In order to control for attentional mechanisms, participants also completed a number of 0-back trials, in which they simply responded to the current stimulus item. Test duration was approximately 15 min.

Participants were trained in the n-back task before beginning the first test session (baseline measurement). Training consisted of 5 trials each for the 4 n-back task levels (0-back, 1-back, 2-back, and 3-back) for a total of 20 trials. Each trial took approximately 30 seconds. The program instructed participants as to what task they were completing (1-back, 2-back, etc) prior to the beginning of each trial. Therefore, if no breaks were taken, overall training time was 10 minutes. Training lasted approximately 15 minutes under normal circumstances. Participants achieved a stable level of performance (mean correct responses of three consecutive trials within 15%), minimizing the effect of learning across sessions.

Behavioral Risk Assessment (Balloon Analogue Risk Task [BART])

The Balloon Analog Risk Task (BART) assessed risk-taking behavior: scores correlated with self-reports of risk-taking, sensation seeking behavior, impulsivity, and drug use (160). The BART also demonstrated acceptable test-retest reliability and stability over time within individual subjects (163). It has been used to assess risk-taking behavior in crack/cocaine dependent individuals (154), 3,4-methylenedioxymethamphetamine (MDMA) users (164) and individuals after

amphetamine administration (165). We are not aware of any studies that utilizing BART to assess risk-taking propensity following acute cannabis or THC administration.

The BART is a computerized task in which participants viewed a computer screen displaying a small, simulated balloon and a simulated balloon pump (Appendix K). Participants used a mouse to inflate the balloon by approximately one-eighth of an inch. With each click of the pump, participants earned 1 cent that was deposited into a temporary cache that could be seen by the participant. Participants are asked to inflate the balloon as much as possible, earning as much money as possible, before the balloon pops. If the balloon pops, the money held in the temporary cache is lost. However, if participants want to stop inflating the balloon, they click on the "Press to Collect $$$" button in order to move money from the temporary cache to a permanent bank. The amount of money in the permanent bank is displayed continuously on the screen. After the balloon pops or the money is collected, the next balloon appears. The task is repeated for a total of 20 balloons ("trials"). The number of pumps, amount of money earned and number of popped balloons serve as primary outcomes for quantifying risk-taking behavior.

In order to make the task as personally relevant as possible, participants earned real money (1 cent/pump, averaging 40 cents/balloon, $8 per session or about $40 over five sessions) as a result of the choices they made. Although we informed subjects that the rewards and penalties they incurred in the task translated into actual cash earned, it was made clear to them that they could not end up owing money to the researchers. Test duration was approximately 5 min for 20 balloons.

Specimen Collection and Analysis

The Chemistry and Drug Metabolism Section has a long history of studying drugs of abuse in both traditional and alternative specimen matrices. The Section has a number of analytical tools for measuring drugs and metabolites in biological matrices, including seven GC-MSs, five of which are equipped to do negative and positive chemical ionization and equipped with a Dean's switch and cryotrapping for 2-D analyses. Additionally, the Section has four liquid chromatography-tandem mass spectrometers (LC-MS/MS). Biological specimens were analyzed for cannabinoid biomarkers by immunoassay, GC-MS and LC-MS/MS.

Whole Blood/Plasma Specimens

Peripheral venous whole blood was collected 0.5 h prior to dosing. Depending on the volume desired, specimens were collected in 4, 6 and/or 10 mL green-top (sodium heparin) Vacutainer® tubes and stored on ice no more than 2 h prior to centrifugation to separate plasma or serum (Appendix D). Specimens were stored at room temperature, refrigerated at 4 °C or frozen at -20 °C until analysis, depending on the specific stability test (Appendix B). Less than 450 mL whole blood was collected during the entire study. No more than 70 mL blood was collected at a single time point.

Oral Fluid Specimens

Oral fluid specimens were collected with Quantisal™ oral fluid collection devices, Draeger DrugTest 5000 test cassettes, and by expectoration into polypropylene tubes. Up to 9 expectoration, Quantisal™ and DrugTest 5000 oral fluid specimens were

collected during the experiment. Specimens were stored refrigerated or at -20 °C until analysis as detailed in the stability experiment.

Urine Specimens

Participants collected all voided urine *ad libitum* in polypropylene bottles during their entire stay on the research unit. Specimens were kept in a refrigerator until retrieved and checked by research staff. Aliquots of urine specimens were stored at room temperature, refrigerated at 4 °C or frozen at -20 °C until analysis, depending on the specific stability test.

Stability Study Analyses

This protocol aimed to determine cannabinoid stability in multiple matrices under variable storage conditions, concentrations, and time intervals. As discussed in the introduction, comprehensive analysis of cannabinoid stability has not, to our knowledge, been undertaken in authentic specimens. This protocol was designed specifically to address this knowledge gap.

Cannabinoid Stability in Whole Blood and Plasma

As described in Appendix D, whole blood and plasma were collected at 0.25, 0.5, 1, 2, 3, 4 and 6 h after the start of cannabis smoking for pharmacokinetic analyses. In addition to the pharmacokinetic specimens taken at these times, additional specimens also were collected at 0.25, 0.5, 1, 2, 3, and 4 h for the stability study. Within 2 h of collection of these additional specimens, whole blood and plasma were combined into two pools for each participant: “high THC” whole blood and plasma pools consisiting of

the additional specimens taken at the 0.25, 0.5, and 1 h time points, and "low THC" pools consisting of the additional specimens taken at the 2, 3, and 4 h time points. After thorough mixing, a portion of these pools was aliquotted into Nunc cryotubes for storage and stability determinations, with a single tube utilized for each determination. Plasma and whole blood (at each concentration for each participant) were stored in the dark at three temperatures (room temperature, 4 °C and -20 °C) for various lengths of time as detailed in Appendix B and quantified on the appropriate day. A significant change in concentration with respect to time was evidence of degradation, and was followed with post-hoc testing to determine which times differed from baseline.

Cannabinoid Stability in Oral Fluid

As described in Appendix D, OF was collected at 0.25, 0.5, 1, 2, 3, 4 and 6 h after the start of cannabis smoking for pharmacokinetic analyses through expectoration and the Quantisal™ OF collection device. While pharmacokinetics analyses were the primary endpoint, we had minimal expectoration and Quantisal™ device specimen volume remaining after these analyses, allowing for a targeted cannabinoid stability study in oral fluid. For each matrix, oral fluid aliquots from all time points were combined into a pool. After thorough mixing, a portion of these pools was aliquotted into Nunc cryotubes for storage and stability determinations, with a single tube utilized for each determination. Oral fluid was stored in the dark at three temperatures (room temperature, 4 °C and -20 °C) for various lengths of time as detailed in Appendix B and quantified on the appropriate day.

Cannabinoid Stability in Urine

As described in Appendix D, urine was collected *ad libitum* throughout the study. Urine was utilized for cannabinoid stability analyses in this matrix as well as for determination of glucuronide biomarker pharmacokinetics. A portion of urine collected was combined into two pools for each participant: a "high THC" pool consisting of urine collected from 0.25-6 h post-smoking and a "low THC" pool consisting of the "high THC" pool diluted with blank urine to approximately 15 μg/L. After thorough mixing, these pools were aliquotted into Nunc cryotubes for storage and stability determination, with a single tube utilized for each determination. Urine was stored in the dark at three temperatures (room temperature, 4 °C and -20 °C) for various lengths of time as detailed in Appendix 2 and quantified on the appropriate day.

Specimen and Data Storage

All clinical and analytical data from Chemistry and Drug Metabolism (CDM) protocols are archived on the CDM server, nida-notation2, with back-ups created on digital analog tapes twice weekly.

Specimens were stored at 4 °C and -20 °C in walk-in refrigerators and freezers on the first (room 01B216B) and fifth floors (room 05A314C) of the BRC Building. After analysis, specimens were returned to the walk-in freezers if adequate specimen remained.

Risks and discomforts

Adverse Events Associated with Smoked Cannabis

Psychological effects

The main feature of recreational cannabis smoking is the euphoria or 'high'. This intoxication may be accompanied with decreased anxiety, alertness, depression and tension and increased sociability. However, cannabis also can produce dysphoric reactions, including severe anxiety and panic, paranoia and psychosis. These reactions are dose-related and more common in naïve users, anxious subjects and psychologically vulnerable individuals. In this study, subjects smoked the cannabis cigarette *ad libitum* so that the dose could be titrated to individual comfort level.

Accompanying the high, and often contributing to it, cannabis produces perceptual changes. Spatial perception is distorted and time perception is impaired. Hallucinations may occur with high doses (166-167). Cannabis may impair cognitive and psychomotor performance. Effects include slowing of reaction time, motor incoordination, deficits in short-term memory, difficulty in concentration and impairment in complex tasks which require divided attention. The effects are dose-related but can be demonstrated after relatively small doses (5-10 mg THC in a joint), even in experienced cannabis users, and have been shown in many studies across a wide range of neurocognitive and psychomotor tests (166).

Systemic effects

Cannabinoids produce a dose-related tachycardia that may reach rates of up to 160 beats/minute or more, although tolerance may develop with around-the-clock use. There is also a widespread vasodilatation and reddening of the conjunctivae, a characteristic sign of cannabis use (168). Postural hypotension and fainting may occur. Chronic cannabis smoking is associated with bronchitis and emphysema (166).

Adverse Events Associated with Study Measures

Blood collection

Venous blood sampling may cause pain, tenderness, bruising, or bleeding at the needle puncture site. Subjects may feel transient lightheadedness or dizziness, or lose consciousness (syncope), because of anxiety and a vasovagal reaction. This risk was minimized by performing venipuncture while subjects were seated and having them remain under staff observation until it was clear that there were no acute adverse effects from the procedure. The risk of infection was negligible because standard sterile technique was used. Placement of indwelling venous catheters posed a risk of infection or thrombophlebitis, which increased with duration of placement. This risk was minimized by use of careful sterile technique with prompt removal if there were clinically significant signs or symptoms such as tenderness, swelling, or redness. The risk of anemia was negligible because subjects had normal hematocrits and the total amount of blood collected (approximately 450 mL) was less than the amount collected during a routine blood donation.

Oral Fluid Collection

The only risk associated with oral fluid collection was dryness of the mouth.

Urine Collection

There were no adverse events associated with collection of urine.

Cognitive Testing

Risks of cognitive testing included boredom and anxiety while taking the tests.

Prevention/Minimization of Adverse Events

Participants were carefully screened medically and psychologically to exclude individuals who may be at increased risk for adverse events. Subjects were carefully monitored by trained staff as described below. Ample opportunity to take breaks during the questionnaire session minimized the risk of restlessness. Any abnormality, adverse event, or matter of concern was promptly evaluated as appropriate.

Participant monitoring

Participants' responses to smoked cannabis were closely monitored by clinical staff and study personnel. A NIDA-IRP physician was present during cannabis smoking and for the following 30 min. Thereafter, the physician was available on-call. In order to initiate dosing, a participant had to be normotensive (SBP < 140 and DBP < 90), and have a resting pulse < 100. Participants remained under staff observation, with heart rate, BP, and respiratory rate assessed every 30 min for 3 h and then hourly for an additional 3

h after smoking. Participants were discharged if vital signs were normal (SBP < 140, DBP < 90, HR < 100) and the post-session neuromotor exam was completed successfully. Participants were withdrawn from the study at their request or if the participant was unable or unwilling to comply with protocol requirements.

Outcome Measures

Primary outcome measures included the correlation between oral fluid and plasma cannabinoid concentrations. Secondary outcome measures included determination of cannabinoid biomarker concentrations in whole blood, plasma, oral fluid and urine; stability of these concentrations over time; performance on neurocognitive tasks; and subjective assessments.

The primary BART outcome measure was the average number of pumps across the 20 balloons (adjusted to include only those pumps that did not result in the balloon popping). Greater numbers of pumps indicated greater riskiness, as the amount of money increased with every pump. Change in the level of riskiness over time was assessed by comparing the average number of pumps across consecutive trials (trials were divided into two blocks of ten for this analysis). A second dependent measure, amount of money earned, produced results similar to those for the pump. The third dependent measure, number of explosions, was an index of the negative consequences of participants' willingness to take risks (pump the balloon more).

Statistical Analyses

Data Analysis

Pharmacodynamic Data Analysis

Pharmacodynamic effects evaluated included subjective (SES and 5 point Likert) and cognitive measures (n-Back, Critical Tracking, Divided Attention and Balloon Analogue Risk Tasks). The analysis consisted of one-way repeated-measures analyses of variance (ANOVA) to assess the effect of time on each outcome. A significant time effect was evidence for impairment, and was followed with post-hoc testing to determine which time points differed from baseline. Probability levels were expressed as two-tailed, and effects were considered significant if $p < 0.05$.

Pharmacokinetic and Specimen Stability Data Analysis

Pharmacokinetic parameters calculated for cannabinoid biomarkers included areas under the curve (AUC), maximum concentrations, time to maximum concentrations, and metabolite/parent ratios over time. To investigate the stability of cannabinoid concentrations after extended storage, a one-way repeated-measures ANOVA was performed to assess the effect of time for each metabolite. A significant time effect was evidence for degradation, and was followed with post-hoc testing to determine which time points differed from baseline (within 24 h of specimen collection).

As the analytical precision of cannabinoid concentrations was limited to ± 20% by the current quantification method, changes in concentration less than 20% from control analysis (within 24 h of specimen collection) were considered insignificant and indicative

of analyte stability. Statistical software (WinNonlin®) was used to calculate pharmacokinetic parameters and conduct ANOVA and post-hoc testing for analyte stability analysis.

Power Analysis

The primary outcomes for this study were the disposition of cannabinoids in oral fluid over time after smoked cannabis and the correlation between oral fluid and blood/plasma cannabinoid concentrations. Others (89) reported a correlation coefficient of approximately 0.85 between oral fluid and serum (log-log concentrations). Assuming an effect size of 0.85, a sample size of 10 subjects, and a two-tailed alpha of 0.05, the G-Power® (Version 3.0.10) power calculator (Universitat Kiel, Germany) estimated power to be approximately 0.94 for within-subject correlations (e.g., comparing plasma and oral fluid cannabinoid concentrations collected at a single time point). Pharmacodynamic effects from smoked THC in dosages less than or similar to those proposed for this study achieved statistical significance with a number of completers similar to that required for the oral fluid-plasma cannabinoid concentration correlation (55, 169-173). Therefore, we believed power was adequate for these secondary analyses based on published results from prior studies.

Accrual Number Request

Based on expected effect sizes and power analysis for primary outcomes, we needed 10 participants to complete this protocol. Assuming a completion rate of 67%, we requested an accrual ceiling of 15 participants.

Human subjects Protection

Equity of Subject Selection

Participants were recruited without regard to ethnic origin, sex, race, or religion. The recruitment target was based on the national population aged 18 years or older who were current (twice in the past month) cannabis users (derived from the 2003 National Household Survey on Drug Use and Health) and the Baltimore City Department of Planning 2000 census (174). Target enrollment was: 65% male, 64% African American, 32% Caucasian, and 9% Hispanic.

Exclusion Justification

Pregnant or nursing women were excluded because of possible risks of smoked cannabis to the fetus or nursing child and because the NIDA-IRP research unit cannot provide appropriate care to such subjects and their children. Subjects must have been at least 18 years old, i.e., adults able to give valid informed consent. Individuals older than 45 years old were excluded because of the increased likelihood of occult medical problems which could be exacerbated by THC. Educationally disadvantaged individuals were eligible as long as they had at least an estimated IQ $\geq$ 85 or an estimated 8^{th} grade reading level as assessed by the Wide Range Achievement Test (WRAT), ensuring adequate comprehension of the consent form and study procedures.

Qualifications of Investigators

Marilyn A. Huestis, Ph.D. - Principal Investigator- Chief, Chemistry and Drug Metabolism Section, Clinical Pharmacology and Therapeutics Research Branch, IRP,

NIDA/NIH; is an experienced clinical investigator and a clinical, analytical and forensic toxicologist with more than 42 years experience in the pharmacodynamics and pharmacokinetics of drugs of abuse and correlations of drug concentrations and behavior. She has experience in conducting drug administration studies of cannabis, THC, Sativex®, cocaine, codeine, MDMA, methamphetamine, methadone, buprenorphine and rimonabant. As principal investigator her responsibilities included, but were not limited to, study design and implementation, oversight of participant selection and training, drug administration, pharmacokinetic analyses, data analysis and interpretation, publication of study results, and obtaining subject consent. As Principal Investigator, she took overall responsibility for the consenting process.

David A. Gorelick, M.D., Ph.D. – Medically Accountable Investigator; is an experienced clinical researcher in the area of drug abuse, and is board-certified in psychiatry and addiction medicine. He served as medically responsible physician, supervised the recruitment and evaluation of participants to ensure compliance with protocol criteria, and had overall responsibility for their medical safety during the study, including drug administration. He contributed to study design and implementation, data analysis and interpretation, publication of study results, and obtaining subject consent.

David M. Schwope, M.S. – Pre-doctoral IRTA, University of Maryland, Baltimore; Chemistry and Drug Metabolism Section, Clinical Pharmacology & Therapeutics Research Branch, IRP, NIDA/NIH; has a B.S. in chemistry, an M.S. in forensic science, and two years of experience in animal performance toxicology. He is currently pursuing his doctorate in toxicology under the direction of Dr. Huestis. He

participated in protocol design, analysis, interpretation, and publication of data, training of subjects, and conducting study sessions.

Erin Karschner, Ph.D. - Post-doctoral fellow, Chemistry and Drug Metabolism Section, Clinical Pharmacology & Therapeutics Branch, IRP, NIDA/NIH; has a dual B.A. degree in biology and chemistry and a doctorate in toxicology. She participated in data analysis, interpretation, and publication of data, training of subjects, conducting study sessions and obtaining subject consent.

Garry Milman, Ph.D. – Post-doctoral IRTA, Chemistry and Drug Metabolism Section, Clinical Pharmacology and Therapeutics Research Branch, IRP, NIDA/NIH. Dr. Milman has a B.S. in chemistry, an M.S. in medicinal chemistry and received his doctorate in medicinal chemistry in 2007. He participated in analysis of study data, presentation of findings, and obtaining subject consent.

David Darwin, B.S. – Research Chemist, Chemistry and Drug Metabolism Section, Clinical Pharmacology & Therapeutics Research Branch, IRP, NIDA/NIH; has a B.S. in chemistry and 30 years of research experience at NIDA. His responsibilities included implementing and coordinating aspects of clinical procedures, training of research participants, conducting study sessions, assisting in IRB communications, and assisting in data publication.

Study Benefits

This study did not offer direct benefit to participants, but was likely to yield generalizable knowledge regarding cannabinoid pharmacokinetics and pharmacodynamics.

Summary of Risk

Participation in this study represented more than minimal risk.

Description of the consent process

Consent was obtained from participants by doctoral level co-investigators (Appendix L). The consent form was read to and discussed with the participant and any questions resolved. Before signing the consent form, participants completed a 10-question true/false test to determine their understanding of the procedures and risks. A score of at least 80% correct was required for enrollment in the study. If participants scored less than 80%, the test was repeated once after the material was reviewed. If participants did not score at least 80% on the second test, they could not participate in the study.

Data and Safety monitoring

The principal investigator and associate investigators were responsible for overall data and safety monitoring.

Reporting Adverse Events

Serious adverse events, unexpected adverse events, and non-serious adverse events were reported according to NIH guidelines.

Alternatives to Participation

Participants did not receive any treatment in this study. The alternative, therefore, was not to participate.

Participant Confidentiality

Confidentiality of participants was maintained at all times. Study records, data, and specimens identified subjects by ID number, not by name or other personally identifiable information. The code linking ID number with subjects' identity was kept confidential by investigators, and kept under lock and key when not in use. A federal certificate of confidentiality was obtained. This allowed investigators to withhold personally identifiable information from outside requestors such as police, courts, and subjects' relatives. No identifiable subject information was released outside the research team without written permission, with the following exceptions: 1) information needed by other clinical personnel or facilities to provide medical care to subjects; 2) audits or reviews of the research study by NIH, FDA, or other authorized federal agencies.

Conflict of interest

NIH guidelines on conflict of interest were distributed to all investigators. There were no conflicts to report.

Participant Remuneration

Volunteers were compensated for time and research-related inconveniences as indicated in Table 1.

	Compensation
Outpatient	
Intravenous catheter	$10
Neurocognitive testing ($20/hour for 10 h)	$200
Inpatient	
Overnight stay ($40/night for 1-2 nights)	$40-$80
Intravenous catheter	$10
Neurocognitive testing ($20/hour for 10 h)	$200
Subtotal	$210-$290
Completion incentive (10%)	$21-$29
BART Compensation ($0-$12.80/trial for 4-5 trials)	$0-$64
Travel expenses (RT to/from NIDA IRP)	$15
Training ($20/hour for 3 h)	$60
Total	$306-$458

Table 1. Protocol 10-DA-N458 Remuneration Table

Chapter 3 – Direct Quantification of Cannabinoids and Cannabinoid Glucuronides in Whole Blood by Liquid Chromatography Tandem Mass Spectrometry

(as published in *Analytical and Bioanalytical Chemistry*, 2011)

Abstract

The first method for quantifying cannabinoids and cannabinoid glucuronides in whole blood by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated. Solid-phase extraction followed protein precipitation with acetonitrile. HPLC separation was achieved in 16 min via gradient elution. Electrospray ionization was utilized for cannabinoid detection; both positive (Δ^9-tetrahydrocannabinol [THC], cannabinol [CBN]) and negative (11-hydroxy-THC [11-OH-THC], 11-nor-9-carboxy-THC [THCCOOH], cannabidiol [CBD], THC-glucuronide and THCCOOH glucuronide) polarity were employed with multiple reaction monitoring. Calibration by linear regression analysis utilized deuterium-labeled internal standards and a $1/x^2$ weighting factor, yielding R^2 values ≥ 0.997 for all analytes. Linearity ranged from 0.5-50 μg/L (THC-glucuronide), 1.0-100 μg/L (THC, 11-OH-THC, THCCOOH, CBD and CBN) and 5.0-250 μg/L (THCCOOH-glucuronide). Imprecision was ≤ 10.5% CV, recovery was ≥ 50.5% and bias within ± 13.1% of target for all analytes at three concentrations across the linear range. No carryover, endogenous or exogenous

interferences were observed. This new analytical method should be useful for quantifying cannabinoids in whole blood and further investigating cannabinoid glucuronides as markers of recent cannabis intake.

Introduction

Cannabis use substantially impacts public safety, as many individuals drive or operate complex equipment soon after self-administration. The National Highway Traffic Safety Administration (NHTSA) reported that in 2007, 8.6% of nighttime drivers tested positive for cannabinoids in blood and/or oral fluid, a rate almost 4 times higher than the percentage of drunk drivers with a blood alcohol concentration ≥ 0.8 g/L (175). While finding cannabinoids in blood or oral fluid does not necessarily imply impairment, windows of drug detection in these matrices are often short for occasional or moderate smokers (57, 176-177), increasing impairment probability.

Δ^9-tetrahydrocannabinol (THC) is the primary psychoactive component in cannabis and is metabolized via cytochrome P450 (CYP) 2C9 and 2C19 isozymes to several phase I metabolites, most prominently 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (THCCOOH) (178-179). THC and its phase I metabolites also undergo UDP-glucuronosyltransferase-catalyzed phase II metabolism to form cannabinoid glucuronides *in vivo* (44, 50, 180), facilitating excretion. Currently, little is known about cannabinoid glucuronide pharmacological activity or detection windows following cannabis intake, although others hypothesized that these glucuronides could serve as markers of recent cannabis intake due to a shorter half-life *in vivo* (181-182). Detection and quantification of these metabolites may provide scientific data permitting

researchers, physicians and law enforcement personnel to document recent cannabis intake.

Analysis of glucuronides by gas chromatography-mass spectrometry (GC-MS) is difficult as chemical derivatization requirements and volatility issues preclude direct detection and quantification. Therefore, analytical procedures for cannabinoids in urine (121, 183-184), blood (43), meconium (137, 185) and oral fluid (186) typically include expensive and time-consuming alkaline and/or enzymatic glucuronide hydrolysis to liberate cannabinoids prior to extraction and GC-MS analysis. However, these hydrolyses introduce multiple confounding issues, including, but not limited to poor chromatography (43) and variable hydrolysis efficiencies of the ether- and ester-linked glucuronide species (36, 43, 138, 187-188).

To circumvent hydrolysis and facilitate direct quantification of phase II cannabinoid metabolites, sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are required. Yet few LC-MS/MS methods are available for cannabinoids in whole blood (109-111), likely resulting from higher limits of quantification (LOQ) than typically achieved by GC-MS. Additionally, to date, these published methods do not included glucuronide metabolites. Furthermore, development of glucuronide analytical methods is hampered by a lack of commercially available native and isotopically-labeled cannabinoid glucuronide standards.

To this end, we developed and validated the first sensitive and specific LC-MS/MS method for simultaneous detection of free and glucuronidated cannabinoids in human whole blood. While whole blood is frequently the specimen collected in driving under the influence of drugs (DUID) cases and other investigations, to our knowledge no

studies directly investigated whole blood pharmacokinetics following smoked cannabis. Therefore, we will utilize this method to investigate *in vitro* cannabinoid stability, evaluate cannabinoid glucuronides as markers of recent cannabis intake and determine whole blood cannabinoid pharmacokinetics in order to provide a scientific database for researchers, clinicians and forensic toxicologists interpreting whole blood cannabinoid concentrations.

Experimental

Clinical Samples

A healthy cannabis smoker provided written informed consent to participate in a study investigating cannabinoid pharmacokinetics, *in vitro* cannabinoid stability and novel markers of cannabis intake following a single smoked cannabis dose. The Institutional Review Board of the National Institute on Drug Abuse, National Institutes of Health approved this protocol. Cannabis cigarettes contained 6.8% THC (*w/w*) or approximately 56 mg THC and were smoked *ad libitum* over a 10 min period following an overnight stay on a secure residential unit. Whole blood was collected with sodium heparin 0.5 h prior to, 0.25 h after and 1.0 h after the start of cannabis smoking. Blood was transferred to polypropylene storage tubes and stored refrigerated until analysis within 24 h.

Instrumentation

All experiments were performed on an AB Sciex 3200 Qtrap triple quadrupole mass spectrometer with a TurboV ESI source (AB Sciex, Foster City, CA). The mass

spectrometer was interfaced with a Shimadzu UFLCxr system consisting of two LC-20ADXR pumps, a SIL-20ACXR autosampler, and a CTO-20AC column oven (Shimadzu Corporation, Columbia, MD). Evaporation under nitrogen was completed using a TurboVap LV evaporator from Zymark (Hopkinton, MA).

Reagents

Standards and deuterated internal standards were purchased from Cerilliant (Round Rock, TX) except for THC-glucuronide that was from ElSohly Laboratories, Inc (Oxford, MS). Ammonium acetate, formic acid and acetonitrile (ACN) were obtained from Sigma-Aldrich (St. Louis, MO). Methanol was from Fisher Scientific (Fair Lawn, NJ). Ammonium hydroxide and glacial acetic acid were from Mallinckrodt Baker (Phillipsburg, NJ). Water was purified in house by an ELGA Purelab Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA). All solvents were HPLC grade or better. 200-mg, 6-mL Bond Elut Plexa solid-phase extraction (SPE) cartridges were utilized for preparing samples (Agilent Technologies, Culver City, CA). Blank human whole blood was evaluated for absence of cannabinoids prior to use.

Preparation of Standard Solutions

Individual stock solutions of 1.0 g/L THC, 11-OH-THC, THCCOOH, CBD and CBN, 100 mg/L THCCOOH-glucuronide and 10 mg/L THC-glucuronide were diluted with methanol to prepare calibration solutions. 11-OH-THC-glucuronide and di-glucuronide metabolites are not commercially available. A stock solution containing 10 mg/L of analytes other than THC-glucuronide was prepared in methanol and stored at -20 °C. Dilutions of the stock solution (adding in THC-glucuronide) created calibrators at

0.5, 1.0, 2.0, 5.0, 10, 20, 50, 100, and 250 µg/L when fortifying 25 µL of standard solution into 500 µL of blank human whole blood.

Quality control samples were prepared in methanol from different vials than utilized for preparing standards. Low-, medium-, and high-quality control samples were prepared across the linear dynamic range of the assay. Whole blood low-, medium-, and high-quality control target concentrations were: THC-glucuronide 1.5, 4.5, and 45 µg/L; 11-OH-THC, CBD, CBN, THC and THCCOOH 2.5, 7.5, and 75 µg/L; and THCCOOH-glucuronide 7.5, 22.5, and 225 µg/L, respectively. All quality control solutions were stored at -20 °C.

Stock internal standard solution was prepared by diluting 100 mg/L solutions of THC-d_3, 11-OH-THC-d_3, THCCOOH-d_9 and CBD-d_3 1:10 with methanol and storing at -20 °C. A 1:50 dilution of internal standard stock solution was prepared in methanol and 25 µL of the diluted solution was added to each 500 µL whole blood sample, providing a final internal standard concentration of 10 µg/L. Deuterated CBN, THCCOOH-glucuronide, and THC-glucuronide are not currently commercially available; THC-d_3 was utilized for CBN quantification and THCCOOH-d_9 for quantification of both glucuronides.

Procedures

Sample Preparation

Blank blood (0.5 mL) was pipetted into a 10-mL conical polypropylene tube (Sarstedt, Newton, NC). 25 µL of internal standard and either 25 µL of standard or quality control solution were added. 25 µL blank methanol was added to authentic

specimens. Ice-cold ACN (1.5 mL) was added drop-wise while vortexing. Tubes were capped and centrifuged (4000*g*, 4 °C) for 5 min. Supernatants were decanted into clean tubes, 4.5 mL 0.2% NH_4OH in de-ionized water (*v/v*) was added, and samples mixed immediately prior to SPE loading.

Solid Phase Extraction

Extraction columns were conditioned with 2 mL methanol and 2 mL de-ionized water. Samples were decanted onto conditioned columns and loaded by gravity. Columns were washed with 2 mL 79:20:1 de-ionized water:acetonitrile:glacial acetic acid (*v/v/v*) and then dried under full vacuum (≥ 30 kPa) for 20 sec. Analytes were eluted with two separate 1.5 mL aliquots of 1% glacial acetic acid in ACN (*v/v*) under gravity. Vacuum was briefly applied after both aliquots were collected. Eluents were collected in a 10-mL conical polypropylene tube and dried under nitrogen at 42 °C in a Zymark TurboVap evaporator. Samples were reconstituted in 150 µL of initial mobile phase (70:30 A:B), vortexed for 15 sec and transferred to 250 µL pulled-point glass inserts in autosampler vials.

Liquid Chromatography

Chromatographic separation was performed with an Ultra Biphenyl column (100 x 2.1 mm, 5 µm) fitted with an Ultra II Biphenyl guard cartridge (10 x 2.0 mm) (Restek Corp, Malvern PA). The autosampler temperature was 4 °C and column oven 40 °C throughout analysis. The injection volume was 25 µL. Gradient elution was performed with (A) 10 mM ammonium acetate in water adjusted to pH 6.15 (± 0.05) with formic acid and (B) 15% methanol in acetonitrile (*v/v*) at a flow rate of 400 µL/min. The initial

gradient conditions were 30% B, hold for 30 sec, then increase to 90% B at 6.0 min. 90% B was maintained for 7.5 min, at which time the column was re-equilibrated to 30% B over 0.75 min and held for 1.75 min. HPLC eluent was diverted to waste for the first 2.5 min and the final 9 min of analysis.

Mass Spectrometry

Mass spectrometric data were acquired with electrospray ionization (ESI). THC-glucuronide, THCCOOH-glucuronide, THCCOOH, 11-OH-THC and CBD were acquired in negative ionization mode while THC and CBN were acquired in positive ionization mode. MS/MS parameter settings (Table 2, compound-specific optimization) were optimized via direct infusion of individual analytes (500 µg/L in initial mobile phase) at 10 µL/min. Optimized source parameters were as follows: Gas (1) 0.31 MPa, Gas (2) 0.48 MPa, Curtain Gas 0.17 MPa, Source Temperature 650 °C. Three acquisition periods were employed, with dwell times of 150 ms for each MS/MS transition in the first, 100 ms for the second and 150 ms for the final period. Unit resolution was used for all experiments.

Data Analysis

Linear regression with $1/x^2$ weighting was employed for all analytes. Peak area ratios of target analytes and their respective internal standards were calculated for each concentration. Analyst Version 1.5 (AB Sciex, Foster City, CA) was utilized for all data collection and processing; statistical calculations were completed with GraphPad Prism 5 for Windows (GraphPad Software, La Jolla, CA).

Analyte	Q_1 Mass (m/z)[b]	Q_3 Mass (m/z)[b]	DP (V)	CE (V)	RT (min)
THCCOOH-glucuronide	**519.0**	**342.9**	-45	-31	3.1
	519.0	299.0	-45	-44	3.1
THC-glucuronide	**489.0**	**313.1**	-55	-38	3.2
	489.0	174.9	-55	-24	3.2
THCCOOH-d_9	**352.2**	**254.2**	-55	-38	4.3
	352.2	194.1	-55	-28	4.3
THCCOOH	**343.0**	**245.1**	-60	-36	4.3
	343.0	191.1	-60	-44	4.3
11-OH-THC-d_3	**332.1**	**271.2**	-50	-32	5.2
	332.1	314.2	-50	-26	5.2
11-OH-THC	**329.0**	**267.9**	-50	-38	5.3
	329.0	311.1	-50	-24	5.3
CBD-d_3	**316.1**	**248.1**	-50	-32	5.7
	316.1	182.2	-50	-26	5.7
CBD	**312.9**	**245.1**	-60	-28	5.8
	312.9	178.9	-60	-26	5.8
CBN	**311.2**	**223.1**	61	27	6.2
	311.2	178.3	61	81	6.2
THC-d_3	**318.3**	**196.2**	70	29	6.2
	318.3	123.1	70	43	6.2
THC	**315.2**	**193.2**	70	29	6.3
	15.2	123.1	70	43	6.3

Table 2. LC-MS/MS Parameters for Cannabinoids and Cannabinoid Glucuronides in Whole Blood

[a] Q_1: Quadrupole 1; Q_3: Quadrupole 3; DP: declustering potential; CE: collision energy; RT: retention time; THC: Δ^9-tetrahydrocannabinol; THCCOOH: 11-nor-9-carboxy-THC; 11-OH-THC: 11-hydroxy-THC; CBD: cannabidiol; CBN: cannabinol.

[b] Bold font denotes quantifier transition.

Validation

Specificity, sensitivity, linearity, intra- and inter-batch imprecision, bias, extraction efficiency, matrix effect, carryover, dilution integrity, endogenous and exogenous interferences and analyte stability were investigated to evaluate method integrity. Specificity was based on relative retention time, precursor mass, and fragment ion. Retention times for QC and authentic specimens were required to be within ± 0.2 min of the mean calibrator retention time. Transition peak area ratios for QC and authentic specimens were required to be within ± 20% of the mean peak area ratios for calibrators of each respective analyte.

Sensitivity was evaluated by determining limits of detection (LOD) and (LOQ). A series of decreasing concentrations of drug-fortified whole blood was analyzed to determine empirically LOD and LOQ. LOD was determined as the concentration with a signal-to-noise ratio of at least 3, transition peak area ratios within 20% of the mean calibrator ratio and acceptable chromatographic retention time and peak shape. LOQ was the lowest concentration with a signal-to-noise ratio of at least 10, acceptable bias and imprecision (within at least 20% of target concentration and relative standard deviation within at least 20%, $n = 6$), transition peak area ratios within 20% of the mean calibrator ratio and acceptable chromatographic retention time and peak shape.

Linearity of the method was investigated by calculation of the regression line by the method of least squares and expressed by the squared correlation coefficient (R^2). A $1/x^2$ weighting factor was applied to compensate for heteroscedasticity as evaluated

through residuals analysis. Linearity of each analyte was determined with at least five concentration levels, not including the blank matrix, on 4 separate days.

Imprecision and bias were evaluated at three QC concentrations spanning the dynamic linear range. Intra-batch imprecision (% CV) was evaluated by six determinations per concentration in 1 day. Inter-batch imprecision (% CV) was evaluated for two replicates per concentration on 10 days (n total = 20). One-way ANOVA was employed to evaluate inter-batch repeatability as detailed by Peters and Maurer (189); $p < 0.05$ indicated significance. Bias was determined comparing the mean measured concentration of six analyses to the target value and was expressed as the percent of target concentration.

Extraction efficiency (%) and matrix effect (%) for each analyte also were determined at low, medium, and high control concentrations according to the design proposed by Matuszewski et al (190). For determination of extraction efficiency, quality control standard solution was added prior to or following SPE. Extraction efficiency, %, was expressed as the mean analyte area of samples with control solution added before SPE (n = 6) divided by the mean analyte area of samples with control solution added after SPE (n = 6). Matrix effect was investigated by comparing analyte peak areas of extracted blank samples that were fortified after SPE versus analyte peak areas of neat samples prepared in initial mobile phase (30:70 A:B) at equivalent concentrations. Matrix effect was computed by dividing the analyte areas of blank samples fortified after SPE by areas of neat samples, expressed as percent.

Carryover was determined by injecting a negative specimen containing internal standard after a specimen containing two times the upper LOQ. As high concentrations

are sometimes observed in blood following cannabis smoking, dilution integrity (1:5 and 1:10) was assessed with three blank blood specimens fortified with high QC solution. Specimens were combined with additional blank whole blood at 1:5 and 1:10 ratios to yield a 500 µL sample. Internal standard was added and specimens were processed as normal.

Interference from endogenous whole blood compounds was assessed by fortifying aliquots from ten blank whole blood pools with low QC solution and evaluating calculated concentrations. Interferences from over 80 illicit and common therapeutic drugs, metabolites and related compounds were evaluated by adding potential interferents into whole blood aliquots fortified with low QC solution. A compound did not interfere if the low QC quantified within 20% of target and had stable retention times and correct transition ratios. All interferences (Table 3) were tested at 1000 µg/L except for the cannabinoids that were tested at 250 µg/L.

Hydrolysis of glucuronides during sample processing was evaluated with blank whole blood fortified to 50 µg/L THC-glucuronide and 250 µg/L THCCOOH-glucuronide. Quantifying THC and THCCOOH formed in these hydrolysis controls allowed the calculation of percent hydrolysis for glucuronide metabolites. THC-glucuronide and THCCOOH-glucuronide standards also were investigated individually for presence of THC and THCCOOH, respectively. Individual neat standards were evaporated, reconstituted in mobile phase and quantified against a neat calibration curve to quantify any free cannabinoids present.

2-ethyl-5-methyl-3,3-diphenylpyrroline	clonazepam	norbenzoylecgonine
3,4-(methylenedioxyphenyl)-2-butanamine	clonidine	norbuprenorphine
3,4-methylenedioxyamphetamine	clonipramine	norcocaethylene
3,4-methylenedioxyethylamphetamine	cocaethylene	norcocaine
3,4-methylenedioxymethamphetamine	cocaine	norcodeine
4-bromo-2,5-dimethoxyphenethylamine	codeine	norcotinine
4-hydroxy-3-methoxyamphetamine	cotinine	nordiazepam
4-hydroxy-3-methoxymethamphetamine	diazepam	norephedrine
6-acetylcodeine	diphenhydramine	norfluxetine
6-acetylmorphine	ethylamphetamine	normorphine
7-aminoclonazapam	flunitrazapam	noroxycodone
7-aminoflunitrazapam	fluoxetine	noroxymorphone
7-aminonitrazapam	flurazepam	oxazepam
8,11-dihydroxy-Δ^9-tetrahydrocannabinol	hydrocodone	oxycodone
8-hydroxy-Δ^9-tetrahydrocannabinol	hydromorphone	oxymorphone
acetaminophen	ibuprofen	paroxetine
acetylsalicylic acid	imipramine	pentazocine
alprazolam	lorazepam	phenycyclidine
amphetamine	methadone	*p*-hydroxyamphetamine
benzoylecgonine	methamphetamine	*p*-hydroxybenzoylecgonine
bromazepam	*m*-hydroxycocaine	*p*-hydroxycocaine
brompheniramine	morphine	*p*-hydroxymethamphetamine
buprenorphine	morphine-3-glucuronide	*p*-hydroxynorephedrine
caffeine	morphine-6-glucuronide	*m*-hydroxybenzoylecgonine
cannabigerol	nicotine	temazepam
cathinone	nitrazepam	*trans*-3'-hydroxycotinine
chlorpheniramine	propoxyphene	
N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine		
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine		

Table 3. Exogenous Interferences Investigated by Fortification into a Low Quality Control Sample

All compounds fortified at 1000 μg/L except cannabinoids fortified at 250 μg/L.

Analyte stability in whole blood (n = 5) was evaluated at three QC concentrations under three conditions: 16 h at room temperature (RT), 72 h at 4 °C and three freeze-cycles at -20 °C (23 h freeze, 1 h thaw at RT). Stability of extracted whole blood samples while in the 4 °C autosampler was evaluated over 24 h. Extracted low, medium, and high QC samples (n = 3 at each level) were analyzed immediately after extraction along with calibration standards. Another set of three low, medium, and high QC

samples were analyzed 24 h after extraction and subsequent storage in autosampler vials at 4 °C. All samples were quantified from the initial calibration curve.

Results and Discussion

Cannabinoids are the most commonly abused illicit drugs, and cannabinoid medications are utilized for an increasing number of indications, documenting the need for accurate, sensitive and robust cannabinoid quantification. Numerous analytical methods are available to quantify cannabinoids in human whole blood, with and without conjugate hydrolysis (130, 153, 191-193). However, these methods are limited to parent THC, phase I metabolites and other minor cannabinoids, and fail to consider implications of phase II metabolites. Specifically, factors such as poor hydrolysis efficiency (43, 138) and glucuronide instability (139) can introduce unnecessary (and potentially substantial) error into quantitative determinations. Direct identification and quantification of glucuronides negates these issues and can yield novel insight into glucuronide pharmacokinetics and glucuronide *in vitro* stability while possibly providing an opportunity to utilize cannabinoid glucuronides as markers of recent cannabis intake. The present method sensitively and specifically quantifies these glucuronides directly in addition to typical cannabinoids of interest, including minor cannabinoids CBD and CBN (Figure 3). Thus, this first analytical method for directly analyzing free and glucuronidated cannabinoids in the same whole blood specimen is a significant advancement in the detection and quantification of this important class of compounds.

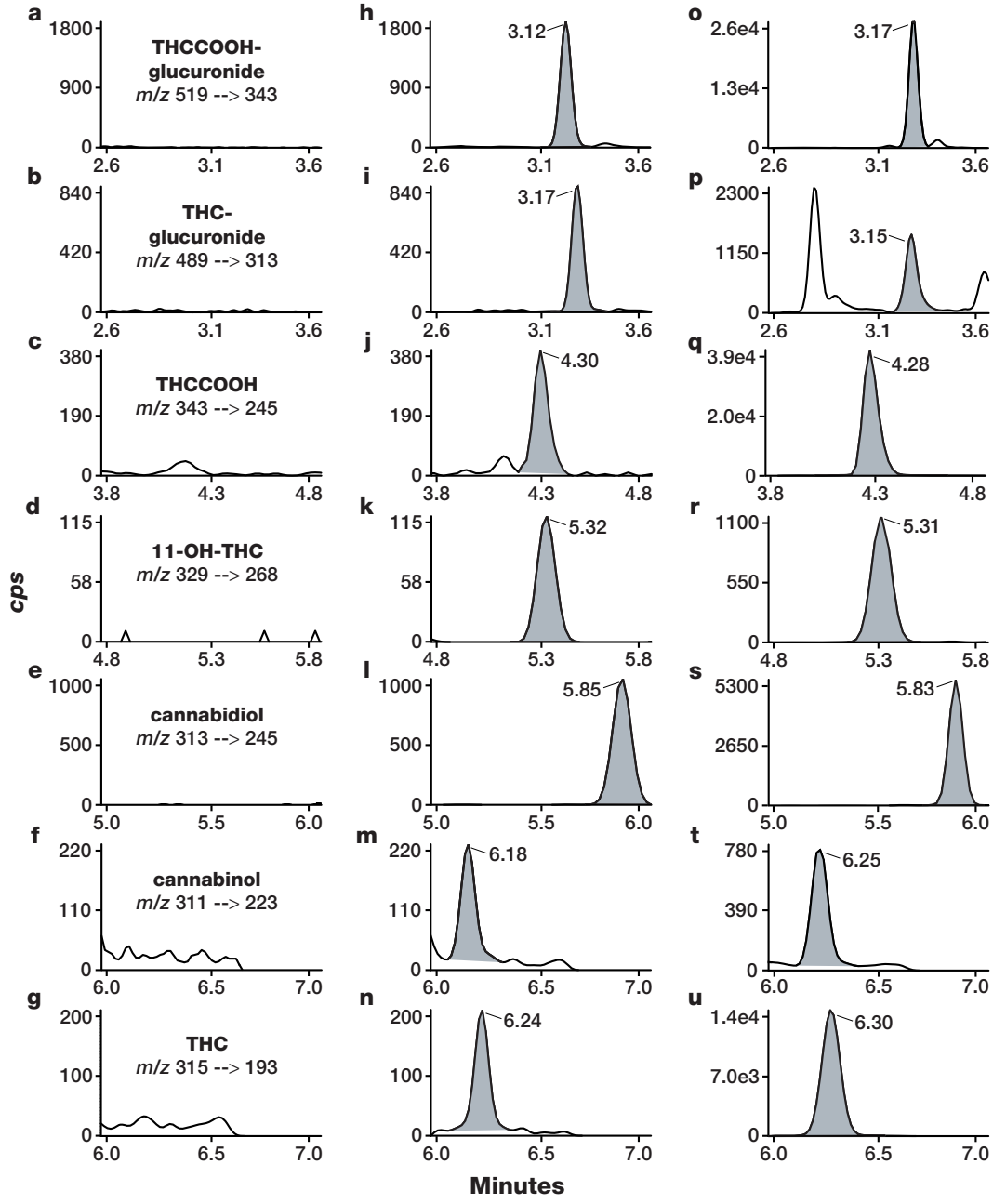


Figure 3. Selected MRM ion chromatograms of (a-g) extracted blank whole blood, (h-n) analytes at limit of quantification, and (o-u) a whole blood specimen 0.25 h after smoking a 6.8% THC (*w/w*) cannabis cigarette.

Limits of quantification (LOQ): Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), cannabidiol, cannabinol 1 μg/L; THC-glucuronide 0.5 μg/L and THCCOOH-glucuronide 5.0 μg/L. Authentic specimen concentrations are detailed in Table 8.

Calibration and Validation

The method was validated according to the criteria described in the Experimental Section. Table 4 details LOD, LOQ and calibration results for each analyte. LOQs were determined empirically through analysis of decreasing concentrations of drug-fortified whole blood and were 1 μg/L for THC, 11-OH-THC, THCCOOH, CBD and CBN with a 0.5 mL whole blood specimen, exceeding cutoff criteria proposed by Farrell et al (194) and meeting the 1 μg/L THC cutoff typically employed for driving under the influence of drugs (DUID) testing (195). To extend the dynamic linear range for THCCOOH-glucuronide and minimize the number of re-extractions that might be required due to high THCCOOH-glucuronide concentrations, a 250 μg/L calibrator was included for this analyte. However, extending the linear range required increasing the LOQ from 2.0 to 5.0 μg/L to meet *a priori* specifications for calibration curve linearity. Linear ranges and R^2 values ($1/x^2$ weighting) were acceptable ($R^2 > 0.990$) for all analytes. Linear ranges were THC-glucuronide 0.5-50 μg/L, THCCOOH-glucuronide 5.0-250 μg/L and THC, 11-OH-THC, THCCOOH, CBD and CBN 1.0-100 μg/L (Table 4); these ranges should prove useful for clinical and forensic casework. Calibrators for THC, 11-OH-THC, THCCOOH, CBD and CBN quantified within ± 15% (± 20% for LOQ and glucuronides) when quantified against the entire calibration curve. We expect that our ongoing clinical studies will help establish the utility of glucuronide metabolites for establishing recency of use, generating wider interest in cannabinoid glucuronide testing. Additional interest might prompt proper deuterated internal standards synthesis, allowing more stringent criteria (± 15%) to be applied to all analytes at concentrations > LOQ.

Analyte	Internal standard	LOD (µg/L)	LOQ (µg/L)	Slope ± SD (n = 4)	y-int ± SD (n = 4)[a]	R^2 ± SD (n = 4)	Linear range (µg/L)
THC[b]	THC-d_3	0.5	1.0	0.115 ± 0.013	0.015 ± 0.022	0.999 ± 0.001	1.0–100
11-OH-THC[c]	11-OH-THC-d_3	1.0	1.0	0.068 ± 0.003	0.002 ± 0.013	0.997 ± 0.002	1.0–100
THCCOOH[d]	THCCOOH-d_9	1.0	1.0	0.114 ± 0.006	0.019 ± 0.015	0.999 ± 0.001	1.0–100
CBD[e]	CBD-d_3	0.25	1.0	0.110 ± 0.010	0.001 ± 0.003	1.000 ± 0.000	1.0–100
CBN[f]	THC-d_3	1.0	1.0	0.180 ± 0.025	-0.016 ± 0.012	0.999 ± 0.001	1.0–100
THC-glucuronide	THCCOOH-d_9	0.25	0.5	0.303 ± 0.036	0.005 ± 0.011	0.999 ± 0.001	0.5–50
THCCOOH-glucuronide	THCCOOH-d_9	1.0	5.0	0.054 ± 0.010	-0.025 ± 0.025	0.998 ± 0.002	5.0–250

Table 4. Limits of Detection (LOD), Limits of Quantification (LOQ), and Calibration Results for Cannabinoids and Cannabinoid Glucuronides in Whole Blood by LC-MS/MS

[a] y-intercept. [b] Δ^9-tetrahydrocannabinol. [c] 11-hydroxy-THC. [d] 11-nor-9-carboxy-THC. [e] cannabidiol. [f] cannabinol.

Deuterium-labeled analogues are not currently commercially available for THCCOOH-glucuronide, THC-glucuronide and CBN. The decision to implement THC-d_3 and THCCOOH-d_9 for CBN and glucuronides, respectively, was based on similarities in extraction efficiency and matrix effects. This choice was not ideal as differences in efficiencies were present and these can vary depending on the matrix pool; nevertheless, *a priori* specifications for sensitivity and linearity were met. Other glucuronide metabolites, including morphine-3-glucuronide-d_3, buprenorphine-glucuronide and mefenamic acyl-β-D-glucuronide-d_3 were investigated as potential internal standards. However, these were either not extracted efficiently (buprenorphine-glucuronide) or not well-retained on our chromatographic system (morphine-3-glucuronide-d_3 and mefenamic acyl-β-D-glucuronide-d_3). While we attempted to minimize matrix effects through sample preparation including solid phase extraction, some matrix effect remained. The matrix effects for glucuronides and their respective internal standards were not identical, but our approach is the best available at this time. Furthermore, we investigated matrix effect in 10 different whole blood pools demonstrating that low QC quantification remained within ± 20% in all 10 whole blood pools. Despite these efforts, differential matrix effect cannot be excluded, and glucuronide quantification could be affected. It should be noted that deuterated glucuronide analogues are recommended should they become available, as improvements in imprecision, bias and reliability could be realized.

Bias and imprecision were evaluated at three concentrations across the linear dynamic range of each analyte. Intra-batch imprecision (% CV) was less than 7.9% for all analytes at all concentrations (n = 6); inter-batch imprecision (% CV) was less than

10.4% (n = 20). Bias, calculated as the percent of target concentrations at low, mid and high QC concentrations for each analyte, ranged from 93.8% to 113.1% of target concentrations (n = 6) (Table 5). One-way ANOVA yielded statistically significant differences in inter-batch repeatability for several analytes; however, differences were less than 10.4% CV and considered clinically insignificant.

Extraction efficiency for native and deuterium-labeled analytes ranged from 50.5% to 93.9% (Table 6). Table 6 also displays ion suppression/enhancement produced by matrix effect; positive values indicate ion enhancement and negative values indicate ion suppression. While substantial matrix effects were observed for 11-OH-THC, similar results were obtained for the corresponding deuterated analogue and quantification was not adversely affected.

Development of an effective sample cleanup that removed matrix interferences while maintaining high extraction efficiency proved to be the greatest challenge during method development. The extraction procedure (reversed-phase polymeric SPE), gentle wash step (20% acetonitrile in water) and polar elution solvent (acetonitrile) yielded high concentrations of phospholipids in extracts as evidenced through a positive precursor ion scan of *m/z* 184 as detailed by Xia and Jemal (196). Extending the 90% acetonitrile hold to 7.5 min during the chromatographic gradient provided effective column washing and removal of phospholipids; forgoing this wash yielded substantial increases in ion suppression for subsequent injections. In addition to high phospholipid concentrations, rapid increases in system backpressure were observed during initial method development with a smaller HPLC column particle size (3 μm) and methanolic mobile phase.

Analyte	Intra-batch imprecision (% CV, *n* = 6)			Inter-batch imprecision (% CV, *n* = 20)			Calculated Concentration (µg/L, *n* = 6)			Bias (% of target, *n* = 6)		
	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
THC[a,b]	4.8	2.8	3.5	8.6	9.8	7.4	2.8	28.0	71.8	110.8	112.1	95.7
11-hydroxy-THC	6.5	6.1	4.3	10.4	8.9	7.4	2.7	27.6	72.4	109.1	110.2	96.5
11-nor-9-carboxy-THC	7.9	4.2	5.3	5.5	7.2	7.4	2.6	26.5	73.4	104.6	106.1	97.8
cannabidiol	5.9	3.0	3.5	4.9	6.4	6.1	2.8	27.2	74.2	111.1	108.7	99.0
cannabinol	3.3	2.5	3.2	9.9	10.1	8.6	2.8	26.6	70.3	111.1	106.5	93.8
THC-glucuronide[c]	5.7	4.4	5.7	5.7	7.6	8.2	1.7	15.8	43.0	110.1	105.3	95.6
THCCOOH-glucuronide[d]	5.2	4.2	6.8	9.1	8.7	8.4	8.3	84.8	237.3	110.0	113.1	105.5

Table 5. Bias and Imprecision Data for Cannabinoids and Cannabinoid Glucuronides in Whole Blood by LC-MS/MS

[a] Δ^9-tetrahydrocannabinol. [b] THC, 11-hydroxy-THC, 11-nor-9-carboxy-THC, cannabidiol and cannabinol low-, mid- and high-quality control target concentrations were 2.5, 25 and 75 µg/L, respectively. [c] THC-glucuronide low-, mid- and high-quality control target concentrations were 1.5, 15 and 45 µg/L, respectively. [d] THCCOOH-glucuronide low-, mid- and high-quality control target concentrations were 7.5, 75 and 225 µg/L, respectively.

Analyte	Extraction efficiency (%, n = 6)			Matrix effect (%, n = 6)		
	Low	Mid	High	Low	Mid	High
THC[a,b]	50.5	57.9	75.6	-9.5	-8.1	0.0
11-hydroxy-THC	71.5	84.6	82.3	-56.4	-60.1	-66.6
11-nor-9-carboxy-THC	66.8	77.1	93.9	-10.5	-20.0	-32.1
cannabidiol	63.2	68.5	83.8	-16.8	-16.1	-30.1
cannabinol	56.4	63.2	76.5	-19.7	-12.8	-3.7
THC-glucuronide[c]	73.2	75.6	87.6	48.1	41.3	1.7
THCCOOH-glucuronide[d]	56.1	58.2	57.3	8.7	9.0	-10.3
THC-d_3	50.6	57.3	71.8	-11.3	-8.2	6.0
11-hydroxy-THC-d_3	79.9	84.2	79.3	-57.6	-59.3	-65.3
11-nor-9-carboxy-THC-d_9	70.9	77.6	87.3	-14.1	-17.8	-26.5
cannabidiol-d_3	63.3	67.0	81.2	-18.0	-16.8	-26.7

Table 6. Extraction Efficiency and Matrix Effect for Cannabinoids and Cannabinoid Glucuronides in Whole Blood by LC-MS/MS

[a] Δ^9-tetrahydrocannabinol. [b] THC, 11-hydroxy-THC, 11-nor-9-carboxy-THC, cannabidiol and cannabinol low-, mid- and high-quality control target concentrations were 2.5, 25 and 75 µg/L, respectively. [c] THC-glucuronide low-, mid- and high-quality control target concentrations were 1.5, 15 and 45 µg/L, respectively. [d] THCCOOH-glucuronide low-, mid- and high-quality control target concentrations were 7.5, 75 and 225 µg/L, respectively.

Backpressure increases were mitigated through replacement of methanol with acetonitrile, increasing column particle size to 5 μm and more frequent replacement of the guard column. Thus, slight decreases in resolution and cost-efficiency were offset by increased column life and a more reliable method.

Carryover in a negative specimen following a specimen containing twice the upper limit of quantification was assessed. No carryover was observed for any analyte; ion transition ratios were not within 20% of calibrators and any signal present was less than LODs. Common therapeutic and illicit drugs and metabolites at concentrations of 1000 μg/L (cannabinoids 250 μg/L) did not interfere with analytes of interest at the low QC concentration. Additionally, ten pools of whole blood were tested for potential endogenous interferences; none were observed in any pool for any analyte. Dilution integrity was maintained up to 10 times dilution with blank whole blood and all analytes quantified within 20% of the theoretical high QC concentration.

Quantification of THCCOOH and THC formed in glucuronide control samples during extraction was conducted (n = 6 each). Mean (SD) percentages of THCCOOH-glucuronide and THC-glucuronide hydrolysis were 0.6 ± 0.05% and 3.7 ± 0.35%, respectively. However, these are both likely artifacts as neat THCCOOH-glucuronide and THC-glucuronide calibrators were determined to contain 0.5 ± 0.1% THCCOOH and 3.2 ± 0.2% THC, respectively (n = 5 each). While the ester-linked THCCOOH-glucuronide was reported as relatively labile (139), we observed minimal hydrolysis of THCCOOH-glucuronide during extraction. The THC impurity has a minor effect on THC quantification that is less than the analytical error for the method and a low LOQ of 1 μg/L was achieved. To confirm a lack of substantial effect on THC quantifications,

samples fortified with only THC at the LOQ (1 μg/L) were quantified against the entire calibration curve containing all analytes. Acceptable quantifications were obtained (± 20%) for these samples, confirming minimal bias resulting from the THC impurity present in the THC-glucuronide standard.

Stability at 4 °C on the autosampler for 24 h was determined for extracted specimens. All analytes at all concentrations (low, mid and high QC) were stable under these conditions, with mean concentrations differing from samples injected immediately (n = 3) by less than -8.3% (Table 7). For fortified whole blood samples, THCCOOH, 11-OH-THC and both glucuronides were stable in under all other conditions tested (three freeze-thaw cycles, 72 h at 4 °C and 16 h at RT). However, losses up to 35.7% were observed for THC after 72 h at 4 °C. Additionally, CBD, CBN and THC demonstrated relative instability under three freeze-thaw cycles, 72 h at 4 °C and 16 h at RT. It should be noted that these losses were observed in fortified samples; losses in authentic specimens may not reflect these findings due to differences in protein binding (43).

Application of Method

Whole blood was collected from a clinical research participant prior to and after smoking a single cannabis cigarette *ad libitum*. Baseline concentrations were less than LOQ for all cannabinoids except THCCOOH and THCCOOH-glucuronide. 15 and 60 min after the start of smoking, blood was collected and concentrations determined by this new analytical method (Table 8). THC-glucuronide quantified at 0.6 μg/L in the first specimen, demonstrating the necessity for the low LOQ that this method achieved.

Analyte	24 h autosampler (% difference, *n* = 3)			72 h 4 °C (% difference, *n* = 3)			16 h RT[b] (% difference, *n* = 3)			3 Freeze/thaw cycles (% difference, *n* = 3)		
	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
THC[c,d]	2.1	0.9	3.6	-32.1	-35.7	-33.0	-32.9	-21.9	-21.9	-30.4	-20.9	-17.8
11-hydroxy-THC	-2.9	-4.0	-3.7	1.5	-4.9	-5.2	-19.5	1.6	0.0	-5.6	1.5	-0.9
11-nor-9-carboxy-THC	-6.9	1.8	3.7	2.1	1.2	1.5	4.7	7.6	10.4	9.2	6.8	7.6
cannabidiol	1.3	0.4	-0.3	-27.6	-29.5	-28.6	-13.5	-14.3	-15.7	-12.1	-17.9	-13.3
cannabinol	4.9	-4.6	-2.0	-5.9	-12.8	-13.2	-14.7	-21.2	-20.6	-8.0	-21.2	-20.0
THC-glucuronide[e]	-8.3	-1.5	4.7	-11.3	-6.2	-9.2	5.3	8.0	5.9	-2.9	-2.2	-2.4
THCCOOH-glucuronide[f]	-6.6	-2.2	3.8	-4.7	5.2	5.0	13.1	14.6	7.3	7.6	10.5	5.3

Table 7. Stability Data for Cannabinoids and Cannabinoid Glucuronides in Whole Blood by LC-MS/MS[a]

[a] Positive values indicate % increase from theoretical, negative values indicate % loss from theoretical. [b] Room temperature [c] Δ^9-tetrahydrocannabinol. [d] THC, 11-hydroxy-THC, 11-nor-9-carboxy-THC, cannabidiol and cannabinol low-, mid- and high-quality control target concentrations were 2.5, 25 and 75 µg/L, respectively. [e] THC-glucuronide low-, mid- and high-quality control target concentrations were 1.5, 15 and 45 µg/L, respectively. [f] THCCOOH-glucuronide low-, mid- and high-quality control target concentrations were 7.5, 75 and 225 µg/L, respectively.

Analyte	Baseline[a] (µg/L)	0.25 h[b] (µg/L)	1.0 h[b] (µg/L)
THC[c]	< LOD[d]	54.8	13.4
11-hydroxy-THC	< LOD	7.2	4.1
11-nor-9-carboxy-THC	12.5	59.2	51.9
cannabidiol	< LOD	2.1	< LOQ[e]
cannabinol	< LOD	2.9	< LOD
THC-glucuronide	< LOD	0.6	< LOQ
THCCOOH-glucuronide	33.0	38.1	96.0

Table 8. Cannabinoids and Cannabinoid Glucuronides Quantified in Whole Blood Collected from a Volunteer during a Controlled Smoked Cannabis Administration Study

[a] Baseline samples collected 0.5 h prior to the start of *ad libitum* smoking of a single 6.8% THC (*w/w*) cannabis cigarette. [b] Time from the start of smoking. [c] Δ^9-tetrahydrocannabinol. [d] Concentration below method limit of detection. [e] Concentration below method limit of quantification.

It should be noted that specimens were analyzed within 24 h of collection, minimizing any potential losses due to analyte degradation. Concentrations suggest THC-glucuronide may serve as possible marker of recent cannabis intake, given that it is detectable following cannabis smoking, albeit at a low concentration. Further research is required to assess detection windows for THC-glucuronide or other minor cannabinoids, such as CBD or CBN, following smoked cannabis.

Conclusions

This method is the first robust, sensitive and specific LC-MS/MS technique for direct detection and quantification of several cannabinoids and two cannabinoid glucuronides in human whole blood, yielding a comprehensive cannabinoid whole blood profile following cannabis intake. The rapid and simple extraction and 16 min analysis are beneficial; however, care should be taken to prevent buildup of phospholipids and other matrix components, leading to increased HPLC backpressure and loss of resolution. This method is utilized for several controlled cannabinoid administration studies and will provide whole blood pharmacokinetic and cannabinoid stability data useful to clinicians and forensic toxicologists interpreting whole blood cannabinoid concentrations often obtained during DUID cases and other investigations. This new analytical method for cannabinoids in whole blood offers advantages in sensitivity and spectrum of cannabinoid analytes included over existing LC-MS/MS and GC-MS assays, and when applied to controlled cannabinoid administration studies, may improve our ability to interpret cannabinoid whole blood results.

Chapter 4 – Identification of Recent Cannabis Use: Whole Blood and Plasma Free and Glucuronidated Cannabinoid Pharmacokinetics Following Controlled Smoked Cannabis Administration

(as published in *Clinical Chemistry*, 2011)

Abstract

BACKGROUND: Δ^9-tetrahydrocannabinol (THC) is the most frequently observed illicit drug in accident and driving under the influence of drugs investigations. THC-glucuronide has been suggested as a marker of recent cannabis use, but there are no blood data following controlled THC administration to test this hypothesis. Furthermore, there are no studies directly examining whole blood cannabinoid pharmacokinetics, although this matrix is often the only available specimen.

METHODS: Participants (9 men, 1 woman) resided on a closed research unit and smoked *ad libitum* one 6.8% THC cannabis cigarette. THC, 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), cannabidiol (CBD), cannabinol (CBN), THC-glucuronide and THCCOOH-glucuronide were directly quantified in whole blood and plasma by liquid chromatography-tandem mass spectrometry within 24 h of collection to obviate stability issues.

RESULTS: Median whole blood (plasma) observed C_{max} were 50 (76), 6.4 (10), 41 (67), 1.3 (2.0), 2.4 (3.6), 89 (190), and 0.7 (1.4) μg/L 0.25 h after starting smoking for THC, 11-OH-THC, THCCOOH, CBD, CBN, and THCCOOH-glucuronide, respectively, and 0.5 h for THC-glucuronide. At observed C_{max}, whole blood (plasma) detection rates were 60% (80%), 80% (90%) and 50% (80%) for CBD, CBN and THC-glucuronide, respectively. CBD and CBN were not detectable after 1 h in either matrix (LOQ 1.0 μg/L).

CONCLUSIONS: Human whole blood cannabinoid data following cannabis smoking will assist whole blood and plasma cannabinoid interpretation, while furthering identification of recent cannabis intake.

Introduction

Cannabis intake substantially impacts public safety since many individuals drive or operate complex equipment soon after self-administration. Indeed, cannabis is the most common illicit substance detected in blood and oral fluid of surveyed drivers (175). Drug presence does not necessarily imply impairment; however, cannabis detection windows in these matrices are relatively short for less than daily cannabis smokers (57, 176-177), increasing impairment probability following a positive test.

The primary psychoactive chemical in cannabis, Δ^9-tetrahydrocannabinol (THC), is metabolized via cytochrome P450 (CYP) 2C9 and 2C19 isozymes to several phase I metabolites, most prominently 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (THCCOOH) (178-179). These analytes undergo phase II metabolism by uridine 5'-diphospho-glucuronosyltransferase (UGT) to produce cannabinoid glucuronides *in*

vivo (44, 50, 180). Glucuronide formation facilitates excretion, but cannabinoid glucuronide pharmacological activity and detection windows following cannabis smoking are unknown, although these metabolites have been hypothesized to be markers of recent (181) or frequent (182) use. Characterization of major and minor cannabinoids in blood or plasma following cannabis smoking may enable researchers, physicians and law enforcement personnel to document recent cannabis intake, identify driving under the influence of cannabinoids (DUID) or determine cannabinoid contributions to accident causation.

No studies, to our knowledge, have directly examined whole blood cannabinoid pharmacokinetics in humans following cannabis smoking, although this matrix is often employed for forensic and DUID work. Additionally, we know of no studies that have examined THC- or THCCOOH-glucuronide in whole blood or plasma following controlled, smoked cannabis administration. Plasma glucuronide-free ratios for THCCOOH following a single oral cannabis dose have been investigated (197), as have free and conjugated plasma THCCOOH concentrations following 5-mg intravenous THC (49). Other studies have examined glucuronides after self-reported administration (182, 198) or oral THC (dronabinol) dosing (43). Glucuronide concentrations typically have been determined indirectly by calculating the difference between total and free concentrations, with total analyte concentrations obtained following enzyme and/or alkaline hydrolysis.

In this study, we directly characterized for the first time free and glucuronidated THC and THCCOOH, 11-OH-THC, cannabidiol (CBD) and cannabinol (CBN) in cannabis smokers following a single smoked cannabis cigarette. Authentic whole blood

and plasma samples were simultaneously collected before and after smoking. These data provide the an accurate and comprehensive cannabinoid metabolic profile in humans following cannabis smoking, yielding further insight into cannabinoid metabolism and documenting windows of drug detection for novel markers of recent cannabis smoking.

Materials and Methods

Participants

Participants provided written informed consent for this Institutional Review Board- approved protocol. Inclusion criteria for participants were age 18-45 y, cannabis smoking at least twice monthly for 3 months prior to entry, positive urine cannabinoid test, normal cardiac function, and veins suitable for intravenous catheter placement. Clinically significant medical or psychiatric disease, physical dependence other than cannabis, pregnancy or nursing (if female), history of cannabis-related psychosis or seizure, drug treatment request or blood donation in the previous 30 d were exclusionary. Participants were admitted to the secure research unit 15-20 h prior to dosing, with no cannabis use restrictions enforced prior to admission.

Smoked Cannabis Administration and Blood Collection

Cannabis cigarettes were obtained through the NIDA Chemistry and Physiological Systems Research Branch and independently assayed to contain 6.8 ± 0.2% THC, 0.25 ± 0.08% CBD and 0.21 ± 0.02% CBN (w/w). Mean cigarette weight was 0.79 ± 0.16 g, yielding total THC, CBD and CBN content of 54, 2.0 and 1.7 mg per cigarette, respectively. Participants smoked a single cannabis cigarette *ad libitum* for 10 min.

Venous blood samples were collected via indwelling intravenous catheter into sodium heparin blood tubes on ice 0.5 h prior to and 0.25, 0.5, 1, 2, 3, 4, and 6 h after the start of smoking. Participants could optionally stay a second night with an additional blood collection at 22 h. Blood collected for plasma was centrifuged (1600*g*, 15 min) and plasma separated within 2 h. All samples were stored in polypropylene tubes at 4 °C until analysis within 24 h of collection.

Cannabinoid Analysis

Cannabinoids were quantified by a previously validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method (199). Briefly, 1.5 mL acetonitrile was added to 0.5 mL specimen (whole blood or plasma) to precipitate proteins. After mixing, the sample was centrifuged and the supernatant diluted and subjected to solid-phase extraction. The eluent was evaporated and reconstituted in mobile phase. Extracts were chromatographed via gradient elution at 400 μL/min and detection was via electrospray ionization. Limits of quantification (LOQ) were 1 μg/L for THC, 11-OH-THC, THCCOOH, CBD, and CBN, 0.5 μg/L for THC-glucuronide and 5 μg/L for THCCOOH-glucuronide.

Calculations and Statistical Analysis

Non-compartmental pharmacokinetic analyses were performed with WinNonlin Professional 5.2 for Windows (Pharsight Software, St. Louis, MO). C_{max} and T_{max} calculations included data only from participants with quantifiable analyte concentrations. One-way ANOVA with Tukey's HSD post-hoc testing for molar glucuronide-free ratios utilized SPSS® 15.0 for Windows (Chicago, IL); all other

descriptive statistical calculations were performed with GraphPad Prism 5.2 for Windows (GraphPad Software, La Jolla, CA).

Results

Samples

Participant demographics and self-reported cannabis use histories are detailed in Table 9. There was a wide range in age (18.5-45.7 years), BMI (18.1-32.0 kg/m^2) and self-reported joints smoked in the previous 14 d (10-168 joints). Ten participants (nine male, one female) completed the protocol; six stayed for an additional night, yielding n = 6 for the 22 h time point. Samples (n = 85 each matrix) were collected without difficulty or appreciable hemolysis in all but participant I, where the 0.25 h specimen was not obtained due to catheter dislodgement.

Cannabinoid Disposition Following Cannabis Smoking

Median (range) baseline whole blood concentrations at 0.5 h prior to smoking were < LOQ (< LOQ-5.9) μg/L for THC, < LOQ (< LOQ-2.0) μg/L for 11-OH-THC, 10.5 (5.2-66) μg/L for THCCOOH, and 39.0 (23-160) μg/L for THCCOOH-glucuronide. Median (range) plasma concentrations at 0.5 h prior to smoking were 1.6 (< LOQ-7.3) μg/L for THC, < LOQ (< LOQ-2.3) μg/L for 11-OH-THC, 17 (8.2-100) μg/L for THCCOOH and 83 (46-450) μg/L for THCCOOH-glucuronide. THC-glucuronide, CBD and CBN were < LOQ for all participants in whole blood and plasma samples prior to smoking.

Subject	Sex	Ethnicity	Age (y)	BMI[a] (kg/m^2)	Mean available dose (µg/kg)[b]	Days since last cannabis	Typical joints/day	Days cannabis use/ last 14	Age 1st cannabis use (y)	22 h sample?
A	M	Caucasian	37.1	18.1	1000	n/a[c]	6	14	22	Yes
B	M	African American	26.5	30.6	560	3	2	10	16	Yes
C	M	Caucasian	45.7	32.0	480	1	5	14	12	Yes
D	F	Caucasian	27.7	22.3	980	1	6	8-9	15	Yes
F	M	Caucasian	34.1	23.0	720	2	1	10	16	No
G	M	Caucasian	22.2	22.9	560	1	12	14	15	No
H	M	African American	18.5	22.3	740	2	6	9	13	Yes
I	M	African American	41.5	25.5	690	3	6	12	19	Yes
K	M	Caucasian	22.5	20.9	710	4	2	13	16	No
L	M	African American	30.7	30.6	590	1	3	9	15	No
		Mean	30.6	24.8	700	2.0	4.9	11.7	15.9	
		Standard Deviation	8.9	4.7	170	1.1	3.2	2.2	2.8	

Table 9. Demographics and self-reported cannabis smoking characteristics for 10 adult cannabis smokers in Protocol 10-DA-N458

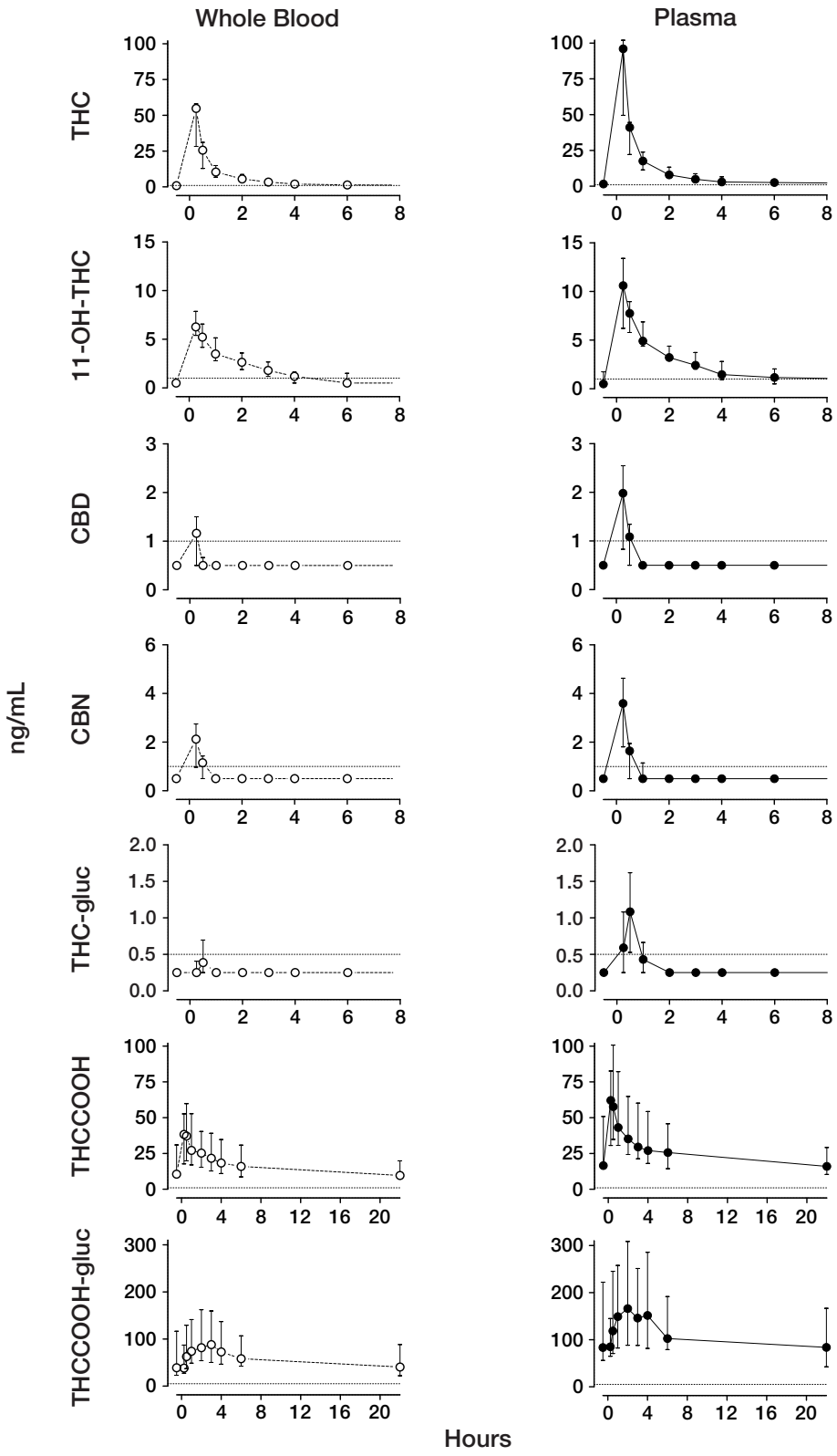
[a] BMI = weight (kg)/height^2 (m). [b] Average amount of THC contained in cigarette (µg) divided by participant weight (kg). [c] Data not collected

Whole blood and plasma concentrations for all analytes were substantially higher than baseline 0.25 h after starting smoking (Figure 4), although there was high inter-individual variability for all analytes due to differences in *ad libitum* smoking topography and/or cannabinoid metabolism. Table 10 details the highest observed concentrations (C_{max}) and times to C_{max} (T_{max}) for each matrix. Minor cannabinoids (CBD, CBN and THC-glucuronide) were not detected in all participants' whole blood or plasma after cannabis smoking; however, when detected, maximum whole blood (plasma) concentrations were 2.1 (3.4), 2.9 (4.7) and 0.8 (2.3) μg/L, respectively.

Following T_{max}, whole blood and plasma concentrations decreased rapidly for all analytes except THCCOOH-glucuronide; concentrations for this analyte remained increased for approximately 3-4 h after smoking. From 6 h post-smoking, median whole blood and plasma THCCOOH-glucuronide concentrations decreased gradually, returning to baseline by 22 h. Within 1 h, median whole blood concentrations for CBD, CBN and THC-glucuronide were < LOQ and median plasma concentrations were < 2 μg/L for these analytes.

Figure 4. Median (inter-quartile range) whole blood (o) and plasma (•) concentrations following smoking of a 6.8% THC cannabis cigarette.

Samples collected at -0.5, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 and 22 h after starting smoking. Dotted lines indicate limits of quantification: 1 μg/L for Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), cannabidiol and cannabinol; 0.5 μg/L for THC-glucuronide (THC-gluc) and 5 μg/L for THCCOOH-glucuronide (THCCOOH-gluc).


Whole Blood
Plasma
THC
11-OH-THC
CBD
CBN
THC-gluc
THCCOOH
THCCOOH-gluc
ng/mL
Hours

Analyte	n[a]	T_{max}[b] (h)	Apparent C_{max}[c] (µg/L)	$C_{1\ h}$[d] (µg/L)	$C_{6\ h}$[e] (µg/L)	$AUC_{0\text{-}6\ h}$[f] (h*µg/L)
Whole Blood						
THC[g]	10	0.26 (0.25-0.50)	50 (13-63)	10 (3.3-25)	1.4 (< LOQ-7.4)	60 (14-110)
11-OH-THC[h]	10	0.26 (0.25-0.50)	6.4 (3.2-8.8)	3.5 (1.5-6.3)	< LOQ (< LOQ-2.0)	11 (3.9-23)
THCCOOH[i]	10	0.40 (0.25-2.0)	41 (19-80)	27 (11-72)	16 (6.4-39)	140 (61-360)
CBD[j]	6	0.25 (0.25-0.50)	1.3 (< LOQ-2.1)	< LOQ[l]	< LOQ	NC[m]
CBN[k]	8	0.27 (0.25-0.50)	2.4 (< LOQ-2.9)	< LOQ (< LOQ-1.4)	< LOQ	NC
THC-glucuronide	5	0.50 (0.48-0.50)	0.7 (< LOQ-0.8)	< LOQ	< LOQ	NC
THCCOOH-glucuronide	10	2.0 (0.98-3.0)	89 (46-220)	74 (30-220)	58 (21-140)	440 (180-1100)
Plasma						
THC	10	0.26 (0.25-0.50)	76 (18-110)	18 (5.2-28)	2.6 (1.0-9.2)	110 (25-135)
11-OH-THC	10	0.26 (0.25-0.50)	10 (4.0-16)	4.9 (2.0-14)	1.2 (< LOQ-3.0)	19 (7.5-42)
THCCOOH	10	0.50 (0.25-0.52)	67 (27-110)	43 (19-87)	26 (9.6-61)	210 (93-480)
CBD	9	0.25 (0.25-0.50)	2.0 (< LOQ-3.4)	< LOQ (< LOQ-1.1)	< LOQ	NC
CBN	10	0.25 (0.25-0.50)	3.6 (< LOQ-4.7)	1.2 (< LOQ-1.7)	< LOQ	NC
THC-glucuronide	8	0.50 (0.48-0.52)	1.4 (< LOQ-2.3)	0.6 (< LOQ-1.0)	< LOQ	NC
THCCOOH-glucuronide	10	2.0 (0.28-4.0)	190 (68-460)	150 (64-420)	100 (48-350)	840 (350-2400)

Table 10. PK Parameters after smoking

[a] Number of samples included in calculation of T_{max} and C_{max}. [b] Time to maximum concentration. [c] Maximum observed concentration. [d] Concentration at t = 1 hour. [e] Concentration at t = 6 hours. [f] Area under the concentration-time curve from 0 to 6 hours. [g] Δ^9-tetrahydrocannabinol. [h] 11-hydroxy-THC. [i] 11-nor-9-carboxy-THC. [j] Cannabidiol. [k] Cannabinol. [l] Below limits of quantification (LOQ): THC, 11-OH-THC, THCCOOH, CBD, CBN 1 µg/L, THC-glucuronide 0.5 µg/L and THCCOOH-glucuronide 5 µg/L. [m] Not calculable - too few points to determine

22 h after smoking, whole blood concentrations (n = 6) for THC, 11-OH-THC, CBD, CBN, and THC-glucuronide were < LOQ for all but Participant I, who had residual THC and 11-OH-THC concentrations of 5.0 and 1.7 μg/L, respectively. Similar results were obtained in plasma, although a 2nd participant also was positive for THC and 11-OH-THC at this time. THCCOOH and THCCOOH-glucuronide concentrations were variable in these extended samples, ranging from 4.4-43 μg/L and 15-190 μg/L in whole blood and 7.0-59 μg/L and 36-180 μg/L in plasma, respectively.

Glucuronide-Free Cannabinoid Ratios

Molar glucuronide-free cannabinoid ratios were determined for THC and THCCOOH in both matrices (Figure 5A, 5B). Throughout the study, THC (Figure 5A) molar glucuronide/free ratios across participants ranged from 0.006 to 0.019 (median 0.013) in whole blood and 0.006 to 0.041 (median 0.019) in plasma, indicating minimal THC-glucuronide formation relative to free THC. THCCOOH (Figure 5B) molar ratios were markedly different, ranging from 0.43 to 5.30 (median 2.05) in whole blood and 0.45 to 10.93 (median 2.39) in plasma across participants and time. Whole blood median ratios were significantly lower immediately following smoking (0.25 and 0.5 h) compared to baseline ($p = 0.009$ and 0.037, respectively); similar non-significant differences were observed in plasma ($p = 0.170$ and 0.330, respectively). As free THCCOOH concentrations decreased and THCCOOH-glucuronide concentrations remained increased 0.5-2 h after smoking, glucuronide-free ratios returned to baseline concentrations in both matrices for the remainder of the session.

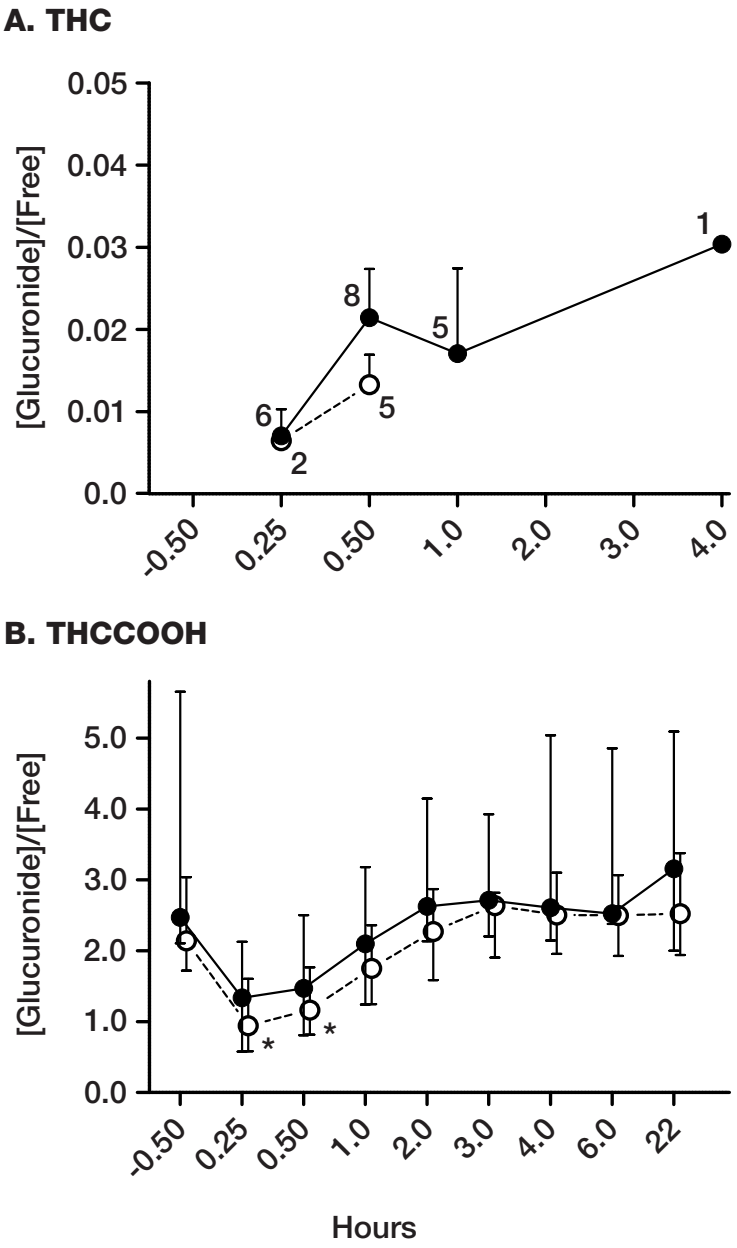


Figure 5A-B. Median (inter-quartile range) molar whole blood (o) and plasma (•) [glucuronide]/[free] ratios for A) Δ^9-tetrahydrocannabinol (THC) and B) 11-nor-9-carboxy-THC (THCCOOH) following a 6.8% THC cannabis cigarette.

n values indicate data points for THC; for THCCOOH n = 9, 6 and 10 at 0.25 h, 22 h, and all other times, respectively. THC-glucuronide was < LOQ (0.5 µg/L) after 1 h in whole blood and 4.0 h in plasma. *$p < 0.05$ compared to baseline, Tukey's HSD.

Cannabinoid Detection Rates

Cannabinoid detection rates were investigated in whole blood and plasma following cannabis smoking (Figure 6A, 6B). THCCOOH and THCCOOH-glucuronide were quantified in all samples. THC and 11-OH-THC were quantified in all whole blood and plasma samples for at least 2 h; after this time detection rates decreased, with 10% detection in whole blood and 30% detection in plasma 22 h after smoking. In whole blood, CBD, CBN and THC-glucuronide were detected 0.25 h after smoking in 60%, 80% and 20% of samples, respectively (LOQs 1.0, 1.0 and 0.5 μg/L). However, by 1 h after smoking, CBN was the only minor cannabinoid detected in whole blood, and only for a single participant. In plasma, CBD, CBN and THC-glucuronide were detected at 0.25 h in 80%, 90% and 70% of samples, respectively, with detection of all three analytes for at least one participant at 1 h. Detection rates for THC-glucuronide were highest in both whole blood and plasma at 0.5 h, corresponding with the observed T_{max} for this analyte, while other analyte detection rates were highest 0.25 h after smoking.

Discussion

This study directly characterizes, for the first time, cannabinoid glucuronides in both whole blood and plasma following controlled smoked cannabis. The minor cannabinoids CBD and CBN also were also included, yielding novel cannabinoid pharmacokinetic data following smoked cannabis administration.

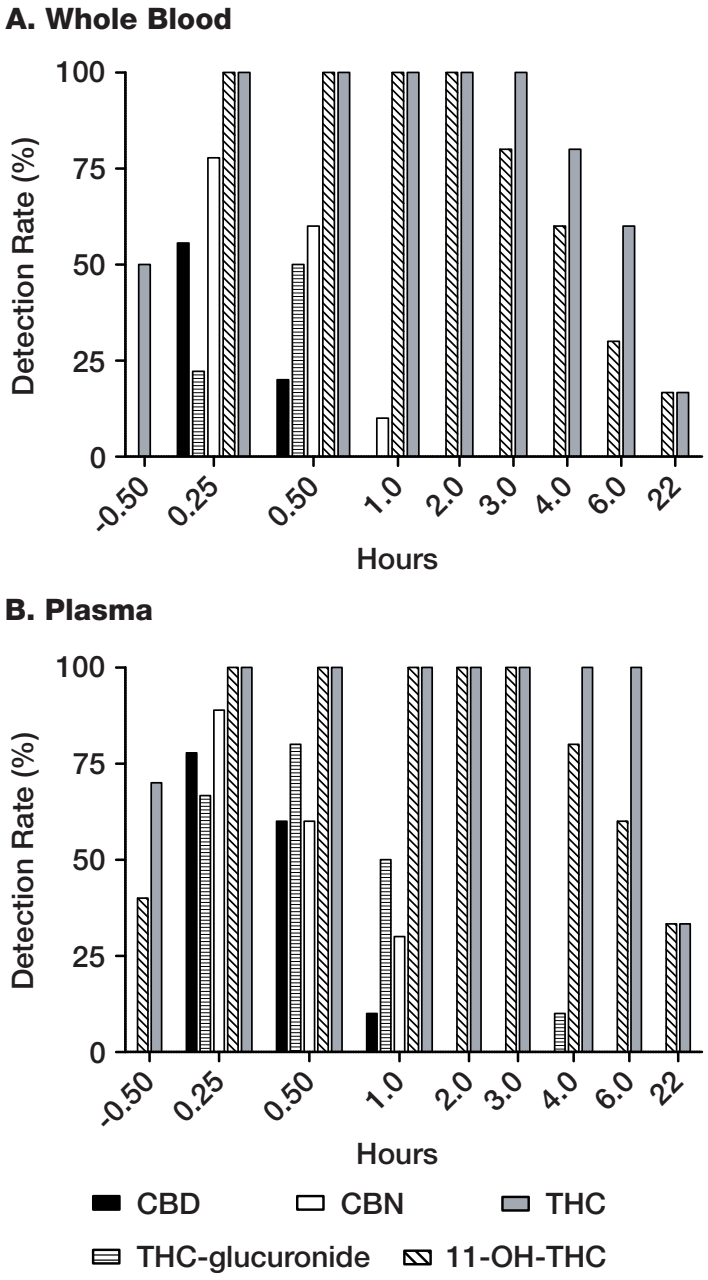


Figure 6A-B. Cannabinoid detection rates following a single smoked 6.8% THC cannabis cigarette in A) whole blood and B) plasma.

n = 9, 6 and 10 at 0.25 h, 22 h, and all other times, respectively. All samples contained THCCOOH and THCCOOH-glucuronide. Limits of quantification: Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), cannabidiol (CBD), cannabinol (CBN), 11-nor-9-carboxy-THC (THCCOOH) 1 µg/L; THC-glucuronide 0.5 µg/L; THCCOOH-glucuronide 5.0 µg/L.

This study describes: 1) directly-determined whole blood cannabinoid and cannabinoid glucuronide concentrations following cannabis smoking, providing a scientific database for interpretation of whole blood forensic and clinical investigations; and 2) whole blood and plasma concentrations for minor cannabinoids THC-glucuronide, CBD and CBN, which indicate that these minor cannabinoids warrant further investigation as markers of recent (< 2 h) smoked cannabis intake.

All samples were collected on ice, processed and transferred to refrigerated storage within 2 h of collection, and analyzed by LC-MS/MS within 24 h to minimize concentration changes due to analyte instability. Many studies investigated cannabinoid analyte instability *in vitro*, with results dependent upon collection technique (200), storage container characteristics (145, 151), and storage time (139) and conditions (139, 147, 153). The ester-linked THCCOOH-glucuronide is susceptible to instability, especially at increased storage temperature and time (139). The present study circumvented these concerns by quantifying potentially unstable acyl glucuronides within 24 h of specimen collection, a period of < 5% spontaneous hydrolysis (139).

Concentrations were variable between participants. Whole blood THC observed C_{max} ranged from 13-63 μg/L (median 50 μg/L) 15 min after starting smoking. Similarly, plasma THC C_{max} ranged from 18-110 μg/L (median 76 μg/L). Plasma concentrations were similar to or slightly lower than those reported previously (57, 59, 176, 201-202). Administration of 23.1% THC (69.4 mg) in cannabis/tobacco cigarettes via a paced smoking procedure over 22 min (59) yielded a mean plasma C_{max} of 190 μg/L (range 24-262 μg/L). Huestis administered 3.55% THC (33.8 mg) in cannabis cigarettes via a

paced procedure over 11.2 min; mean (range) peak plasma THC concentration for 6 participants was 162 μg/L (76-267 μg/L) (57).

Smoking topography (i.e. number of puffs, depth of inhalation, hold time, and time between puffs) and side-stream smoke production (58) during *ad libitum* smoking introduced variability in whole blood and plasma cannabinoid concentrations, similar to that observed in other well-controlled paced (57, 59, 89) and non-paced (203) smoking studies. Ethical considerations currently prohibit paced smoking protocols, due to the possibility of forcing participants to consume more THC and at a faster rate than when self-administered. *Ad libitum* dosing, while more variable, is ideal as it more closely represents authentic smoking behavior, providing more realistic concentrations while minimizing adverse events. Indeed, only two adverse events were reported in the present study, neither of which led to study withdrawal. Participants stopped smoking as they reached their desired "high," limiting higher THC concentrations and untoward effects. The slight discrepancy in concentrations between this and prior studies is likely attributable to study design: both Huestis (57) and Hunault (59) collected plasma more closely to the onset of smoking. *Actual* C_{max} and T_{max} likely occurred prior to the last puff (57) and data at these times were not captured in the present study.

Few prior studies examined cannabinoid glucuronide concentrations in any matrix (43, 49, 181, 197-198, 204); none investigated whole blood concentrations. Skopp measured THCCOOH-glucuronide directly (without hydrolysis) in serum 24-48 h after self-reported cannabis intake (198). Concentrations ranged from 9-1048 μg/L for "heavy" users (> 1 joint per day), 35-368 μg/L for "moderate" users (> 1 joint per week but < 1 joint per day) and 10-204 μg/L for "light" users (< 1 joint per week). In the

present cohort, plasma THCCOOH-glucuronide concentrations were 35-460 μg/L over the entire study, with self-reported cannabis intake on more than half the days preceding study sessions. The highest observed THCCOOH-glucuronide concentrations at each blood collection were from the heaviest smoker (Participant G, 12 joints/day on 14/14 days), while the lowest observed concentrations were often from Participant K, who self-reported last smoking 4 days prior to dosing. While lower THCCOOH-glucuronide concentrations generally were observed with decreasing self-reported intake, we could not differentiate between light, moderate or heavy cannabis smokers solely on the basis of an individual blood specimen. This concurs with Skopp (198) who observed variable cannabinoid concentrations, precluding accurate frequency of use determinations.

Median THCCOOH-glucuronide concentrations were substantially higher than free THCCOOH concentrations in whole blood and plasma in the present study, although the glucuronide-free ratio was variable within and between participants. This ratio was > 5.0 in both matrices on several occasions, indicating extensive ester-linked glucuronidation. However, we observed significant and non-significant glucuronide-free ratio decreases in whole blood and plasma, respectively, 15-30 min after smoking as a result of rapid THCCOOH formation. UGT-catalyzed formation of THCCOOH-glucuronide from free THCCOOH is likely the rate-limiting step: THCCOOH concentrations increase more rapidly than THCCOOH-glucuronide concentrations immediately after smoking, decreasing the ratio. As time passes, remaining acid metabolite is glucuronidated and the ratio returns to baseline within 1-2 h post-smoking in both matrices, remaining constant thereafter. This phenomenon was previously reported by Kelly (49), who also observed a brief decline in glucuronide-free ratios in

plasma for up to 30 min following cannabis smoking. Further examination of this ratio may provide a marker of recent cannabis intake; however, one potential limitation with this approach is the frequent lengthy delay between cannabis smoking and sample collection during DUID and other investigations. Based on present and past data, samples obtained 2-3 h after smoking would not be useful because THCCOOH glucuronide-free ratios may have already returned to baseline.

The ether glucuronide of THC was previously proposed as a marker of recent cannabis intake (181, 197), yet few prior studies examined this metabolite in blood. Skopp (182) observed low THC-glucuronide concentrations in urine from heavy and moderate cannabis users, but not occasional users. Schwilke (43) observed significant increases of approximately 9.5% ($p < 0.001$) and 170% ($p < 0.001$) in plasma THC and THCCOOH concentrations, respectively, following *E. coli* β-glucuronidase hydrolysis in samples collected during extended oral THC (dronabinol) administration. Our study reported here documented 2% median (0.6-4.1%) glucuronide-free molar ratios for THC and 240% median (45-1000%) glucuronide-free molar ratios for THCCOOH in plasma. The discrepancies in observed glucuronidation rates with Schwilke et al (43) are likely due to different routes of administration (smoked and oral) for THC and are likely methods-based for THCCOOH. Enteric glucuronidation following oral dronabinol potentially yielded higher THC glucuronidation rates, while inefficient enzymatic THCCOOH-glucuronide hydrolysis (121) may explain the discrepancy in observed THCCOOH glucuronidation rates.

THC-glucuronide concentrations were low in our study, with peak whole blood and plasma concentrations of 0.8 and 2.3 μg/L, respectively. We are not aware of any *in*

vitro or *in vivo* studies evaluating whether this metabolite possesses pharmacological activity similar to THC. Still, it is likely that observed concentrations would yield little or no effect on THC-induced impairment, as the glucuronide is only present immediately after smoking when whole blood concentrations of THC are typically > 10 μg/L. Additionally, this minor metabolite was not observed in all participants; maximum detection rates were 50% and 80% in whole blood and plasma, respectively. Concentrations decreased to < LOQ in all but one specimen by 2 h after smoking.

Observed detection rates render THC-glucuronide an inclusionary, but not exclusionary, marker for recent cannabis intake at a 0.5 μg/L LOQ. CBD and CBN had similar detection windows in whole blood and plasma, with CBN more prevalent than CBD between 0.25 and 1 h. These analytes were not detected beyond 2 h after smoking, rendering them possible candidates for markers of recent cannabis smoking. CBD and CBN are amenable to GC-MS analysis, are often readily extracted by current mixed-mode SPE procedures and have commercially available deuterium-labeled internal standards, unlike cannabinoid glucuronides. However, concentrations of these analytes in cannabis vary depending upon strain (205) and storage time and conditions (206), potentially altering detection rates. Additionally, these cannabinoids are present in cannabis smoke (206-207) and, unlike THC-glucuronide, could possibly be detected following passive exposure. If detection limits improve for these minor cannabinoids, further study could suggest potential cutoffs and analytical approaches for confirming these analytes as markers of recent cannabis intake.

These data advance our understanding of THC metabolism following smoked cannabis. No data on 11-OH-THC-glucuronide, ether-linked THCCOOH-glucuronide or

di/bis-THCCOOH-glucuronide were collected since standards for these analytes are not currently available; however, ester-linked THCCOOH-glucuronide is the predominate species (43, 208). THC, THCCOOH and THCCOOH-glucuronide were observed in baseline samples due to the relatively short (15-19 h) residential stay prior to dosing. The aim of the residential stay was to eliminate potential intoxication or impairment, not fully eliminate previously self-administered cannabinoids from biological samples. Since baseline concentrations of THC and 11-OH-THC were negligible compared to concentrations after dosing, error resulting from residual cannabinoids was minimal for these analytes. Candidates for markers of recent cannabis intake were < LOQ in all participants in the baseline specimen, enabling accurate detection window determination. Finally, varying cannabis composition could yield increased or decreased CBD or CBN content, leading to altered intake and detection windows. Cannabinoid pharmacotherapy such as Sativex® also complicates interpretation, as low CBD concentrations are likely following intake (209).

To our knowledge, these are the first directly measured authentic human whole blood and plasma cannabinoid glucuronide data following cannabis smoking. Furthermore, we provide simultaneous THC, 11-OH-THC, THCCOOH, CBD and CBN concentrations. Observed peak cannabinoid concentrations were variable, likely due to inter-individual differences in smoking topography and prior cannabis smoking history. Direct glucuronide detection provided three primary benefits: 1) THCCOOH-glucuronide detection may improve interpretation of results since high baseline THCCOOH-glucuronide concentrations may indicate chronic, daily cannabis smokers, precluding utilization of recent cannabis use predictive models (140); 2) detection of THC-

glucuronide provides a potential new marker for recent cannabis intake because concentrations rapidly fall below LOQ in whole blood and plasma; 3) direct LC-MS/MS detection precludes time- and resource-intensive specimen hydrolysis and sample derivatization, yielding savings over typical GC-MS procedures and improving the accuracy of measurement. Minor amounts of CBD and CBN were detected in some, but not all participants' samples for up to 2 h following smoking, rendering these cannabinoids additional potential markers of recent cannabis intake. These data provide valuable information for interpreting whole blood cannabinoid concentrations in forensic DUID accident investigations and clinical monitoring scenarios, while furthering our understanding of potential markers of recent cannabis intake.

Chapter 5 – Psychomotor performance, subjective and physiological effects and whole blood Δ^9-tetrahydrocannabinol concentrations in heavy, chronic cannabis smokers following acute smoked cannabis

(as submitted to *Psychopharmacology*, 2011)

Abstract

Rationale: Δ^9-tetrahydrocannabinol (THC) is the illicit drug most frequently observed in accident and driving under the influence of drugs (DUID) investigations. Whole blood is often the only available specimen collected during such investigations, yet few studies have examined relationships between cannabis effects and whole blood concentrations following cannabis smoking.

Objectives: This study aims to examine cannabinoid concentration-effect relationships in heavy, chronic cannabis smokers following *ad libitum* smoking of higher-potency cannabis cigarettes and evaluate a model (cannabis influence factor [CIF]) for determining impairment from cannabinoid blood concentrations.

Methods: Nine male and one female heavy, chronic cannabis smokers resided on a closed research unit and smoked *ad libitum* one 6.8% THC cannabis cigarette. THC, 11-hydroxy-THC and 11-nor-9-carboxy-THC were quantified in whole blood and plasma. Assessments were performed prior to and up to 6 h after smoking, including

subjective (visual analog scales [VAS] and Likert scales), physiological (heart rate, blood pressure, respirations) and psychomotor (critical tracking and divided attention tasks) measures.

Results: THC significantly increased VAS responses and heart rate, with concentration-effect curves demonstrating counter-clockwise hysteresis. No significant differences were observed for critical tracking or divided attention task performance in this cohort of heavy, chronic cannabis smokers. CIF was not suitable for quantifying psychomotor impairment following cannabis consumption and was not precise enough to determine recent cannabis use with accuracy.

Conclusions: These data inform our understanding of impairment and subjective effects following acute smoked cannabis and interpretation of whole blood cannabinoid concentrations in forensic DUID and other accident investigations.

Introduction

An estimated 125-203 million persons aged 15-64 worldwide smoked cannabis at least once in the previous year (210), with16.7 million Americans and 18.1% of individuals aged 18-25 years smoking cannabis in the previous 30 days (5). Additionally, cannabis is the most common illicit substance detected in blood and oral fluid of nighttime drivers (175). While impairment cannot be assumed from drug presence, detection windows in these matrices are relatively short for less than daily cannabis smokers (57, 176-177), increasing impairment probability following a positive test.

The primary psychoactive chemical in cannabis, Δ^9-tetrahydrocannabinol (THC), is metabolized *in vivo* to several phase I metabolites, most prominently the equipotent 11-

hydroxy-THC (11-OH-THC) and non-psychoactive 11-nor-9-carboxy-THC (THCCOOH) (178-179). Several studies investigated cannabinoid metabolism and urine, plasma and oral fluid pharmacokinetics following acute and chronic oral, smoked and intravenous THC administration (43, 52, 64, 66, 89, 211). Recently, we investigated whole blood cannabinoid pharmacokinetics with direct liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis for the first time, providing valuable data regarding cannabinoid detection in this matrix following cannabis smoking (212).

THC is detectable in plasma within seconds after the first puff of a cannabis cigarette, with dose-related tachycardia, subjective "high" and conjunctival injection (52, 59, 64, 66). Cannabis impairs psychomotor performance, cognition, and driving ability in both driving simulators and on-the-road driving tests (31, 74-75, 87, 213-217). Hunault et al examined cognitive and psychomotor effects following smoked cannabis containing up to 69 mg THC and observed significant, dose-related impairing effects following paced smoking in primarily occasional cannabis smokers (59). Papafotiou examined cannabis-induced impairment in a driving simulator in cannabis smokers with unknown histories after one week of abstinence; significant impairment was observed 80 but not 30 min after smoking a 1.74% or 2.93% THC cannabis cigarette (91). However, Ramaekers et al studied effects of cannabis smoking (13% THC, 500 μg/kg) in heavy (> 4 days/week) and occasional (≤ 1x/week) smokers and found significant impairment in tracking performance, divided attention and inhibitory control in occasional, but not heavy smokers (90).

The cannabis influence factor (CIF), described by Daldrup as a predictor of cannabis-induced impairment, is calculated as molar blood [THC] + [11-OH-THC]

divided by [THCCOOH] (218). A CIF > 10 was proposed to predict cannabis-induced impairment analogous to a 0.11 g/dL blood alcohol concentration. Menetrey et al examined this model following controlled oral cannabis decoction administration, yet none, to our knowledge, examined this following controlled cannabis smoking.

The present study examined relationships between whole blood cannabinoid concentrations and pharmacodynamic effects in heavy, chronic cannabis smokers. Cannabis-induced effects were determined in critical tracking and divided attention task performance, subjective and physiological measures, as well as the CIF, following cannabis smoking. These data should facilitate interpretation of whole blood cannabinoid results, often the only specimen available in driving under the influence of drugs (DUID), accident and other forensic investigations.

Materials and Methods

Participants

Participants provided written informed consent for this National Institute on Drug Abuse Institutional Review Board-approved protocol. Inclusion criteria were cannabis use at least twice monthly for 3 months prior to study entry, positive urine cannabinoid test, 18-45 years old, normal cardiac function and veins suitable for intravenous catheter placement. Clinically significant medical or psychiatric disease, history of clinically significant adverse event associated with cannabis intoxication, current interest in drug abuse treatment, pregnancy or nursing, or blood donation in the previous 30 days were exclusionary. Participants were heavy (> 4 days/week) and chronic (> 2 years) cannabis

smokers admitted to the secure research unit 15-20 h prior to dosing; no cannabis use restrictions were enforced prior to admission.

Smoked Cannabis Administration and Blood Collection

The NIDA Chemistry and Physiological Systems Research Branch supplied cannabis cigarettes containing 6.8 ± 0.2% THC, 0.25 ± 0.08% CBD and 0.21 ± 0.02% CBN (w/w). Mean cigarette weight was 0.79 ± 0.16 g, yielding total THC, CBD and CBN content of 54, 2.0 and 1.7 mg per cigarette, respectively. Participants smoked a single cigarette *ad libitum* for 10 min while seated with legs elevated, and remained in this position during the data collection period. Whole blood and plasma were collected on ice into sodium heparin blood tubes via indwelling intravenous catheter 0.5 h prior to and 0.25, 0.5, 1, 2, 3, 4, and 6 h after the start of smoking. Blood collected for plasma was centrifuged (1600 x g, 15 min) and plasma separated within 2 h.

Cannabinoid Analysis

Cannabinoids were quantified by a previously validated LC-MS/MS method (199). Briefly, 1.5 mL acetonitrile was added to 0.5 mL specimen to precipitate proteins. After mixing and centrifugation, the supernatant was diluted and subjected to solid-phase extraction (SPE). Extracts were evaporated, reconstituted and chromatographed; detection was via electrospray ionization. Imprecision was < 10.5% CV, recovery was > 50.5% and bias within ± 13.1% of target across the linear range. Limits of quantification (LOQ) were 1.0 μg/L for THC, 11-OH-THC, and THCCOOH.

Subjective Scales

Visual analog scales (VAS) were presented on a computer screen -0.5, 0.25, 0.5, 1, 2, 3, 4 and 6 h after the start of smoking. Participants indicated the magnitude of "Good Drug Effect," "High," "Stoned," "Stimulated," "Sedated," "Anxious" and "Restless" on a 160-mm line anchored with "not at all" and "most ever." Position on the scale was converted to a percentage between 0 and 100.

5-point Likert scales for "Difficulty concentrating," "Altered sense of time," "Slowed or slurred speech," "Body feels sluggish or heavy," "Feel hungry," "Feel thirsty," "Shakiness/tremulousness," "Nausea," "Headache," "Palpitations," "Dizzy," and "Dry mouth or throat" were presented on a computer screen immediately following VAS. Participants selected the response best characterizing their condition: 1 none; 2 slight; 3 mild; 4 moderate; or 5 severe.

Cardiovascular Measures

Blood pressure (systolic and diastolic), heart rate and respiratory rate were measured before and after smoking at -0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 6 h.

Cannabis Influence Factor

CIF was calculated as 100*([THC] + [11-OH-THC]) / [THCCOOH] (218-219) for all whole blood and plasma samples; analytes quantifying below LOQ were considered to have 0.0 nmol/mL.

Impairment Assessments

The “critical tracking task” (161) measures a participant’s ability to control a displayed error signal in a first-order compensatory tracking task. Error is displayed as a horizontal deviation of a cursor from the midpoint on a horizontal, linear scale. Compensatory joystick movements null the error by returning the cursor to the midpoint. The frequency of cursor deviations, and, therefore, its velocity, increases as a stochastic, linear function of time. Control is lost at the point where the response lags the cursor’s last movement by 180 degrees. The response frequency at this point is defined as the critical frequency or lambda-c (λ_c). The test includes five trials. The average of the middle three scores (i.e., deleting the highest and lowest) is taken as the final score. This task was performed before smoking and at 1.5, 3 and 5.5 h after starting smoking.

The “divided attention task” (162) measures a participant’s ability to divide attention between two simultaneous tasks. The primary task is the same as the critical tracking task described above, with the exception that the velocity of the error signal is kept constant at 50% of the participant’s optimal performance ($\lambda_c/2$). Tracking error is measured by the absolute distance (mm) between the cursor's position and the center of the scale. The secondary task involves monitoring 24 single-digit numbers (0-9) displayed in the four corners of a central screen that change asynchronously every 5 seconds. The participant is required to remove his/her foot from a pedal-switch as quickly as possible any time the target numeral "2" appears. Mean absolute tracking error (mm), number of correct detections (hits) and number of control losses are the primary performance measures. This task was performed before smoking and at 1.6, 3.1 and 5.6 h after starting smoking.

Participants were trained before the study session to achieve stable task performance and minimize practice effects. Critical tracking task training continued until participants performed with < 10% variance from the average measured over three trials. The divided attention task was practiced for 12 minutes, regardless of final performance.

Calculations and Statistical Analysis

SPSS® 15.0 for Windows (SPSS, Chicago, IL) was utilized for statistical evaluations. GLM repeated-measures ANOVA were conducted to investigate changes in participants' VAS scores over time, with a Huynh-Feldt epsilon correction applied to counter sphericity violations. Bonferroni post-hoc testing determined differences between specific time points. Wilcoxon signed-rank tests evaluated differences between whole blood and plasma CIF values at each time point. Areas within concentration-effect curves were calculated in a manner similar to Galeazzi et al (220) by subtracting the total area under the lower limb of the curve from that under the upper limb, determined by the trapezoidal rule. P values ≤ 0.05 were considered significant. Statistical calculations were performed with GraphPad Prism 5.2 (GraphPad Software, La Jolla, CA).

Results

Samples

Participant demographics and self-reported smoked cannabis histories are detailed in Table 9 (Chapter 4). Ten participants (nine male, one female) completed the protocol. Age ranged from 18.5-45.7 years and BMI ranged from 18.1-32.0 kg/m^2. Mean [SD] self-reported joints smoked in the previous 14 days was 11.7 [2.2], with a range of 10-

168 joints. Only one scheduled blood sample (Participant I at 0.25 h) was not collected, due to catheter dislodgement.

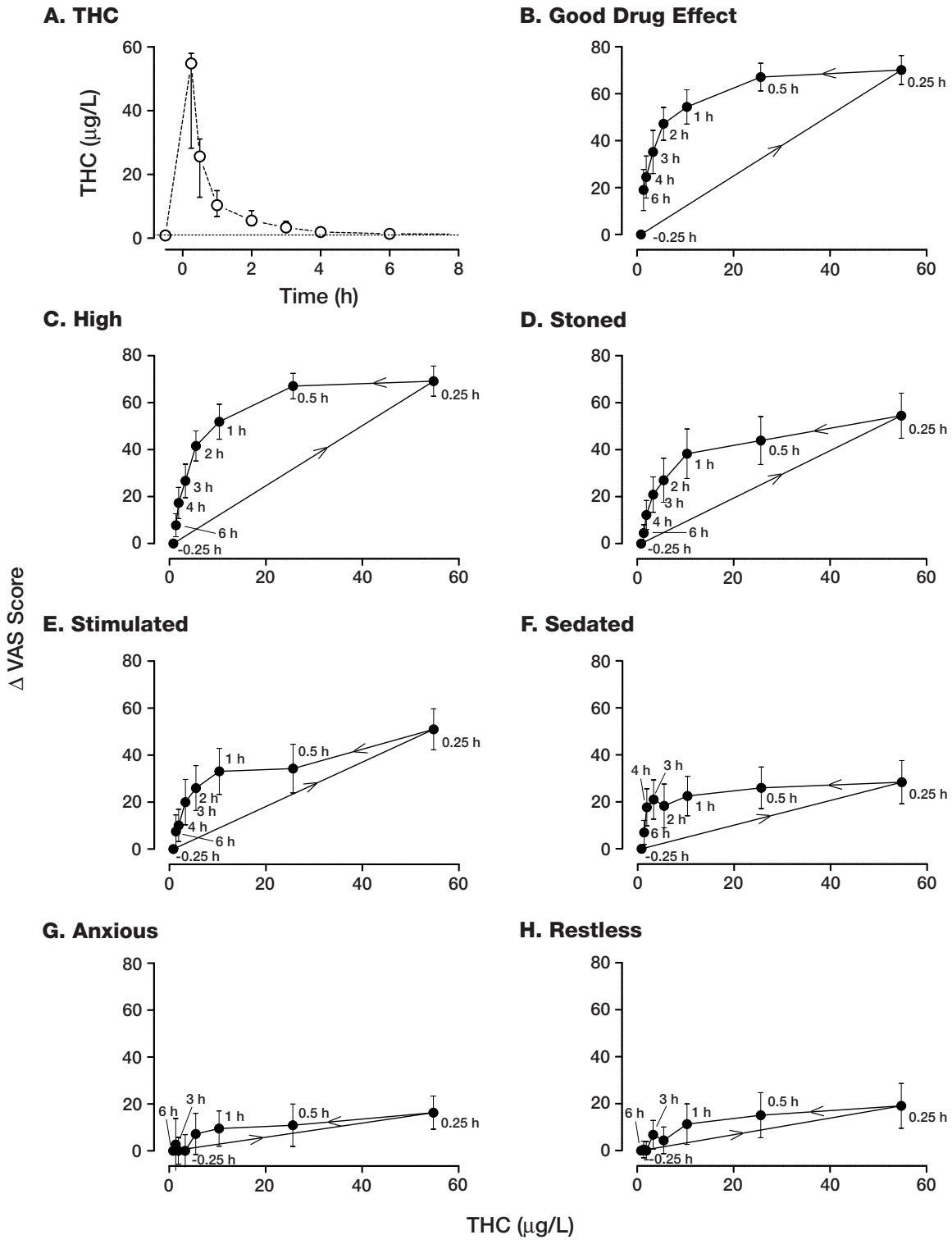
Subjective Effects Following Smoked Cannabis

Cannabis smoking significantly increased "Good drug effect" ($F(2.37, 21.36) = 17.6$, $p = 0.000$), "High" ($F(2.78, 25.02) = 26.03$, $p = 0.000$), "Stoned" ($F(2.94, 26.49) = 13.29$, $p = 0.000$), "Stimulated" ($F(3.25, 29.30) = 8.54$, $p = 0.000$) and "Sedated" ($F(4.70, 42.34) = 3.35$, $p = 0.014$), but did not significantly change "Anxious" ($F(4.82, 43.35) = 1.15$, $p = 0.349$) or "Restless" $F(2.55, 22.91) = 2.58$, $p = 0.086$).

Mean blood concentration-effect curves for each of 7 VAS scales are shown in Figure 7A-H. For all scales, observed peak THC blood concentration and peak VAS scores occurred 0.25 h after starting smoking, at the time of the first blood collection. Blood THC concentrations decreased rapidly, although subjective effects persisted. Starting about 1 h after smoking, subjective effects decreased linearly through 6 h, with THC concentrations changing more slowly during this period. This pharmacodynamic-pharmacokinetic relationship described a counterclockwise hysteresis for all VAS measures, demonstrating the lack of correlation between blood concentrations and observed effects until after the initial distribution phase.

Figure 7A-H. Median (inter-quartile range) whole-blood THC concentrations (A) and mean (SEM) hysteresis plots for seven VAS scales (B-H) following smoking of a 6.8% THC cannabis cigarette (n = 10).

VAS scores are calculated as change from baseline (-0.5 h). Samples collected at -0.5, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 after starting smoking. Dotted line in THC plot indicates limit of quantification (1 μg/L). Arrows in hysteresis plots indicate samples collected over increasing time.

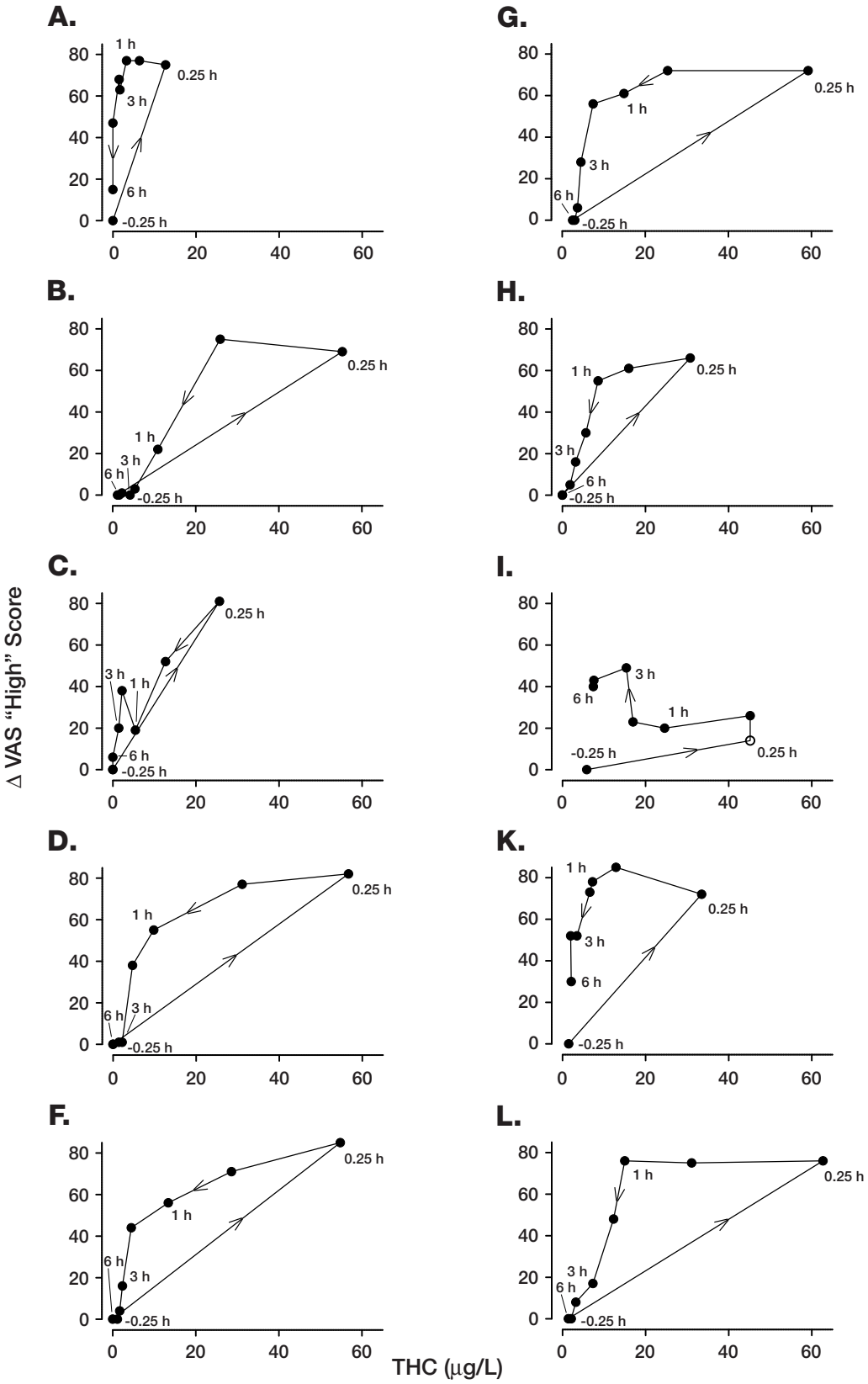

A. THC
THC (μg/L)
Time (h)
B. Good Drug Effect
C. High
D. Stoned
E. Stimulated
F. Sedated
G. Anxious
H. Restless
Δ VAS Score
THC (μg/L)
0.25 h
0.5 h
1 h
2 h
3 h
4 h
6 h
-0.25 h

While group mean blood concentration-VAS relationships yielded consistent hysteresis effects, concentration-effect curves for individual subjects varied substantially (Figure 8A-L). All participants (except I) reported peak subjective "High" between 66 and 85; with peak whole blood THC concentrations at the time of these responses ranging from 13 (Participant A) to 63 μg/L (Participant L). There was no apparent relationship between hysteresis areas and age, sex or self-reported frequency or chronicity of cannabis intake.

5-point Likert scales displayed varied effects. Participants reported significant increases compared to baseline for "Slowed or slurred speech" ($F(2.10,18.9) = 4.35$, $p = 0.026$), "Feel Hungry" ($F(5.86, 52.7) = 22.0$, $p = 0.000$), "Feel thirsty" ($F(5.37, 48.3) = 19.2$, $p = 0.000$), "Shakiness/tremulousness" ($F(1.81, 16.3) = 4.19$, $p = 0.037$) and "Dry mouth or throat" ($F(6.34, 57.0) = 20.6$, $p = 0.000$), with no significant increases observed for other Likert scales.

Figure 8A-L. Individual (n = 10) hysteresis plots for "High" VAS scales following smoking of a 6.8% THC cannabis cigarette.

VAS scores are calculated as change from baseline (-0.5 h). Samples collected at -0.5, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 after starting smoking. Arrows in hysteresis plots indicate direction of plot.


A.
B.
C.
D.
F.
G.
H.
I.
K.
L.
Δ VAS "High" Score
THC (μg/L)
0
20
40
60
80
1 h
3 h
6 h
0.25 h
-0.25 h

Cardiovascular Measures

Cannabis smoking significantly increased heart rate only at 0.5 h ($F(1,9) = 11.99$, $p = 0.007$). Diastolic blood pressure decreased significantly only from 0.5 to 1 h, ($F(1,9) = 8.95$, $p = 0.015$). Systolic blood pressure and respiratory rate were unaffected at all times during the session.

Cannabis Influence Factor

CIF increased substantially immediately following cannabis smoking, reaching a median peak (range) of 150 (93-250) in whole blood and 170 (79-270) in plasma 0.25 h after starting smoking (Figure 9). CIF then rapidly decreased, although the rate of change lessened over time. Median (range) whole blood CIF was 13 (0.0-32) 4 h after smoking and 8.0 (0.0-27) 6 h after smoking; plasma CIF was 14 (6.9-34) at this final observation. 6 h plasma CIF was significantly higher than baseline ($F(1, 9) = 19.24$, $p = 0.002$), although whole blood CIF was not ($F(1, 9) = 4.35$, $p = 0.067$). No significant differences were observed between plasma and whole blood CIF at any time other than 6 h ($T = 2$, $p = 0.037$). Converting plasma cannabinoid concentration to whole blood concentrations with the suggested 0.5 whole blood to plasma concentration ratios yielded no changes in calculated CIF values.

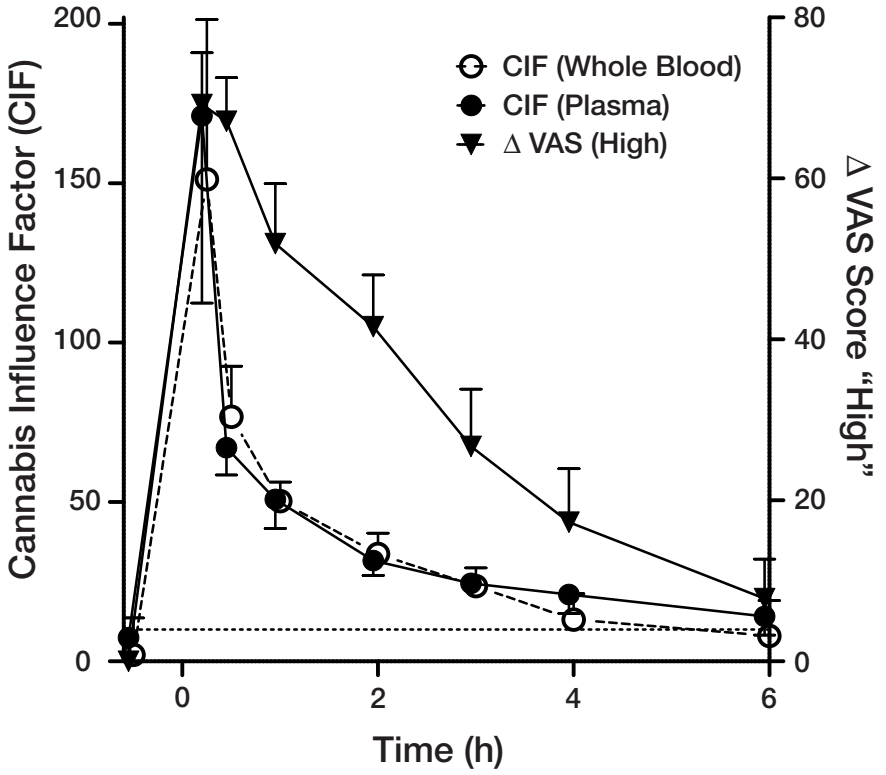


Figure 9. Mean (SEM) Cannabis Influence Factor (CIF) and VAS "High" score following *ad libitum* smoking of a 6.8% THC cannabis cigarette (n = 10).

VAS score is calculated as change from baseline (-0.5 h). CIF calculated from molar whole blood concentrations as 100*([THC] + [11-OH-THC]) / [THCCOOH]. Samples collected at -0.5, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 after starting smoking. Dotted line at CIF = 10 indicates "impaired" as proposed by Daldrup.

Critical Tracking Task/Divided Attention Task

Cannabis smoking had no significant effects on critical tracking task performance in this population of almost-daily, chronic cannabis smokers. For the divided attention task, significant differences were observed between baseline and 3 h, $F(3,27) = 2.4$, $p = 0.022$ for the number of correct identifications (hits) in the secondary peripheral stimuli task, although no significant differences were observed 1.5 h after smoking. No

significant effects due to cannabis smoking were observed for control losses, reaction time, or tracking error (Figure 10).

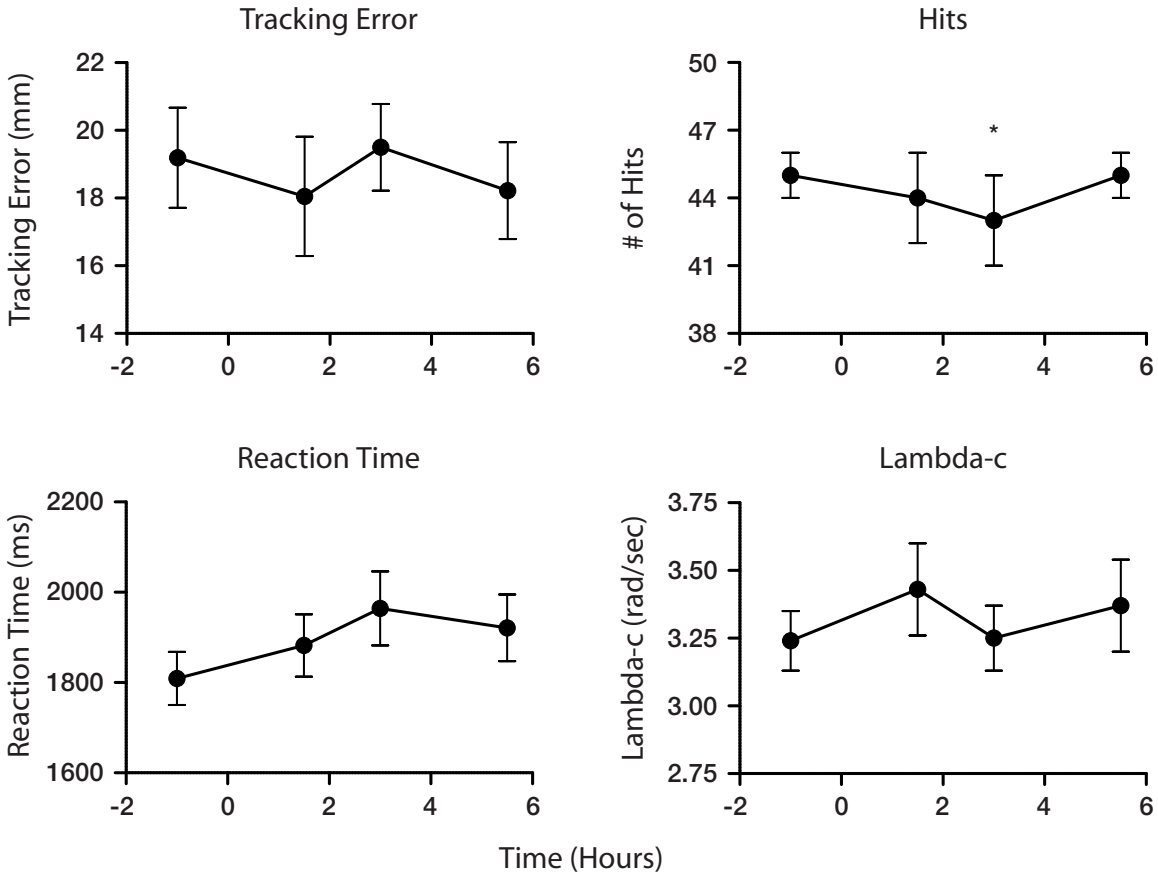


Figure 10. Mean (SEM) values (n = 10) for lambda-c in the critical tracking task (CTT) and tracking error, hits and reaction time in the divided attention task (DAT) as a function of time after smoking *ad libitum* a single 6.8% THC cannabis cigarette.

Samples collected after smoking at 1.5, 3 and 5.5 h for CTT and 1.6, 3.1 and 5.6 h for DAT. * $p<0.05$ compared to baseline, repeated-measures ANOVA.

Discussion

The present study characterizes acute cannabis effects over 6 h after smoking a 6.8% THC cigarette containing approximately 54 mg THC in a sample of heavy, chronic cannabis smokers. Robust subjective and cardiovascular effects were observed, while psychomotor performance changes were modest in this group of heavy, chronic smokers. THC potency was selected to closely represent current mean illicit cannabis potency in seized US drugs, which increased from 3.4% in 1993 to 8.8% in 2008 (4) , thus providing insight into the pharmacodynamics and pharmacokinetics of the higher-potency cannabis currently prevalent in the US.

As expected, smoking a single cannabis cigarette significantly increased presumably positive effects such as "Good drug effect," "High," "Stoned," "Stimulated" and "Sedated," while having no significant effect on negative responses such as "Anxious" or "Restless." There are several possibilities that may account for these results. The *ad libitum* study design and/or frequency and chronicity of cannabis smoking in our population may have minimized adverse events. Ethical considerations precluded paced smoking procedures in the current study, as this could lead to THC consumption at a faster rate than is typical during self-administration.

When VAS subjective effects were compared to simultaneously collected whole blood THC concentrations, counter-clockwise hystereses were observed. Cocchetto et al (92) first detailed hysteresis following cannabis smoking, indicating a delay in the onset of subjective effects as compared to plasma concentrations. Barnett et al (93) also observed a counter-clockwise hysteresis for heart rate and THC plasma concentrations,

and Chiang et al (94) described differences in hysteresis areas for oral, smoked and intravenous THC based on differences in administration route or cannabis potency. In 1993, Cone and Huestis detailed hysteresis for "How much drug effect do you feel" (measured by VAS) following smoked cannabis (66, 95). That study well characterized the absorption phase portion of the hysteresis, including data immediately after the first cigarette puff, throughout smoking and up to 12 h after cannabis smoking. Following the absorption and distribution phases, linear decreases in mean VAS score changes were observed from 1 to 12 h.

Counter-clockwise hystereses are typically observed for drugs with a larger volume of distribution, drugs with active metabolites, or an indirect mechanism of drug action (96). THC, with 2- or 3-compartment pharmacokinetics and an equipotent metabolite (11-OH-THC), is a likely candidate for a counter-clockwise hysteresis. Here, for the first time, we report hystereses for whole blood THC concentrations and several VAS scores, improving our understanding of subjective THC effects following *ad libitum* smoked cannabis. Yet, as demonstrated in Figure 8, inter-individual variability is high for these concentration-effect curves. Hystereses may vary substantially depending on dose, experimental setting or prior chronicity or frequency of use (96).

Our finding of minimal impairment on tracking and divided attention tasks from smoking a single cannabis cigarette, while observing robust subjective effects, suggests that there may be differential tolerance to the acute psychomotor and subjective effects of cannabis. Ramaekers et al (90) observed significant impairment differences between frequent and occasional smokers following 500 μg/kg smoked cannabis, surmising tolerance to acute THC-induced impairment of tracking and divided attention in the

heavy smoker group, similar to the findings of Hart et al (214). In the present study, mean *available* dose was 702 μg/kg (range 478-1000) (Table 9), with minimal performance changes in critical tracking and divided attention tasks 1.5-5.5 h after smoking. Most participants in our study had smoked cannabis almost daily for a mean [SD] of 7.2 [5.3] years, with several self-reporting numerous smoked joints per day. These findings appear to confirm those of Ramaekers and Hart, documenting significant subjective response and minimal impairment in driving-related psychomotor tasks in heavy, chronic cannabis smokers. However, further study is required to evaluate whether psychomotor tasks more sensitive to cannabis impairment and more similar to complex driving scenarios (such as a stop-signal task (90)) may show impairment in heavy, chronic users.

Although originally developed to determine cannabis impairment utilizing serum cannabinoid concentrations, we evaluated CIF with whole blood and plasma data to examine its usefulness in these matrices. Menetrey et al investigated whole blood CIF following a cannabis decoction in milk with 45.7 mg THC (219), achieving a mean peak CIF of approximately 50 following oral dosing, decreasing to approximately 18 after 5.5 h, and 5 after 24 h. The present study examined CIF in whole blood and plasma following smoked cannabis intake, yielding peak median CIF of 150 and 170, respectively. Following peak, CIF in whole blood and plasma were similar (Figure 9). Absolute CIF values decreased much more quickly than subjective "high." This is expected, as blood cannabinoid concentrations decreased much more rapidly than subjective response (as observed in Figure 7). Median CIF in these chronic, daily cannabis smokers was > 10 through 4 h in both matrices after cannabis smoking,

suggesting that the original cutoff proposed by Daldrup could be considered a rough determination of recent cannabis intake. However, we observed minimal impairment on tracking and divided attention tasks during this period. Therefore, this model does not appear suitable for quantifying impairment following cannabis consumption in heavy, chronic cannabis smokers. Finally, although spending > 12 h on a closed clinical unit, four participants had whole blood CIF > 10 in the baseline sample (0.5 h prior to smoking), precluding use as a definitive marker for recent cannabis intake.

Two participants (I and K) had 6 h whole blood CIF values of 26 and 27, respectively. Interestingly, these participants reported increased "High" "Stoned" and "Stimulated" VAS response at this time point, indicating residual drug effect correctly identified by the CIF. Yet, other individuals had a CIF > 10 in whole blood and plasma at 6 h, with no increase over baseline in any VAS measure. While other cutoffs could yield improved accuracy, caution is warranted in utilizing this model, as subjective effect may be incorrectly assumed for chronic cannabis smokers with high residual THC concentrations.

As described by Schwope et al (212), Mareck et al (181), and Kelly et al (49), detection of minor cannabinoids, CBD, CBN or THC-glucuronide may be beneficial in determining recent cannabis intake. Based on present data, detection of these markers would likely occur during the period of strong subjective effects and potential impairment. However, the frequent lengthy delay between cannabis smoking and sample collection during DUID and other investigations may preclude this approach, especially with whole blood (212). Further research may provide additional confidence in utilizing these analytes as markers of cannabis-induced impairment.

Cannabis smoking significantly increased dry mouth and thirst. As law enforcement, clinicians and workplace drug testing personnel consider oral fluid as an alternative matrix for detection of cannabis intake, cannabis-induced xerostomia may reduce the ability to collect oral fluid samples immediately after cannabis smoking. Furthermore, OF stimulation through citric acid candy or manual stimulation may lead to artificially lowered OF cannabinoid concentrations, confounding interpretation (221). However, oral fluid cannabinoid concentrations immediately following smoking are high (often > 1 mg/L) (222) increasing detection. Discarding low volume oral fluid samples without testing should be discouraged, as these samples are often positive (223).

Concentration-effect curves for whole blood THC and subjective effects produce counter-clockwise hysteresis following *ad libitum* smoking of a single 6.8% THC cannabis cigarette. Despite whole blood THC concentrations of 13-63 μg/L 15 min after the start of cannabis smoking, little psychomotor impairment was observed, while robust cardiovascular and subjective responses were seen. These data suggest differential tolerance to the psychomotor, but not other, effects of THC in heavy, chronic cannabis smokers, as previously reported by others (90, 214). CIF did not accurately predict impairment or subjective response following a single cannabis cigarette, but a high value (≥ 20) may suggest recent cannabis intake. These data advance our understanding of whole blood pharmacodynamic-pharmacokinetic relationships following cannabis smoking in heavy, chronic cannabis smokers. Additional research is needed to provide insight into pharmacodynamic-pharmacokinetic relationships in occasional cannabis smokers, as impairment may differ with less frequent exposure.

Chapter 6 – Conclusions

Research Summary

The major aims of this research were: 1) Develop and validate the first LC-MS/MS analytical method for simultaneous detection and quantification of free THC, 11-OH-THC, THCCOOH, CBD, and CBN and THC- and THCCOOH-glucuronide metabolites in human whole blood, allowing direct investigation of glucuronide pharmacokinetics and *in-vitro* cannabinoid stability. 2) Directly characterize, for the first time, cannabinoid and cannabinoid glucuronide pharmacokinetics in human whole blood and plasma following smoked cannabis. 3) Evaluate relationships between human whole blood cannabinoid (THC) concentrations and subjective effects (assessed with VAS and Likert scales), performance impairment (assessed with critical tracking/divided attention psychomotor tasks) and physiological effects (assessed through cardiovascular changes) following smoked cannabis.

We developed a controlled human smoked cannabis protocol designed to address these questions, facilitating interpretation in forensic DUID and accident investigations and clinical monitoring scenarios where whole blood is the only available sample. The primary findings of this research are detailed in Table 11.

Research Question	Finding	Chapter(s)
Can physiologically relevant cannabinoid glucuronide concentrations in human whole blood and/or plasma be quantified with LC-MS/MS?	0.5 mL of human whole blood or plasma was analyzed by liquid chromatography-tandem mass spectrometry for physiologically relevant concentrations of major and minor cannabinoids, and phase I and phase II cannabinoid metabolites in a single extraction and analytical run.	3
What are detection windows for cannabinoid glucuronides and minor cannabinoids in whole blood and plasma following cannabis smoking?	Generally, THC-glucuronide, CBD and CBN are only detectable for < 2 h in plasma and whole blood following cannabis smoking. THCCOOH-glucuronide is present at concentrations higher than free THCCOOH, and is detectable for up to 22 h following smoking in heavy, chronic cannabis smokers.	4
Are minor cannabinoids CBD and CBN valid markers of recent smoked cannabis intake?	Possibly, but not likely with current analytical limitations. CBD and CBN are not detectable after 2 h, and will likely be below LOQ before forensic specimens are collected, especially in whole blood. However, detection would confirm recent exposure, potentially during the impairment window.	4
What is the relationship between whole blood cannabinoid concentrations and subjective and neurocognitive effects following cannabis smoking?	The relationship between whole blood cannabinoid concentrations and subjective and neurocognitive effects following cannabis smoking is multi-phasic. Plotting visual analog scale results against THC whole blood concentrations yields a counter-clockwise hysteresis. Neurocognitive deficits were modest for heavy, chronic cannabis smokers, even in the presence of strong subjective effects, indicating potential tolerance for psychomotor effects.	5
Does the Cannabis Influence Factor (CIF) provide an accurate measure of concurrent impairment and/or recent cannabis intake following cannabis smoking?	Not definitively. CIF was not suitable for quantifying psychomotor impairment following cannabis consumption. Additionally, it was not precise enough to accurately determine recent cannabis use in this population of chronic smokers as several had CIF > 10 in baseline samples. However, a high value (> 30) may indicate recent use.	5
What is the *in vitro* stability of cannabinoid metabolites in authentic human whole blood and plasma specimens during storage?	Biological matrix, storage temperature and storage time affect *in vitro* stability. Generally, frozen specimens appeared to have stable concentrations. Decreases in THCCOOH-glucuronide and increases in free THCCOOH were noted in 4 °C specimens after 2 weeks, indicating instability in the ester glucuronide linkage.	6

Table 11. Primary Research Findings

Cannabinoids are the most commonly abused illicit drugs, and cannabinoid medications are utilized for an increasing number of indications, documenting the need for accurate and sensitive cannabinoid quantification. Many analytical methods are available to quantify cannabinoids in human whole blood (130, 153, 191-193); however, none include THC, phase I metabolites, other cannabinoids in the cannabis plant and THC- and THCCOOH-glucuronides. Most methods determined total cannabinoids, relying on enzymatic hydrolysis to release cannabinoid glucuronides. Multiple problems exist for obtaining reproducible total concentrations including poor hydrolysis efficiency (43, 187). In addition, cannabinoid glucuronide instability (139) can introduce unnecessary error into quantitative determinations.

Direct glucuronide identification and quantification within 24 h of collection addressed these issues and provided novel insight into glucuronide pharmacokinetics and glucuronide *in vitro* stability. Furthermore, direct glucuronide analysis enabled evaluation of this marker as an indicator of recent cannabis intake. The whole blood method presented in Chapter 3 sensitively and specifically quantified these glucuronides directly, in addition to minor cannabinoids CBD and CBN (Figure 3). Thus, this method for directly analyzing free and glucuronidated cannabinoids in whole blood was a significant advancement in the quantification of these compounds. Additionally, this method was easily adaptable to other matrices. It was adapted without modification for quantifying plasma cannabinoids following cannabis smoking and should be useful to extract and quantify cannabinoids in human urine or oral fluid with only minor modifications.

Chapter 4 detailed the first directly measured authentic human whole blood and plasma cannabinoid glucuronide data following cannabis smoking. Furthermore, simultaneous THC, 11-OH-THC, THCCOOH, CBD and CBN concentrations were provided after a single smoked cannabis cigarette. Observed peak cannabinoid concentrations were variable, likely due to inter-individual differences in smoking topography and prior cannabis smoking history. Direct glucuronide detection provided several benefits. THCCOOH-glucuronide detection may improve interpretation of cannabinoid results, as high baseline THCCOOH-glucuronide concentrations may be a marker for chronic, daily cannabis smoking, precluding utilization of predictive models for estimating time of last cannabis intake (140). Additionally, detection of THC-glucuronide provided a potential new marker for recent cannabis intake, as concentrations rapidly fell below the LOQ in whole blood and plasma. Minor amounts of CBD and CBN were detected in some participants' samples for up to 2 h following cannabis smoking, rendering these cannabinoids additional potential markers of recent cannabis intake. These novel data provide valuable information for interpreting whole blood cannabinoid concentrations in forensic investigations and clinical monitoring scenarios, while furthering understanding of potential markers of recent cannabis intake.

Data presented in Chapter 5 advanced our understanding of whole blood pharmacodynamic-pharmacokinetic relationships following cannabis smoking in heavy, chronic cannabis users. High-potency cannabis smoked *ad libitum* yielded significant increases in heart rate and subjective effects. However, minimal impairment was observed for psychomotor task performance in this heavy, chronic cannabis smoking cohort. While several VAS scores were significantly increased following smoking,

adverse scores for “Anxious” and “Restless” were minimal, likely due to *ad libitum* dosing and substantial cannabis smoking history.

Detection of minor cannabinoids (THC-glucuronide, CBD and CBN) at ≥ 1 μg/L LOQ (199) achieved narrow whole blood detection windows (< 1 h), most likely unhelpful for recent cannabis intake identification. However, significant subjective effects were observed when CBN and CBD exceeded 1 μg/L, suggesting that identification of these minor cannabinoids might be useful for assessing impairment.

Whole blood THC concentrations had a counter-clockwise hysteresis when plotted against subjective effects following *ad libitum* smoking of a single 6.8% THC cannabis cigarette. However, few psychomotor effects were observed in this cohort. Similar to other studies (90, 214), these data suggest potential tolerance to the psychomotor impairing effects of THC, although not to cardiovascular and subjective effects in heavy, chronic cannabis users. In contrast to Daldrup’s postulation, CIF was not suitable for quantifying impairment following cannabis consumption or reliably identifying recent cannabis intake. However, a high value (> 30) provided a rough estimate of recent intake (< 2 h). These data furthered our understanding of impairment and subjective effects following acute smoked cannabis intake.

Preliminary Findings - *In Vitro* Cannabinoid Stability

As detailed in Chapter 1, *in vitro* cannabinoid analyte instability could substantially impact forensic and clinical analyses. To better characterize *in vitro* cannabinoid stability and provide confidence in previously determined whole blood and

plasma cannabinoid concentrations (including those in the present work), we determined free and glucuronide cannabinoid stability in authentic whole blood and plasma specimens for up to 28 days after controlled cannabis smoking. Baseline specimens were analyzed within 24 h of collection.

Procedures were completed as described in the 10-DA-N458 protocol (Chapter 2), with baseline specimens analyzed in triplicate within 24 h of collection and remaining aliquots analyzed in duplicate after 7, 14 and 28 days at -20 °C and 4 °C, and after 7 days at RT. Specimens with concentrations ≥ LOQ were included in calculations; concentrations within ± 20% of the fresh analysis were considered stable.

10 cannabis smokers (9 M, 1 F) participated. The number of cannabinoid analyte concentrations ≥ LOQ (whole blood low and high pools; plasma low and high pools) were: THC (10, 10; 10, 10), 11-OH-THC (10, 10; 10, 10), THCCOOH (10, 10; 10, 10), CBD (0, 2; 0, 6), CBN (0, 6; 0, 8), THC-glucuronide (0, 3; 0, 7) and THCCOOH-glucuronide (10, 10; 9, 9). Mean THC, 11-OH-THC, CBD, CBN, and THC-glucuronide concentrations were stable (< 20% change) after 4 weeks at -20 °C and 4 °C, and 1 week at RT. In the high-THC whole blood pool, mean THCCOOH concentrations also were stable at -20 °C and 4 °C for 4 weeks. THCCOOH-glucuronide was stable in whole blood at -20 °C for 4 weeks, unstable at 4 °C for 4 weeks (mean decreases of -24.1%), and markedly unstable at RT with mean concentration changes of -40.2% and -45.3% at 1 week for low and high pools, respectively. Under these conditions (1 week, RT), mean THCCOOH whole blood concentrations increased by 21.7% and 11.8%, respectively (Table 12).

Plasma cannabinoid concentrations were stable at -20 °C for all analytes, including the glucuronides; however, after 4 weeks at 4 °C mean THCCOOH-glucuronide concentrations decreased by 66.7% and 74.5%, while THCCOOH concentrations increased by 25.7% and 15.6% in the low and high pools, respectively. Free and conjugated THCCOOH concentrations were unstable after 1 week at RT in both plasma pools, although other analytes were stable under these conditions.

Extensive protein binding in authentic whole blood and plasma specimens may improve stability of cannabinoids in these fluids reducing adsorption to container surfaces and precipitant. Frozen specimen storage (-20 °C) provided maximum stability, although short-term (28 day) refrigerated storage (4 °C) may be acceptable for whole blood determinations where analysis of THCCOOH-glucuronide is not required. Refrigerated plasma storage is not recommended for longer than 2 weeks when free THCCOOH concentrations are required due to deconjugation and/or other breakdown or rearrangement of THCCOOH-glucuronide. However, THC concentrations were stable in both matrices under all conditions tested, limiting impact on impairment determinations.

Table 12. *In vitro* whole blood and plasma cannabinoid stability (% concentration change from baseline [1 day])

[a] Concentration. [b] Room Temperature. [c] Concentration too low to determine stability.

WHOLE BLOOD

		- 20 °C				4 °C				RT[b]	
Analyte	Conc[a]	1 day	1 week	2 weeks	1 month	1 day	1 week	2 weeks	1 month	1 day	1 week
THCCOOH-glucuronide	High	0.0	4.3	0.0	-1.2	0.0	-3.3	-14.3	-24.1	0.0	-45.3
	Low	0.0	-5.3	1.5	-3.9	0.0	-5.4	-5.9	-22.6	0.0	-40.2
THC-glucuronide	High	0.0	-0.3	-0.3	2.6	0.0	-3.0	-3.1	-0.1	0.0	-0.7
	Low	n/a[c]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
THCCOOH	High	0.0	2.4	4.1	2.4	0.0	0.4	-0.2	3.2	0.0	11.8
	Low	0.0	0.9	-0.4	-0.5	0.0	2.4	6.9	10.8	0.0	21.7
11-OH-THC	High	0.0	4.9	-0.3	5.8	0.0	-0.8	-1.2	-4.3	0.0	-5.3
	Low	0.0	2.7	2.6	4.9	0.0	-0.6	1.2	-0.2	0.0	-5.0
CBD	High	0.0	-0.3	2.5	2.5	0.0	0.4	2.3	2.4	0.0	1.7
	Low	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
CBN	High	0.0	2.6	7.0	0.9	0.0	1.8	0.4	6.9	0.0	5.2
	Low	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
THC	High	0.0	1.1	2.7	-0.2	0.0	1.0	-1.3	-0.1	0.0	-3.6
	Low	0.0	1.8	-2.2	0.6	0.0	-0.7	3.7	-1.2	0.0	-0.9

PLASMA

		- 20 °C				4 °C				RT	
Analyte	Conc	1 day	1 week	2 weeks	1 month	1 day	1 week	2 weeks	1 month	1 day	1 week
THCCOOH-glucuronide	High	0.0	-3.0	13.9	3.3	0.0	-26.5	-43.0	-74.5	0.0	-47.7
	Low	0.0	1.4	0.1	1.5	0.0	-20.7	-36.9	-66.7	0.0	-73.0
THC-glucuronide	High	0.0	21.7	13.8	-1.6	0.0	1.8	-8.7	-6.2	0.0	-4.1
	Low	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
THCCOOH	High	0.0	0.5	-0.4	0.7	0.0	4.3	8.3	15.6	0.0	36.6
	Low	0.0	3.1	0.8	2.8	0.0	6.1	11.3	25.7	0.0	48.4
11-OH-THC	High	0.0	1.5	1.0	2.3	0.0	5.3	3.0	4.6	0.0	-6.1
	Low	0.0	-6.6	1.2	1.0	0.0	0.4	3.7	0.7	0.0	-11.0
CBD	High	0.0	-0.5	-1.0	-0.8	0.0	1.1	0.0	-0.8	0.0	6.9
	Low	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
CBN	High	0.0	-0.8	-4.0	-1.9	0.0	4.8	6.8	11.4	0.0	2.1
	Low	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
THC	High	0.0	0.5	-1.0	-0.1	0.0	0.2	0.1	1.9	0.0	-0.5
	Low	0.0	-1.2	-0.1	-2.8	0.0	-2.1	-1.3	0.6	0.0	0.3

Future Work

Chapter 2 details several experiments for Protocol 10-DA-N458 that were not addressed in this dissertation. These analyses are expected to continue well beyond the scope of the present research. Additionally, a second part of the protocol was designed and will be undertaken by NIDA IRP CDM in the coming months. This is expected to refine further cannabinoid glucuronide concentrations following cannabis smoking while investigating additional alternative matrices for detecting cannabis intake. Several logical future projects are detailed herein. While this list is not all-inclusive, the major hypotheses are the most feasible and of greatest importance to the scientific community.

Improving Method Sensitivity

As detailed in Chapter 4, minor cannabinoids, including THC-glucuronide, CBD and CBN were detected < 2 h after smoking. As commonly encountered with minor metabolites following drug administration, method sensitivity was the limiting factor in analyte detection. Limits of quantification were 0.5, 1.0 and 1.0 μg/L for THC-glucuronide, CBD and CBN, respectively. As instrument technology (and sensitivity) improves, it is possible these minor metabolites could be detected for longer periods following cannabis administration. This improved sensitivity could lead to formation of a useful cutoff (e.g. 0.25 or 0.1 μg/L) that enables forensic, clinical and research toxicologists to document recent (< 4 h) cannabis use, within the subjective effects and potential impairment window. Improved sensitivity would also permit detection of other minor cannabinoids following intake, such as the THC precursor Δ^9-tetrahydrocannabinolic acid A (THCA-A). Sensitivity improvements may be achieved

with further improvements in instrumentation. However, targeting analyses to only THC-glucuronide or only CBD/CBN could quickly improve extraction efficiency and minimize matrix effects, providing improved sensitivity utilizing current instrumentation (3200 QTrap, AB Sciex). As detailed in Chapter 4, CBD/CBN are likely the more prudent choice, given the current low cost and wide availability of standards, availability of deuterium-labeled analogues and wealth of existing extraction techniques.

Examining Impairment in Occasional Cannabis Smokers

Cannabis use history may have an impact on the behavioral response to a single smoked cannabis cigarette (90, 214). The present work only investigated cannabis-induced impairment in heavy, chronic cannabis smokers. We attempted to dichotomize the current cohort to "heavier" and "lighter" use groups based on self-reported histories; however, no difference was observed. Presumably, as the "lightest" user still smoked ≥ 4x/week, no truly occasional smokers were included. Examination of more occasional (≤ 1 joint/week) cannabis smokers is recommended, as previous research (90) suggests increased impairment may be observed in the occasional cannabis-smoking group. Further characterization of impairment effects between- and within-groups will help elucidate cannabinoid pharmacology and improve interpretation of cannabis-induced impairment as it relates to DUID and accident investigations.

In addition to critical tracking/divided attention task impairment, working memory and risk-taking behavior were investigated in the first part of Protocol 10-DA-N458 as described in Chapter 2. Further characterization of cannabis-induced risk taking following cannabis smoking will provide additional insight into impulsivity and

reward/punishment constructs, allowing for greater understanding of mechanisms responsible for cannabis abuse and resulting choices detrimental to public health. These studies will continue in the second part of this protocol, providing additional statistical power and allowing examination of potential differences between heavy and occasional cannabis smokers.

Cannabinoids in Alternative Matrices Following Cannabis Smoking

Some alternative matrices for cannabinoid detection include breath, sweat and oral fluid (OF). Currently OF is being evaluated for driving under the influence of drugs, drug treatment, workplace and pain management drug testing, and for clinical trials (176, 211, 224-225). Originally, OF THC detection was considered most useful, rather than 11-OH-THC or THCCOOH detection. However, recent research (with ng/L THCCOOH detection (226-227)) demonstrates improved detection rates using THCCOOH rather than THC following oral THC administration (211). Additionally, as THCCOOH is not present in cannabis smoke, utilization of this metabolite minimizes passive contamination concerns. Previous studies did not investigate or were unable to quantify the presence of THC metabolites or alternative cannabinoids in OF following controlled smoking. Protocol 10-DA-N458 is expected to provide essential data characterizing OF cannabinoid concentrations following cannabis smoking. The first phase evaluated several OF collection techniques, including expectoration, Quantisal™ OF collection device and Intercept™ OF collection device. These devices will characterize the disposition of THC and its metabolites in oral fluid following smoked administration and determine whole blood and plasma/oral fluid ratios, thereby contributing to a

pharmacokinetic database useful to law enforcement and clinical and forensic toxicologists utilizing OF to document cannabis intake.

As OF testing gains acceptance in DUID, workplace and clinical monitoring scenarios, validation of robust, sensitive, and cost-effective screening assays will allow detection of illicit drug use while reducing confirmatory demands on personnel and instrumentation. To this end, the we detailed an extensive validation of the Immunalysis Sweat/OF THC Direct ELISA, documenting sensitive, precise and accurate screening for THC in OF (228) (Appendix M). Evaluation of screening devices continued in Protocol 10-DA-N458 with the Drager DrugTest® 5000, an immunoassay-based point-of-collection testing device (POCT). The DrugTest 5000 demonstrated excellent sensitivity and efficiency, and good specificity in the first phase of the protocol, with 91% sensitivity, 75% specificity and 87% efficiency at the 2 µg/L THC SAMHSA confirmation cutoff. This was the first full evaluation of the newest DrugTest 5000 assay and data were greatly improved over other versions.

The second phase of Protocol 10-DA-N458 is expected to continue evaluation of the Draeger DrugTest 5000, as well as additional OF collection devices. Furthermore, other alternative matrices, including breath and urine, will be evaluated following cannabis smoking. It is expected these alternative matrices will provide further insight into cannabinoid disposition following smoked cannabis and provide toxicologists with additional less-invasive matrices useful for cannabinoid detection and determination of recent cannabis intake.

Continuing *In Vitro* Cannabinoid Stability Studies

In vitro stability studies from Protocol 10-DA-N458 are ongoing, and will continue through 1 year from initial (baseline) quantification. The present work details stability through 1 month. As current pharmacokinetic specimens were analyzed within this time with no freeze-thaw cycles, adequate stability characterization was achieved. However, characterization of cannabinoid stability in authentic specimens is needed, as specimens are often stored for up to a year in clinical, forensic and research settings. Therefore, characterization of *in vitro* stability over this time will be useful to toxicologists interpreting concentrations obtained days, weeks, or months after specimen collection. Stability studies will be extended for authentic oral fluid and initiated for authentic urine specimens collected after smoked cannabis. As THCCOOH-glucuronide is thought to predominate in urine, ester-linked glucuronide instability may significantly increase free THCCOOH concentration in this matrix, necessitating stability characterization. It is expected these results will comprise the most accurate and most comprehensive body of work for *in vitro* cannabinoid stability to date; it is expected the present author will publish whole blood and plasma results following the completion of the stability analyses.

Finis

Generally, whole blood is collected as the specimen of choice in DUID and accident investigations. Therefore, directly-determined whole blood cannabinoid concentrations (including phase II glucuronide metabolites), whole blood pharmacodynamic-pharmacokinetic relationships and whole blood *in vitro* cannabinoid

stability in authentic samples are significant findings that advance our understanding of cannabinoid metabolism and effects in humans. Minor cannabinoids, such as THC-glucuronide, CBD and CBN are possible inclusionary markers of recent smoked cannabis intake and are likely only detected in whole blood during the impairment window at a 1.0 µg/L LOQ. However, improved detection limits would provide a greater detection window for these minor metabolites, improving efficacy as markers of recent cannabis intake. Further research is needed to elucidate the potential lack of correlation between robust observed subjective effects and minimal psychomotor impairment in heavy, chronic cannabis smokers following cannabis smoking. Specifically, studies examining cannabis effects in heavy *and* occasional cannabis smokers may help characterize observed differential impairment, as could instruments with greater sensitivity for detecting psychomotor impairment. Regardless, proper specimen collection and storage remain paramount, as failure to preserve cannabinoid analyte concentrations can confound forensic and clinical toxicological interpretation.

Appendix A – Protocol 10-DA-N458 Screening Procedures

All applicants for this protocol will undergo the following screening procedures to determine study eligibility and to characterize their baseline condition

1. Medical history
2. Psychiatric evaluation including Wide Range Achievement Test (WRAT), SCID, and SCL-90R
3. Drug use patterns as assessed with the Drug Use Survey, Addiction Severity Index, and urine drug screen
4. Physical examination
5. Clinical laboratory tests (CBC with differential, NIDA Chemistry Panel, urinalysis, and serum pregnancy [females with reproductive potential only])
6. ECG and 3-minute rhythm strip

Appendix B – Protocol 10-DA-N458 Stability Chart

Whole Blood, Plasma and Urine

Storage Temp	Stability Time Points						
	< 1 day	7 days	14 days	1 month	3 months	6 months	1 year
Room Temperature	X	X					
4 °C	X	X	X	X	X	X	
-20 °C	X	X	X	X	X	X	X

Oral Fluid

Storage Temp	Stability Time Points			
	< 1 day	7 days	1 month	6 months
Room Temperature	X	X		
4 °C	X	X	X	
-20 °C			X	X

Notes

1. Whole blood, plasma, and urine analyzed at one low and one high concentration (based on pooling) for each stability time point.
3. Oral fluid for stability testing was collected by expectoration and the Quantisal™ OF collection device
4. All specimens stored in the dark in Nunc Cryotubes.

Appendix C – Protocol 10-DA-N458 Neuromotor Exam

ARC #:_____Date:_____

Mental Status:

Pre:			Post:		
Alert	Y	N	Alert	Y	N
Drowsy	Y	N	Drowsy	Y	N
Oriented to:			Oriented to:		
Person	Y	N	Person	Y	N
Place	Y	N	Place	Y	N
Time	Y	N	Time	Y	N
Situation	Y	N	Situation	Y	N

Neuromotor Skills

Pre:		Post:	
Walk and Turn:	(circle one)	Walk and Turn:	(circle one)
Can't keep balance	Normal	Can't keep balance	Normal
Abnormal		Abnormal	
Stops walking	Normal	Stops walking	Normal
Abnormal		Abnormal	
Steps off line	Normal	Steps off line	Normal
Abnormal		Abnormal	
Misses heel /toe	Normal	Misses heel /toe	Normal
Abnormal		Abnormal	
Raises arms	Normal	Raises arms	Normal
Abnormal		Abnormal	

Pre-Dosing

Evaluator's Signature_____Date:_____Time_____

Comments:_____

___

Post-Dosing

Evaluator's Signature_____Date:_____Time_____

Comments:_____

___

Appendix D – Protocol 10-DA-N458 Pharmacokinetic Experimental Plan

Day	Nominal Theoretical Time (from start of dose)	Event
1	-0.5	OF/PL/WB
1	0	DOSE (10 min)
1	0.25	OF/PL/WB
1	0.5	OF/PL/WB
1	1	OF/PL/WB
1	2	OF/PL/WB
1	3	OF/PL/WB
1	4	OF/PL/WB
1	6	OF/PL/WB
2	22.5*	OF/PL/WB

Note: All urines collected *ad libitum* by participants during stay

KEY

*	Optional collection if participant stays overnight after dosing
OF	Oral fluid (Quantisal™, DrugTest 5000 and/or expectoration collection methods)
PL	Plasma (sodium heparin Vacutainer)
WB	Whole blood (sodium heparin Vacutainer)
DOSE	One 6.8% THC cannabis cigarette with *ad libitum* smoking

Appendix E – Protocol 10-DA-N458 Risk Perception Questionnaire

ARC#: ___ ___ ___ ___ ___ ___ Date of Rating: __ __/__ __/__ __ __ __

Subject ID: _____ Subject Initials: ___ ___ Rater ID: ___ ___ Rater Initials: ___ ___

NOTES:

_____

_____

Was Form Completed? ____ (1=Yes,2=No) If Not, Please Specify:

_____

Much less likely	Less likely	A little less likely	Equally likely	A little more likely	More likely	Much more likely		Missing data
-3	-2	-1	0	1	2	3		9

I. Form: A

II. "Compared to other adults of your age and gender, how likely is it that you will <fill in event here> at some point in your lifetime?"

1. _____ Lose a large amount of money while gambling
2. _____ Suffer from frostbite
3. _____ Get sick due to toxic gas in your home
4. _____ Get a cut, while preparing food, severe enough to need treatment
5. _____ Experience a fatal overdose of a prescription painkiller
6. _____ Develop asthma
7. _____ Get robbed on the street
8. _____ Become anemic
9. _____ Get sick from exposure to hazardous waste
10. _____ Experience an earthquake

11. _____ Experience non-fatal heat stroke
12. _____ Contract a non-fatal STD (Sexually Transmitted Disease)
13. _____ Experience asbestos poisoning
14. _____ Develop diabetes
15. _____ Experience a fatal overdose of aspirin or Tylenol
16. _____ Be injured by debris falling from space
17. _____ Be injured in a car as a result of not wearing a seatbelt
18. _____ Accidentally drown
19. _____ Contract a case of the common cold
20. _____ Be pressured by friends or family to commit a crime
21. _____ Experience a fatal fall
22. _____ Contract lung cancer
23. _____ Get sick as the result of a vaccination
24. _____ Contract a non-fatal case of influenza
25. _____ Develop cataracts
26. _____ Experience obesity
27. _____ Be the victim of a non-fatal violent crime
28. _____ Develop glaucoma
29. _____ Get sick as a result of drinking water contamination
30. _____ Develop arthritis
31. _____ Experience a non-fatal case of appendicitis
32. _____ Develop gum disease
33. _____ Witness a crime being committed
34. _____ Be involved in an automobile crash as a passenger
35. _____ Get a dog bite that requires medical treatment
36. _____ Be sent to jail
37. _____ Contract a non-fatal case of pneumonia
38. _____ Get sick from using someone else's toothbrush
39. _____ Undergo an organ transplant operation
40. _____ Get sick from exposure to chemical fertilizer

Appendix F – Protocol 10-DA-N458 Self-Assessments of Risk Perception, Risk Taking/Impulsivity and Sensation Seeking

RISK PERCEPTION SCALE

How likely is it that something bad would happen to you if you:

	Score*
1. drove over the speed limit?	_____
2. drove while drunk?	_____
3. drove without a seat belt?	_____
4. drank a lot?	_____
5. had sex with someone you just met?	_____
6. got drunk and had sex with someone you just met?	_____

*1 – 5 scale: 1=Very unlikely; 5=Very likely.

RISK-TAKING AND IMPULSIVITY SCALE

How much does each of the following statements describe you?

	Score*
1. I often act on the spur of the moment without stopping to think.	_____
2. I get a real kick out of doing things that are a little dangerous.	_____
3. You might say I act impulsively.	_____
4. I like to test myself every now and then by doing something a little chancy.	_____
5. Many of my actions seem to be hasty.	_____

*1 – 4 scale: 1=Not at all; 4=Quite a lot.

SENSATION-SEEKING SCALE

How much does each of the following statements describe you?

	Score*
1. I'm always up for a new experience.	_____
2. I like to try new things for the excitement.	_____
3. I go for the thrills in life when I get a chance.	_____
4. I like to experience new and different sensations.	_____

*1 – 4 scale: 1=Not at all; 4=Quite a lot.

Appendix G – Protocol 10-DA-N458 Impulsive Sensation-Seeking Subscale from the Zuckerman-Kuhlman Personality Questionnaire

DIRECTIONS: If you agree with a statement or decide that it describes you, answer TRUE. If you disagree with a statement or feel that it is not descriptive of you, answer FALSE. Answer every statement either True or False even if you are not entirely sure of your answer.

1. (I) I tend to begin a new job without much advance planning on how I will do it.
6. (I*) I usually think about what I am going to do before doing it.
14. (I) I often do things on impulse.
19. (I) I very seldom spend much time on the details of planning ahead.
24. (SS) I like to have new and exciting experiences and sensations even if they are a little frightening.
29. (I) Before I begin a complicated job, I make careful plans.
34. (SS) I would like to take off on a trip with no preplanned or definite routes or timetable.
39. (I) I enjoy getting into new situations where you can't predict how things will turn out.
45. (SS) I like doing things just for the thrill of it.
50. (SS) I tend to change interests frequently.
55. (SS) I sometimes like to do things that are a little frightening.
60. (SS) I'll try anything once.
65. (SS) I would like the kind of life where one is on the move and traveling a lot, with lots of change and excitement.
70. (SS) I sometimes do "crazy" things just for fun.
75. (SS) I like to explore a strange city or section of town by myself, even if it means getting lost.
79. (SS) I prefer friends who are excitingly unpredictable.
84. (I) I often get so carried away by new and exciting things and ideas that I never think of possible complications.
89. (I) I am an impulsive person.
95. (SS) I like "wild" uninhibited parties.

Appendix H – Protocol 10-DA-N458 Barratt Impulsiveness Scale

(Version 11)

Instructions: Please tick the response that best indicates your usual style (Rarely/Never, Occasionally, Often, Almost Always/Always).

1. I plan tasks carefully.
2. I do things without thinking.
3. I make-up my mind quickly.
4. I am happy-go-lucky.
5. I don't "pay attention."
6. I have "racing" thoughts.
7. I plan trips well ahead of time.
8. I am self-controlled.
9. I concentrate easily.
10. I save regularly.
11. I "squirm" at plays or lectures.
12. I am a careful thinker.
13. I plan for job security.
14. I say things without thinking.
15. I like to think about complex problems.'
16. I change .jobs.
17. I act "on impulse."
18. I get easily bored when solving thought problems.
19. I act on the spur of the moment.
20. I am a steady thinker.
21. I change residences.
22. I buy things on impulse.
23. I can only think about one problem at a time.
24. I change hobbies
25. I spend or charge more than I earn.

26. I often have extraneous thoughts when thinking.
27. I am more interested in the present than the future.
28. I am restless at the theater or lectures.
29. I like puzzles.
30. I am future oriented.

Appendix I – Protocol 10-DA-N458 Melbourne Decision-Making Questionnaire

Instructions: People differ in the way they go about making decisions. Please indicate how you make decisions by ticking for each question the response which best fits your usual style (Possible responses: "True for me," "Sometimes true," and "Not true for me").

1. I like to consider all of the alternatives.
2. I try to find out the disadvantages of all alternatives.
3. I consider how best to carry out a decision.
4. When making decisions I like to collect a lot of information.
5. I try to be clear about my objectives before choosing.
6. I take a lot of care before choosing.
7. I avoid making decisions.
8. I do not make decisions unless I really have to.
9. I prefer to leave decisions to others.
10. I do not like to take responsibility for making decisions.
11. If a decision can be made by me or another person I let the other person make it.
12. I prefer that people who are better informed decide for me.
13. I waste a lot of time on trivial matters before getting to the final decision.
14. Even after I have made a decision I delay acting upon it.
15. When I have to make a decision I wait a long time before thinking about it.
16. I delay making decisions until it is too late.
17. I put off making decisions.
18. Whenever I face a difficult decision I feel pessimistic about finding a good solution.
19. I feel as if I am under tremendous time pressure when making decisions.
20. The possibility that some small thing might go wrong causes me to swing abruptly in my preference.
21. I cannot think straight if I have to make a decision in a hurry.
22. After a decision is made I spend a lot of time convincing myself it was correct.

Appendix J – Protocol 10-DA-N458 Subjective Effects Scales

Visual-analogue scales (100 mm anchored with “not at all” and “most ever”)

Good drug effect
High
Stoned
Stimulated
Sedated
Anxious
Restless

5-Point Likert scales (none, slight, mild, moderate, severe)

Difficulty concentrating
Altered sense of time
Slowed or slurred speech
Body feels sluggish or heavy
Feel hungry
Feel thirsty
Shakiness/tremulousness
Nausea
Headache
Palpitations
Upset stomach
Dizzy
Dry mouth or throat

Appendix K – Protocol 10-DA-N458 Balloon Analogue Risk Task (BART)

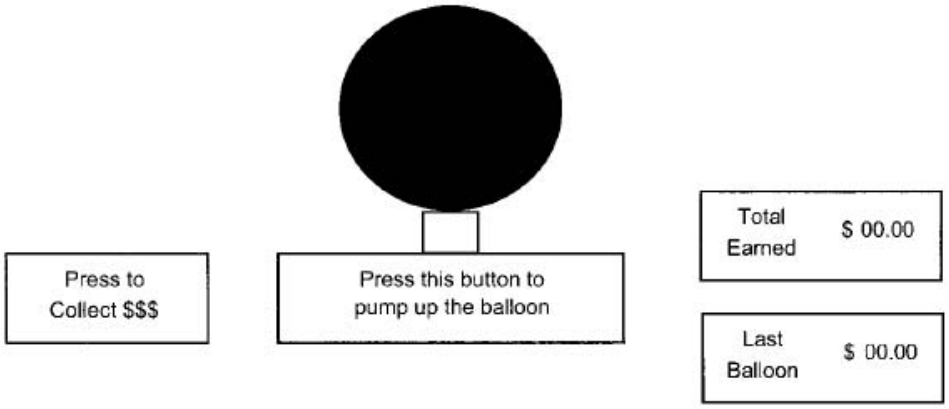


Figure 1. Diagram of the Balloon Analogue Risk Task.

Appendix L – Protocol 10-DA-N458 Consent Form

CLINICAL INVESTIGATION CONSENT FORM

The National Institute on Drug Abuse

Division of Intramural Research Program

Date: November 24, 2010

Protocol No: 10-DA-N458

Title of Research Project: Oral Fluid, Plasma and Whole Blood Pharmacokinetics and Stability Following Smoked Cannabis

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Explanation of Research Project to Subject:

INTRODUCTION

We invite you to take part in a research study at the National Institutes of Health (NIH).

First, we want you to know that:

Taking part in NIH research is entirely voluntary.

You may choose not to take part, or you may withdraw from the study at any time. In either case, you will not lose any benefits to which you are otherwise entitled. However, to receive care at the NIH, you must be taking part in a study or be under evaluation for study participation.

You may receive no benefit from taking part. The research may give us knowledge that may help people in the future.

Second, some people have personal, religious or ethical beliefs that may limit the kinds of medical or research treatments they would want to receive (such as blood transfusions). If you have such beliefs, please discuss them with your NIH doctors or research team before you agree to join the study.

Now we will describe this research study. Before you decide to take part, please take as much time as you need to ask any questions and discuss this study with anyone at NIH, or with family, friends or your personal physician or other health professional.

Purpose of study:

The aim of this study is to learn how your body handles delta-9-tetrahydrocannabinol (THC) after smoking a marijuana cigarette.

Background:

THC is the active chemical in marijuana that produces its effects. We are studying people's reactions, thinking abilities, and behavior after they smoke marijuana. We are also collecting blood and saliva. This will allow us to learn better ways to detect drugs in in a person's body and to study how long THC can be found in blood and saliva.

Study population:

About 15 people who use marijuana will take part in this study.

Who can be in this study:

You may be eligible for this research study if you

1. use marijuana and have no plans of stopping it,
2. are healthy,
3. are between ages 18 and 45,
4. have veins in your arms that we can draw blood from without difficulty.

Who may not be in this study:

You may not be eligible for this research study if you

1. have certain medical or psychiatric problems such as hypertension, diabetes, or schizophrenia,
2. have had a bad reaction to marijuana,
3. are pregnant or nursing,
4. have donated blood within the past 30 days,
5. have ever been treated for drug abuse or if you are interested in receiving drug abuse treatment.

Procedures:

Study overview:

You had tests to determine if you are eligible for this study under another NIDA protocol, Protocol #415. Under that protocol, you had a history, a physical examination, an electrocardiogram, and blood and urine tests.

This study involves 2 visits to NIDA. On the first visit, you will come to NIDA for approximately 3 h practicing the tests that are described below and answering surveys about how you think and act. During the second visit, you will smoke one marijuana cigarette, provide blood, saliva and urine several times, and perform the tests you practiced.

For your 2nd visit, you have the choice of participating as an outpatient or being admitted to the Johns Hopkins Behavioral Pharmacology Research Unit (BPRU). If you wish to be an outpatient, you will need to come to NIDA by 7 a.m. on the morning of your session. You will be here for approximately 10 h. If you are an outpatient, you must have a negative urine drug screen on the morning of your 2nd visit. If your drug screen is positive, you will be sent home. You may be rescheduled once only.

If you choose to stay overnight at BPRU the night before your second visit, you will not have to have a negative urine drug screen on the morning of the study session. If you are admitted to BPRU, you will stay the evening before your session and have the option of staying the following night. If you choose to stay at BPRU only the night before your session, you will be here for about 26 h. If you choose to spend both the nights before and after the session, you will be at NIDA for about 44 h.

Study procedures:

First visit: You will be taught how to do different tests of thinking, memory and concentration. You will sit at a computer and practice three tasks. One will be a memory task, one will test your concentration and muscle control, and one will test how you think about risk. You will practice the tasks so that you are familiar with them for your 2nd visit. You will also complete questionnaires about how you normally think and act. There are 7 questionnaires that should take about one hour to complete.

Second visit: On the morning of the second visit, we will draw blood and collect samples of your saliva and urine as described below. The samples will be tested for THC and other marijuana components. If you are female, you will be given a urine pregnancy test to check that you are not pregnant. You will fill out questionnaires on how you are feeling and how marijuana affects you. This will take about one hour. Then you will smoke one marijuana cigarette at your own pace for about 10 minutes. After smoking, we will again collect blood, saliva and urine. You will answer questions about how you are feeling, repeat the tests of thinking, memory and risk-taking that you had at the first visit, and have your heart rate, blood pressure, and breathing checked.

If you choose not to spend the night at BPRU, you will be discharged from the study later the same day. We do not expect you to be impaired at the end of the day. However, if we think you are still impaired from the smoking session or if we have any concerns about your health or safety, we may request that you spend the night. If you choose to leave the same day, you will not be allowed to drive yourself home. You can have a friend or relative pick you up, or we will provide a taxi for you.

If you choose to spend the night at BPRU after your session, you will be brought to the BPRU where you will have dinner, relax, and spend the night. You will be discharged before 11 a.m. the next day.

You will be searched prior to admission to the BPRU and you will be expected to follow the rules of the unit. For example, you may not receive visitors, and if you leave, you will not be allowed to return to the unit.

Study drug and procedures:

Marijuana: Marijuana is an illegal drug. It is being used in this study for research purposes only with the permission of the FDA. You will smoke a single cigarette

containing about 6.8% or 54 mg THC. This is slightly less than the average concentration of THC in marijuana that has been confiscated in the United States recently.

Blood collection: We will draw blood 8 times during this study if you participate as an outpatient or if you stay at BPRU the night before your session. If you stay at BPRU the night after your session, we will draw blood one additional time the following morning. Less than 15 ounces (450 mL) of blood will be taken during the entire study, and no more than 70 mL at one time. This is less than the amount taken in a normal blood donation. Blood will be drawn by inserting a needle into a vein in your hand or arm. A tube (intravenous catheter) may be inserted into the vein and left there during the study. This allows us to collect blood without sticking you with a needle each time. Each blood collection will take about five minutes.

Urine collection: You will need to collect all of your urine in bottles each time that you go to the bathroom during the time that you are at NIDA and/or BPRU. The urine will be tested for THC and other marijuana components. If you are staying at BPRU, you will have to store your urine samples in a refrigerator.

Saliva collection: Your saliva will be collected several times. We will collect your saliva by having you suck on an absorbent pad on a plastic stick for less than 5 minutes. We may also ask you to spit into a tube. It will take about ten minutes to collect saliva.

Risks/discomforts:

Marijuana: The most common effects of smoked marijuana include lightheadedness, dizziness, irritability, paranoia, drowsiness, and trouble thinking. Other common effects are poor memory, nervousness, difficulty walking, confusion, and hallucinations. You may have increased heart rate, low blood pressure, and bloodshot eyes. These effects usually go away by themselves within about six hours.

Your urine could test positive for marijuana for several weeks after you smoke the marijuana cigarette.

In case of a medical emergency, the physicians responsible for your care will follow the emergency procedures in place at NIDA IRP or BPRU. These may include evaluation at a hospital emergency department.

Blood collection and intravenous catheter placement: Adverse events related to blood collection and the tube in your arm may include pain, feelings of faintness, nausea, and bruising or bleeding where the blood was drawn. Rarely, infection, and/or thrombophlebitis (inflammation of the vein due to blood drawing) may occur.

Urine and saliva collection: There are no medical risks from urine or saliva collection. You may have dry mouth after saliva collection.

Questionnaires: Answering the questionnaires could cause you distress or embarrassment. You can take a break from answering at any time. You do not have to answer any question if it makes you uncomfortable.

Cognitive testing: There are no medical risks from cognitive testing. You may become bored after cognitive testing.

Pregnancy risks: Pregnant and nursing women cannot take part in this study.because marijuana could be dangerous for the fetus or baby. If you are female, you will be given a urine pregnancy test to check that you are not pregnant when you come for the 2nd study visit. If the test is positive or uncertain, you will not be allowed to take part. You should know that a negative pregnancy test does not rule out very early pregnancy. If you think that you could be pregnant, you should tell us, and you should not participate, to protect your baby.

Benefits:

There are no direct benefits to you from taking part in this study. The findings of this study may help us better understand the effects of marijuana. They may also help improve laboratory testing for marijuana. This may result in future benefits to marijuana users and to society.

Right of withdrawal and conditions for early withdrawal:

You may withdraw from the study at any time and for any reason. You will not lose benefits or privileges to which you are otherwise entitled. The investigators can remove you from the study at any time. This may occur if she or he believes that continuing is not in your best medical interest. This may also occur if you are unable to comply with the requirements of the study.

The Principal Investigator, Dr. Marilyn Huestis, reserves the right to end your participation if you do not follow the rules of the the study or of the research unit. The investigators can remove you from the study at any time.

Results from this study:

The information we obtain from this study will not provide information on your health. You will not receive any individual results. We will share with you any new information that may relate to your willingness to continue to participate in this study.

Alternatives to participation:

This study does not provide treatment. The alternative is not to participate.

Confidentiality:

When results of an NIH research study are reported in medical journals or at scientific meetings, the people who take part are not named and identified. In most cases, the NIH will not release any information about your research involvement without your written permission. However, if you sign a release of information form, for example, for an insurance company, the NIH will give the insurance company information from your medical record. This information might affect (either favorably or unfavorably) the willingness of the insurance company to sell you insurance.

We will keep the study information private to the extent possible by law. We have a Certificate of Confidentiality from the Department of Health and Human Services (DHHS). This certificate will protect us from being forced to release any research data in which you are identified, even under a court order or subpoena.

However, we will report certain contagious diseases, information about child abuse, and threats to self or others. Information needed for your care during a medical emergency

may be given to the medical people who are treating you. In addition, under certain conditions, people responsible for making sure that the research is done properly may review your study records. This might include people from the National Institutes of Health, the Food and Drug Administration, authorized hospital accreditation organizations, or the sponsoring company (if any). All of these people are also required to keep your identity confidential. Otherwise, the information that identifies you will not be given out to people who are not working on the study, unless you give permission**.**

Compensation and travel costs:

The amount of payment to research volunteers is guided by the National Institutes of Health policies. In general, patients are not paid for taking part in research studies at the National Institutes of Health. Reimbursement of travel and subsistence will be offered consistent with NIH guidelines.

There is no cost to you for study procedures. You will be paid for your time and inconvenience based on your study participation. This includes $40 for each night on the research unit and $20 per hour for task training and completion. It also includes $10 for intravenous catheter placement. If you complete all parts of the study, you will receive a completion incentive. This is 10% of your total study earnings, excluding any winnings from the computerized task. The maximum amount you can earn, including the completion incentive, is about $394 if you stay for 2 nights. The maximum amount you can earn if you do not stay either night is $306. You may receive some additional money, depending on how well you do on the computerized task (pumping up a balloon without making it burst). You may keep any winnings from the computerized task during the second visit. The amount of money you may win ranges from $0-$64. The money will not be given to you all at once. You will get up to $200 when you leave the research unit. The remaining money will be paid out in amounts of no more than $200 at one time. You will not be paid more often than every other day. You also can have money spent on your behalf. This can be to buy something or pay a bill. This can be done at any time. There is no limit, up to the total amount you have earned for the study. You may withdraw from the study before completion. In this case, you will be compensated for all the days you have participated. There will be no completion incentive if you withdraw. If you do not follow all study procedures, your payment may be reduced. If you end your participation in the study early or are discharged due to inappropriate behavior, you will be paid what you earned until that time, without a completion incentive.

Conflict of interest:

The National Institutes of Health reviews NIH staff researchers for conflicts of interest at least once a year. Details on this process are at http:// ethics.od.nih.gov/forms/ Protocol-Review-Guide.pdf. You may ask study personnel for further details or for a copy of the Protocol Review Guide.

One or more researchers in this study may have up to $15,000 of stock in the supplier of a product(s) used in this study. Under federal rules, this is allowed and does not create a conflict of interest.

This protocol may have researchers who are not NIH employees. Non-NIH investigators are expected to adhere to the principles of the Protocol Review Guide. However, they are not required to report their personal financial holdings to the NIH.

If you are hurt by being in the study:

If you think you have been hurt by being in the study, or if you feel you have not been treated fairly, you should call the Clinical Director. The phone number is 443-740-2360.

NIDA will provide short-term medical care for any physical injury resulting from your participation in this study. In general, no long-term medical care or financial compensation for research-related injuries will be provided by NIDA, NIH or the Federal Government. However, you have the right to pursue legal action if you believe your injury justifies legal action.

Joining of your own free will (volunteering for the study):

You do not have to join this or any research study. You may decline or quit the study at any time. All normal treatment options will still be available to you regardless of whether you join the study. If you wish to undergo treatment for your marijuana use, you will be referred to a counselor. He or she will evaluate you, and based on these results, refer you to a proper facility.

Questions you may have about the research study:

This consent form explains the research study. Please read it carefully. Ask questions about anything you do not understand. If you do not have questions now, you may ask later. During the study, you will be told any new facts that could affect whether you want to stay in the study. If the study relates to a health problem you have, we will explain what other treatment could be given outside the research. You should understand these treatment options before you sign this form. If you have any problems or questions about the study, or about your rights as a research participant, or about any research-related injury, you should call the Principal Investigator, Dr. Marilyn Huestis, at 443-740-2524 or the Medical Advisory Investigator, Dr. David Gorelick, at 443-740-2526.

What your signature means:

Your signature below means: 1) you have been given the opportunity to ask any questions; 2) you have had the time to consider fully whether you want to join the study, and 3) you agree to participate.

NOT VALID WITHOUT IRB STAMP OF CERTIFICATION

Signature of Participant Date

Print name (participant)

Signature of Parent or Guardian (when applicable) Date

Print name (Parent/Guardian)

Witness to Participant's signature* Date

* Optional unless subject is illiterate, or unable to sign

Print name (Witness)

Signature of Investigator Date

Print name (Investigator)

Appendix M – Validation of an enzyme immunoassay for detection and semi-quantification of cannabinoids in oral fluid

(as published in *Clinical Chemistry*, 2010)

Abstract

BACKGROUND: Oral fluid (OF) is gaining prominence as an alternative matrix for monitoring drugs of abuse in workplace, criminal justice, and driving under the influence of drugs programs. It is important to characterize assay performance and limitations of screening techniques for Δ^9-tetrahydrocannabinol (THC) in OF.

METHODS: OF specimens were collected with the Quantisal™ OF collection device from 13 daily cannabis users following controlled oral cannabinoid administration. All specimens were tested with the Immunalysis Sweat/OF THC Direct ELISA and confirmed by two-dimensional gas chromatography-mass spectrometry (2D-GC-MS).

RESULTS: The limit of detection was < 1 μg/L THC equivalent and the assay demonstrated linearity from 1-50 μg/L, with semi-quantification to 200 μg/L. Intra-plate imprecision (n = 7) ranged from 2.9-7.7% CV while inter-plate imprecision (n = 20) was 3.0-9.1%. Cross-reactivities at 4 μg/L were: 11-hydroxy-THC, 198%; Δ^8-tetrahydrocannabinol, 128%; 11-nor-9-carboxy-THC (THCCOOH), 121%; THC (target),

98%; cannabinol, 87%; THCCOOH-glucuronide, 11%; THC-glucuronide, 10%; and cannabidiol, 2.4%. Of 499 tested OF specimens, 52 confirmed positive (THC 2.0-290 μg/L), with 100% diagnostic sensitivity at the proposed Substance Abuse and Mental Health Services Administration 4 μg/L cannabinoid screening cutoff and 2 μg/L THC GC-MS cutoff. 47 specimens screened positive but were not confirmed by 2D-GC-MS, yielding 89.5% diagnostic specificity and 90.6% diagnostic efficiency. 31 of 47 unconfirmed immunoassay positive specimens were from one individual and contained THCCOOH > 400 ng/L, potentially contributing to cross-reactivity.

CONCLUSIONS: The Immunalysis Sweat/OF THC Direct ELISA is an effective screening procedure for detecting cannabinoids in OF.

Introduction

Illicit use of smoked and oral cannabis continues to be a public health concern, as shown in a 2007 Substance Abuse and Mental Health Services Administration (SAMHSA) survey, where 5.8% of the US population aged 12 y and older smoked cannabis in the past month (229). While potentially detrimental to the individual, cannabis also has a substantial impact on public safety, as many elect to operate motor vehicles soon after self-administration. The National Highway Traffic Safety Administration recently reported that in 2007, 8.6% of nighttime drivers were positive for cannabinoids in blood and/or oral fluid (OF), almost 4 times higher than the percentage of drunk drivers with a blood alcohol concentration ≥ 0.8 g/L (175). While finding cannabinoids in blood or OF does not necessarily imply impairment, windows of drug detection in these matrices are short, increasing impairment probability.

Recently, OF was identified as a suitable alternative matrix for monitoring driving under the influence of drugs (DUID) in addition to clinical, workplace, and drug treatment settings. In 2004, SAMHSA proposed revisions to the 1988 mandatory guidelines for federal workplace drug testing programs specifying guidelines for OF testing (230). An OF cannabinoid screening cutoff of 4 μg/L and a Δ^9-tetrahydrocannabinol (THC) confirmation cutoff of 2 μg/L were proposed. At that time, only THC had been detected in OF following cannabis consumption. A potential problem with measurement of the parent drug in OF was whether active cannabis intake could be differentiated from passive exposure to THC-laden cannabis smoke (230).

Therefore, simultaneous collection of a urine sample also was proposed, negating the usefulness of OF testing.

Indeed, prior OF research reported no psychoactive 11-hydroxy-THC (11-OH-THC) or inactive 11-nor-9-carboxy-THC (THCCOOH) in OF by gas chromatography-mass spectrometry (GC-MS) at limits of quantification (LOQ) of 0.5 μg/L (231). Additionally, THC and metabolites were not detected in OF following IV administration of radiolabeled THC (56). However, in 2005, Day et al quantified THCCOOH in authentic OF specimens by a gas chromatography-tandem mass spectrometry (GC-MS-MS) procedure (232). THCCOOH is not present in cannabis smoke; therefore, identification of this metabolite in OF provides conclusive evidence of active cannabis consumption. Moore et al also verified the presence of THCCOOH in OF and achieved a 2 ng/L LOQ with two-dimensional GC-MS (2D-GC-MS) (226). More recently, Milman et al achieved an empirically-determined 7.5 ng/L THCCOOH LOQ and simultaneously quantified THC, 11-OH-THC, cannabidiol (CBD), and cannabinol (CBN) in a single OF extraction (227).

As part of on-going controlled cannabinoid administration research, a reliable, sensitive and specific immunoassay was needed for screening OF. The Immunalysis Sweat/OF THC Direct ELISA and Immunalysis Quantisal™ OF collection device were selected due to a reproducible OF collection volume of 1.0 ± 0.1 mL (233), THC recovery of 81.3-91.4% (234), and THC stability in the collection buffer (235). Following immunoassay validation, we screened authentic OF specimens following controlled oral THC administration and utilized 2D-GC-MS to confirm the presence of THC and several other cannabinoids, including THCCOOH at ng/L concentrations. This

enabled us to determine assay diagnostic sensitivity, specificity, and efficiency for authentic OF specimens collected with the Quantisal™ OF collection device, while also identifying potential contributions from other cross-reacting cannabinoids.

Materials and Methods

Specimens

OF was collected from 12 healthy male participants with a self-reported history of daily cannabis smoking. Participants were administered oral synthetic THC (Marinol® (dronabinol), Unimed Pharmaceuticals, Marietta, GA, USA) daily as part of a protocol to study cannabis tolerance and spontaneous and antagonist-elicited cannabis withdrawal. Double-encapsulated 20-mg oral THC doses were administered with increasing frequency (every 4-8 h) for total doses of 40-120 mg/day for eight days. Participants were admitted to the secure research unit 20 h before the first THC dose and were discharged 24 h after the final dose. Study design was previously described (43). Written informed consent was obtained and the Institutional Review Boards of the National Institute on Drug Abuse, the University of Maryland, and the State of Maryland Department of Health and Mental Hygiene approved the protocol.

OF specimens were collected 3 times daily with the Quantisal™ device that was placed in the mouth until adequate specimen was collected. The device consists of a cellulose pad affixed to a stem with a 1 mL (± 0.1 mL) volume adequacy indicator. Pads were placed into tubes containing 3 mL of Quantisal™ transport buffer solution for 24-72

h at room temperature to elute drugs from the pad, simulating transport conditions. Stems were removed, pads squeezed dry, and solutions transferred to Nunc Cryotubes for storage at -20 °C until analysis.

Blank OF (n = 9) was obtained from healthy volunteers with no self-reported cannabis use in the previous 180 days. OF was obtained by expectoration into 50 mL polypropylene centrifuge tubes, storage at -20 °C for a minimum of 24 h, and centrifugation at 7500 x g for 10 min prior to utilization. Specimens were confirmed negative by 2D-GC-MS (THC LOQ 0.5 μg/L) and combined in equal parts to create a blank OF pool for preparation of cannabinoid calibrators, quality control and method validation samples. OF also was obtained from these volunteers through direct sampling with the Quantisal™ device by the procedure detailed above.

Reagents and Consumables

All native and deuterated standards were obtained from Cerilliant (Round Rock, TX) with the exception of THC glucuronide, which was from ElSohly Laboratories (Jackson, MS). HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ) and water was purified in-house. ELISAs (Catalogue #224 Sweat/OF THC Direct ELISA [STHC]) and Quantisal™ OF collection devices were from Immunalysis (Pomona, CA).

ELISA Screening

All unknown OF specimens were screened singly according to assay instructions provided by Immunalysis (236). Spectrophotometric analysis at 450 nm yielded

absorbance that was inversely proportional to the quantity of cannabinoids present. All raw absorbances (B) were normalized to the mean negative control absorbance for the plate (B_0). A qualitative positive sample required a B/B_0 below the mean B/B_0 of the 4 µg/L cutoff calibrator.

In addition to the 4 µg/L cutoff calibrator, each assay included a negative synthetic OF and OF fortified at 2 µg/L THC (low control) and 8 µg/L THC (high control) that was pre-diluted 1:4 with Quantisal™ extraction buffer. All calibrators and controls were assayed at the beginning and end of the plate to evaluate assay performance. In-house OF controls at 1.0, 3.0, 5.0, and 6.0 µg/L THC were prepared weekly, diluted 1:4 with Quantisal™ buffer and run in duplicate interspersed throughout specimens.

Validation Procedures

Blank volunteer expectorant samples (n = 9) were fortified with THC at 2.0 and 8.0 µg/L, sampled with the Quantisal™ device, and analyzed in triplicate to determine effects of matrix on quantification.

Limits of Detection

Limit of detection (LOD) absorbance values were calculated with negative buffered expectorant and Quantisal™ OF samples (n = 9 for each matrix) by subtracting three times absorbance standard deviation (SD) from mean absorbance of the 9 negative OF samples (A_0) for each matrix. Thus, LOD is a concentration of THC that yields an absorbance of $A_0 - 3SD$.

Linearity

Response linearity was investigated by regression line calculation by the method of least squares and expressed as the coefficient of determination (r^2). Linearity was evaluated with the following THC concentrations: 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10, 15, 20, 25, 30, 50, 75, 100, 125, 150, and 200 µg/L in pooled negative OF. Samples (diluted 1:4 in Quantisal™ buffer) were run in triplicate and mean B/B_0 values were plotted against concentration on a log-logit plot to characterize linearity. Logit values (L) were calculated as $L = \ln[(B/B_0)/(1-(B/B_0))]$.

Imprecision

Imprecision was determined with in-house controls at 1.0, 3.0, 5.0, and 6.0 µg/L THC. For intra-plate imprecision, 7 replicates at each concentration were assayed on a single plate. This plate was read five times over 25 min to determine any variation in absorbance after addition of stop solution (inter-read imprecision). For inter-plate imprecision, 2 replicates of controls at each concentration were assayed on 10 plates (n = 20) over six weeks.

Plate Drift and Control Charting

Variation in absorbance as a function of physical location on the 96-well plate (drift) was determined. Imprecision was monitored across all plates for calibrators and controls through control charting of individual and mean B/B_0. A priori re-analysis cutoffs were 25% CV (intra-plate) or ± 25% of the running mean at any control level (inter-plate).

Cross-Reactivity

Cross-reactivity was characterized by analyzing 11-OH-THC, CBD, CBN, Δ^8-tetrahydrocannabinol (Δ^8-THC), THCCOOH, THCCOOH-glucuronide, and THC-glucuronide in triplicate at 0.1, 1.0, 4.0, 400, and 1000 μg/L. Other commonly encountered drugs that were evaluated included acetaminophen, 6-acetylmorphine, acetylsalicylic acid, alprazolam, amphetamine, benzoylecgonine, caffeine, cathinone, cocaethylene, cocaine, codeine, dextromethorphan, diazepam, flunitrazepam, flurazapam, hydrocodone, hydromorphone, ibuprofen, imipramine, lorazepam, methadone, methamphetamine, 3,4-methylenedioxymethamphetamine, morphine, nicotine, nitrazepam, oxazepam, pentazocine, phencyclidine and temazepam at 1000 μg/L. Cross-reactivity (%) was determined as:

$$\frac{100*(\text{apparent THC concentration from the calibration curve})}{(\text{analyte concentration})}$$

Interferences

Several common products were evaluated for cross-reactivity or matrix effects including Listerine® mouthwash, orange juice, toothpaste, brewed coffee, soy milk, and tap water (control). For all products other than toothpaste, 50 mL was swirled in the mouth of a volunteer for 15 seconds, and then swallowed (orange juice, soy milk, coffee, and water) or expectorated (mouthwash). In addition, potential interference from toothpaste was evaluated by brushing the teeth for 60 seconds with 500 mg toothpaste, rinsing the mouth once with 50 mL tap water, and expectoration to waste. OF specimens were collected with the Quantisal™ device 5 and 30 min after product exposure. One

replicate was analyzed "as-is," the other fortified at 32 μg/L THC to evaluate signal enhancement or suppression.

Hook Effect

High THC concentrations were assayed to ensure lack of a "hook effect." A hook effect (so named for the characteristic shape of the analytical dilution curve) has been reported for some (primarily single-step) immunoassays when an extraordinarily high antigen concentration was present, yielding an artificially high absorbance (i.e. negative result) (237). To test for this effect, pooled negative OF was fortified to 1000 μg/L THC and assayed in triplicate.

2D-GC-MS Confirmation

Cannabinoids in oral fluid specimens were quantified by a previously validated 2D-GC-MS method (227). LOQs were 0.5, 0.5, 0.5, 1.0, and 0.0075 μg/L for THC, 11-OH-THC, CBD, CBN, and THCCOOH, respectively.

Diagnostic Efficiency

Diagnostic sensitivity and specificity were evaluated by ELISA screening (cutoff 4 μg/L) of 499 authentic OF specimens collected with the Quantisal™ device followed by 2D-GC-MS confirmation. Diagnostic sensitivity, the calculated true-positive rate, was determined from total true positives (TP) and false negatives (FN), with sensitivity = 100*TP/(TP+FN). Diagnostic specificity was based on the number of true negatives (TN) and unconfirmed positives (FP), with specificity = 100*TN/(TN+FP). Diagnostic efficiency was 100*(TP+TN)/total specimens. To evaluate plate performance at various

GC-MS cutoffs, efficiency was calculated at 0.5, 1, 2, 3, 4, 5, and 6 µg/L THC cutoffs. Plate performance was also evaluated at THCCOOH GC-MS cutoffs of 20, 50, 100, 200, 300, 400 and 500 ng/L.

Statistical Analysis

Statistical calculations were performed with SPSS® 15.0 for Windows (SPSS Inc, Chicago, IL). A paired T-test determined potential differences ($p < 0.05$) between matrix responses at specific concentrations ($n = 9$). As variances were unequal, the Welch test was utilized for evaluating omnibus differences ($p < 0.05$) during interference characterization. Mean ($n = 2$) calibrator B/B_0 values were utilized for cutoff determinations and diagnostic specificity and sensitivity calculations. Individual cross-reactivity samples quantifying less than the negative control were included in means as having 0% cross-reactivity.

Results

Specimens

499 authentic OF specimens were collected with the Quantisal™ device, including 184 duplicate OF samples obtained simultaneously (both devices in the mouth at the same time) to investigate inter-device variability. At the time of collection, negative control OF samples had variable viscosity, color, and mucus content. However, following frozen storage and centrifugation, a slightly viscous, clear, homogeneous solution was obtained.

Validation

At 2 µg/L, no differences in absorbance were observed between fortified OF diluted directly with buffer and fortified OF sampled with the Quantisal™ device ($t(52) = 1.00$, $p = 0.322$, $r = 0.14$). However, a statistical difference was observed with samples prepared in the same manner at 8 µg/L ($t(52) = -4.845$, $p = 0.0000160$, $r = 0.56$). Despite the difference, both of the 8 µg/L fortified samples quantified greater than the manufacturer's control at 8 µg/L and were qualitatively positive.

Limits of Detection

On five different days, LODs of 0.30, 0.23, 0.24, 0.27, and 0.23 µg/L were obtained for directly buffered expectorant and 0.88, 0.77, 0.82, 0.91, and 0.77 for negative samples collected with the Quantisal™ device. Thus, LODs were 0.3 µg/L for centrifuged, buffered expectorant and 0.91 µg/L for samples collected with the Quantisal™ device.

Linearity

Evaluation of immunoassay linearity from 1-50 µg/L THC (Figure 11) yielded a logarithmic regression line of $y=-1.552 \log(x) + 0.277$ ($r^2 = 0.9936$). The limit of quantification was 1 µg/L based on the correlation coefficient and triplicate concentrations within 26.2% of target. Further testing demonstrated acceptable linearity ($r^2 = 0.9835$) up to 200 µg/L (data not shown) for estimating required dilutions for GC-MS confirmation.

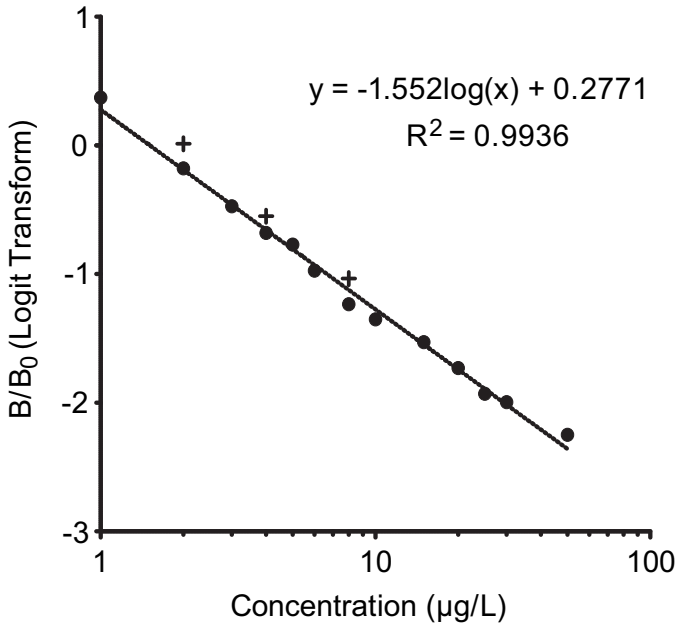

Figure 11. Immunalysis Sweat/Oral Fluid Δ^9-tetrahydrocannabinol (THC) Direct ELISA linearity.

Linearity determined with in-house calibrators (•) prepared by fortifying centrifuged, buffered oral fluid with THC. Evaluation from 1 to 50 µg/L THC yielded a logarithmic regression line in good accordance with manufacturer-provided calibrators and controls (+). Logit values (*L*) were calculated as $L=\ln[(B/B_0)/(1-(B/B_0))]$.

Imprecision

Imprecision was assessed at 1, 3, 5, and 6 µg/L THC. Intra-plate, inter-plate, and inter-read imprecision are detailed in Table 13. Intra-plate imprecision (n = 7) was less than 7.7% CV for all concentrations. Inter-plate (n = 20) and inter-read imprecisions (n = 5) were less than 9.1% and 1% CV, respectively.

	Intra-Plate (n = 5)			Inter-Plate (n = 20)			Inter-Read[a] (n = 5)		
Control Level (µg/L THC)	Mean B/B_0[b]	SD[c]	CV	Mean B/B_0	SD	CV	Mean B/B_0	SD	CV
1	56.4%	1.6%	2.9%	60.3%	1.8%	3.0%	56.6%	0.3%	0.5%
3	37.2%	2.9%	7.7%	38.5%	2.0%	5.1%	37.4%	0.3%	0.9%
5	30.0%	1.1%	3.6%	30.6%	2.8%	9.1%	30.1%	0.2%	0.8%
6	27.1%	2.0%	7.4%	27.8%	1.3%	4.7%	27.3%	0.2%	0.9%

Table 13. Assay imprecision around the 4 µg/L screening cutoff for Immunalysis Sweat/Oral Fluid Δ^9-tetrahydrocannabinol (THC) Direct ELISA

[a] Variation in absorbance after addition of stop solution
[b] Raw absorbance values (B) normalized to the mean negative control absorbance value (B_0)
[c] Standard deviation of mean B/B_0

Plate Drift and Control Charting

Mean intra-plate drift (n = 20) was calculated at each control concentration. Statistically significant drift was observed for the 2 µg/L control, with controls at the end of the plate having an overall higher mean ± SD B/B_0 (52.2 ± 3.51) than at the beginning (49.4 ± 3.34), $t(40) = -2.66$, $p = 0.011$, $r = 0.39$. Additionally, a significant similar plate drift was observed for the 8 µg/L control, with a higher B/B_0 (28.5 ± 2.61) at the end as compared to the beginning (25.3 ± 2.41), $t(40) = -4.00$, $p = 0.00026$, $r = 0.53$. However, all 2 and 8 µg/L controls produced appropriate negative and positive qualitative results. No significant drift was observed for the 4 µg/L calibrator, $t(40) = -1.08$, $p = 0.288$, $r = 0.17$.

Control charting within a single assay lot generally demonstrated an increase in mean B/B_0 absorbance over time within the lot, similar to other commercial immunoassays. However, the test continued to perform within qualitative and quantitative a priori specifications and no re-analyses were required.

Cross-Reactivity

Immunoassay cross-reactivities at 4 μg/L were 11-hydroxy-THC, 198%; Δ^8-tetrahydrocannabinol, 128%; 11-nor-9-carboxy-THC (THCCOOH), 121%; THC (target), 98%; cannabinol, 87%; THCCOOH-glucuronide, 11%; THC-glucuronide, 10%; and cannabidiol, 2.4%. As detailed in Figure 12, cannabinoid cross-reactivities varied substantially across concentrations. Due to loss of linearity at higher concentrations, cross-reactivities typically decreased with increasing cannabinoid concentration. Large ranges were often observed; however, this variability is expected when samples quantify below LOD (1 μg/L) or beyond the assay's upper limit of linearity (200 μg/L). Cross-reactivities for the 30 common drugs or chemicals tested at 1000 μg/L were less than 0.05% and samples were qualitatively negative in all cases. The 1000 μg/L hook effect sample quantified at approximately 260 μg/L THC.

Interferences

Utilizing a Welch test, blank OF assayed after product exposure demonstrated no significant differences from control (OF after water rinse) at 5 or 30 min after exposure ($p = 0.274$ and 0.073, respectively). In all cases, samples quantified below LOQ (1 μg/L); thus, these product exposures did not affect assay performance.

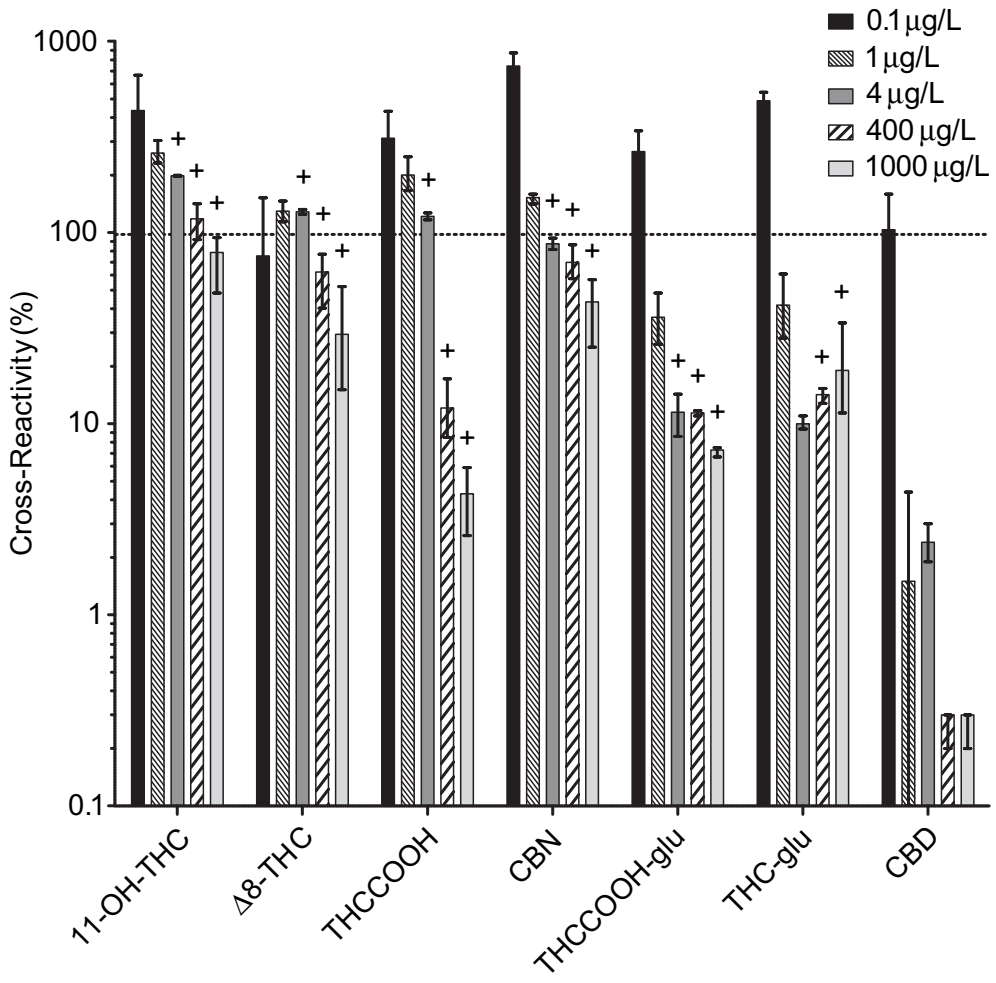

Figure 12. Mean (*n*=3) (range) cannabinoid cross-reactivity in fortified authentic oral fluid in the Immunalysis Sweat/Oral Fluid Δ^9-tetrahydrocannabinol (THC) Direct ELISA.

Dashed line represents 4 µg/L THC (target) cross-reactivity (98%). Individual cross-reactivity samples quantifying less than negative control were included in means as having 0% cross-reactivity. +, sample qualitatively positive. 11-OH-THC: 11-hydroxy-Δ^9-tetrahydrocannabinol; Δ^8-THC: Δ^8-tetrahydrocannabinol; THCCOOH: 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol; CBN: cannabinol; CBD: cannabidiol.

OF also was fortified at 32 μg/L THC after product exposure to characterize any effects of these products on semi-quantification. Product-exposed OF at 32 μg/L THC was not significantly different from control 5 min ($p = 0.233$) or 30 min after exposure ($p = 0.331$).

Diagnostic Efficiency

When utilizing the SAMHSA-proposed 4 μg/L cannabinoids screening cutoff and THC GC-MS 2 μg/L confirmation cutoff, 52 of 499 authentic OF specimens confirmed positive (THC concentrations: 2.0 to 293 μg/L). No false negatives were reported, yielding 100% diagnostic sensitivity. However, 47 samples screened positive above the 4 μg/L cutoff, but were not confirmed by 2D-GC-MS, yielding an overall diagnostic specificity of 400/477 = 89.5% and diagnostic efficiency of 452/499 = 90.6%. Median (range) THC concentrations of these unconfirmed positive specimens were 5.8 μg/L (4.1-9.9) by immunoassay and 0.5 μg/L (none detected-1.8) by 2D-GC-MS (EI). Interestingly, THCCOOH concentrations ranged from 7.5-1118 ng/L by 2D-GC-MS (NCI). Table 14 details a comparison of diagnostic efficiencies with various GC-MS THC confirmation cutoffs. Generally, diagnostic sensitivity increased and specificity decreased as the GC-MS cutoff concentration increased. Diagnostic efficiency peaked at a confirmation cutoff of 1 μg/L THC, although false negative samples increased at (and below) this cutoff.

With the recent identification of THCCOOH in OF (226, 232), THCCOOH immunoassay detection could become important if this analyte is selected as conclusive evidence of cannabis intake, removing the possibility of passive contamination.

GC-MS THC Cutoff Concentrations (µg/L)

	0.5	1	2	3	4	5	6
True Positives[a]	73	**65**	52	44	32	21	15
True Negatives[b]	362	389	400	400	400	400	400
Unconfirmed Positives[c]	26	34	47	55	67	78	84
False Negatives[d]	38	11	0	0	0	0	0
Diagnostic Sensitivity	65.8%	85.5%	100%	100%	100%	100%	100%
Diagnostic Specificity	93.3%	92.0%	89.5%	87.9%	85.7%	83.7%	82.6%
Diagnostic Efficiency	87.2%	**91.0%**	90.6%	89.0%	86.6%	84.4%	83.2%

Table 14. Immunoassay performance (4 µg/L screening cutoff) in 499 authentic oral fluid specimens at varying Δ^9-tetrahydrocannabinol (THC) gas chromatography-mass spectrometry (GC-MS) confirmation cutoffs

[a] True Positives: immunoassay positive and confirmed positive by GC-MS. [b] True Negatives: immunoassay negative and confirmed negative by GC-MS. [c] Unconfirmed Positives: immunoassay positive but confirmed negative by GC-MS. [d] False Negatives: immunoassay negative but confirmed positive by GC-MS.

Table 15 highlights the capability of this immunoassay to screen for OF THCCOOH With a manufacturer-recommended 4 µg/L cannabinoid screening cutoff, employing a 400 ng/L THCCOOH confirmation cutoff provided acceptable diagnostic sensitivity, specificity and efficiency. However, the assay currently provides better performance for THC than THCCOOH, with 65 true positives at maximum diagnostic efficiency (1 µg/L) for THC versus only 44 at maximum THCCOOH diagnostic efficiency (400 ng/L). These data suggest an antibody with greater THCCOOH cross-reactivity could improve THCCOOH sensitivity in OF.

	GC-MS THCCOOH Cutoff Concentrations (ng/L)						
	20	50	100	200	300	400	500
True Positives[a]	90	75	63	57	54	**44**	35
True Negatives[b]	75	155	249	350	375	392	397
Unconfirmed Positives[c]	9	24	36	42	45	55	64
False Negatives[d]	325	245	151	50	25	8	3
Diagnostic Sensitivity	21.7%	23.4%	29.4%	53.3%	68.4%	84.6%	92.1%
Diagnostic Specificity	89.3%	86.6%	87.4%	89.3%	89.3%	87.7%	86.1%
Diagnostic Efficiency	33.1%	46.1%	62.5%	81.6%	86.0%	**87.4%**	86.6%

Table 15. Immunoassay performance (4 µg/L screen cutoff) in 499 authentic oral fluid specimens at varying 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) gas-chromatography-mass spectrometry (GC-MS) confirmation cutoffs.

[a] True Positives: immunoassay positive and confirmed positive by GC-MS. [b] True Negatives: immunoassay negative and confirmed negative by GC-MS. [c] Unconfirmed Positives: immunoassay positive but confirmed negative by GC-MS. [d] False Negatives: immunoassay negative but confirmed positive by GC-MS.

Discussion

As OF testing gains acceptance in DUID, workplace and clinical monitoring scenarios, validation of robust, sensitive, and cost-effective screening assays will allow detection of illicit drug use while reducing confirmatory demands on personnel and instrumentation. Extensive validation of the Immunalysis Sweat/OF THC Direct ELISA documented sensitive, precise and accurate screening for THC in OF.

As this validation demonstrates, control B/B_0 values vary as a function of physical location on the plate, yielding intra-plate drift. While drift had no effect on qualitative

results, it can be moderated with use of automated equipment (multi-channel pipettes, automatic plate washers and dispensers). Additionally, mean values for calibration curves and cutoff absorbance determinations could be applied if one set is assayed at the beginning and another at the end of the plate. This does not appear to be necessary or cost-effective as all controls below and above the cutoff calibrator assayed appropriately.

The 1000 μg/L "hook effect" sample quantified lower than expected (approximately 260 μg/L), suggesting deviation from linearity above 200 μg/L. Linearity failure is expected as absorbance approaches zero and should have little effect on qualitative analysis at a 4 μg/L cutoff, although estimating dilutions for GC-MS confirmation may not be accurate in specimens with these high concentrations.

Metabolite cross-reactivity in some authentic specimens may have contributed to positive immunoassay tests that were not confirmed by 2D-GC-MS, reducing specificity to 89.5%. This is similar to other reported THC ELISA specificities (133, 177). Further analysis of metabolite quantifications indicated that 31 of 47 unconfirmed positives (GC-MS cutoff 2 μg/L THC) were from a single participant and had remarkably high (> 400 ng/L) THCCOOH concentrations. In these cases, THCCOOH (and potentially sub μg/L 11-OH-THC) cross-reactivity likely contributed to positive B/B_0 absorbance.

Diagnostic specificity, sensitivity and efficiency are markedly influenced by changes in the GC-MS confirmation cutoff. Employing the SAMHSA-proposed 2 μg/L confirmation cutoff yields improved plate performance over a 4 μg/L cutoff with an expected increase in TP and decrease in FP. Lowering the cutoff to 1 μg/L THC further increases TP; however, increased FN are observed when utilizing this cutoff. Generally, screening cutoffs greater than confirmation cutoffs are desired when multiple metabolites

may cross-react in the assay. On the other hand, performing unnecessary, expensive and time-consuming confirmation testing is not efficient and delays reporting of negative results. The current Immunalysis cannabinoid ELISA provides acceptable diagnostic sensitivity, specificity and efficiency for a SAMHSA GC-MS cutoff of 1 or 2 μg/L THC.

With 198% cross-reactivity at 4 μg/L, 11-OH-THC presence could contribute to unconfirmed positive specimens for this study. First-pass metabolism of orally administered cannabinoids (as in this study) yields higher relative plasma 11-OH-THC concentrations compared to smoked administration (238), although based on previous research (239), we did not expect to observe OF 11-OH-THC concentrations greater than 0.5 μg/L. For this study, OF also was analyzed for 11-OH-THC by 2D-GC-MS (LOQ 0.5 μg/L); all specimens were below LOQ for this analyte. Further research is needed, as detection of 11-OH-THC at ng/L concentrations may provide additional evidence of active cannabis consumption.

This immunological test is a diagnostically sensitive screening procedure to detect cannabinoids in OF using a screening cutoff of 4 μg/L cannabinoids and a confirmation cutoff of 2 μg/L THC. The test requires about 2.5 h for 80 unknowns assayed singly with manual pipetting. The assay is effective for measuring cannabinoids in expectorated OF as well, although dilution of expectorated OF with buffer prior to analysis is required. As OF testing is accepted as a reliable means of detecting cannabis consumption, this OF screening assay should prove useful in clinical and forensic applications. However, if approval of THCCOOH in OF as evidence of active cannabis intake occurs, improved performance should be achieved with an antibody directly targeting THCCOOH.

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