CANNABINOID DISTRIBUTION AND POSTMORTEM REDISTRIBUTION IN RABBITS FOLLOWING CONTROLLED CANNABIS ADMINISTRATION

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Abstract: Millions of people in the world use *Cannabis sativa* for its mood-altering properties. The main psychoactive component of cannabis, Δ^9-tetrahydrocannabinol (THC) is commonly detected in forensic toxicology laboratories handling motor vehicle and plane crash fatalities. As few studies characterized cannabinoid distribution and postmortem redistribution, we developed a study to investigate postmortem cannabinoid concentrations in rabbits following controlled cannabis administration via a smoking machine. Five rabbits were exposed to cannabis smoke and a broad array of biological specimens were collected immediately upon death. High THC concentrations were observed in lungs, moderate concentrations were seen in the brain, heart, and kidneys, and low concentrations were noted in the liver. A physiologically based pharmacokinetic (PBPK) model was constructed to describe blood and tissue THC concentrations in rabbits following the administration of smoked cannabis by inhalation. The results showed similar THC concentrations in blood and tissues between the predicted and experimental data. Building upon the disposition of cannabinoids in various postmortem fluids and tissues in the rabbits, we evaluated time- and temperature-dependent changes in the concentrations of cannabinoids after rabbit carcasses were stored under room temperature or refrigerated conditions for various times after death. No significant differences in THC concentrations were observed between rabbits stored at room temperature versus under refrigerated conditions. When THC concentrations from various postmortem intervals (PMI) were compared to those observed at PMI 0 h, significant THC concentration changes in heart blood and peripheral blood were noted only in refrigerated samples collected at 2 and 16 h after death. Mean central:peripheral and liver:peripheral ratios for THC were 1.6 and 2.9, respectively. These data indicate that THC was not prone to substantial postmortem redistribution in the rabbit. This was the first study to consider either cannabinoid distribution or postmortem redistribution after inhaled cannabis administration using an animal model. The findings add substantially to our understanding of postmortem cannabinoid concentrations and can aid forensic toxicologists in the interpretation of cannabinoid findings in death investigations.

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INTRODUCTION

Epidemiology

Cannabis sativa (marijuana, sinsemilla, hashish)LIN is the most commonly abused drug worldwide with an estimated 192 million (3.9%) past-year users (1). Effects include euphoria, relaxation, altered perception of time, impairment in memory and learning and decrements in psychomotor performance (2). According to the 2019 National Survey on Drug Use and Health, approximately 48.2 million (17.5%) Americans aged 12 and older consumed cannabis in the previous year (3). Cannabis use is popular among American teenagers with 1.3%, 4.8% and 6.4% of 8^{th}, 10^{th} and 12^{th} graders, respectively, using cannabis daily in 2019. Additionally, the study found that vaping cannabis increased significantly in 8^{th}, 10^{th} and 12^{th} graders with 3.9%, 12.6% and 14% reporting past month vaping (4).

Since the late 1990s, the United States (US) saw a change in state laws allowing medical and recreational cannabis. Cannabis remains illegal under federal regulations of the Controlled Substances Act, but numerous states decriminalized cannabis in the last

two decades. To date, 33 states allow cannabis for medical purposes and 14 approved cannabis for recreational purposes (5). In addition, cannabis potency significantly increased in the US, raising concerns over the health and behavioral consequences of cannabis use. The average content of Δ^9-tetrahydrocannabinol (THC), the main psychoactive cannabinoid in cannabis, increased from 8.9% to 17.1% in confiscated samples from 2008 to 2017, respectively (6).

Due to recent changes in legalization and increasing cannabis use prevalence, cannabinoids are a common finding in forensic laboratories that handle casework for driving under the influence of drugs (DUID) or aviation crashes. The 2013-2014 National Roadside Survey showed that 12.6% of drivers in the US were THC-positive, increasing from 8.6% in 2007 (7). Reports show that cannabis is frequently detected in drivers involved in motor-vehicle crashes and acute cannabis consumption increases the risk of a fatal collision (8). The Federal Aviation Administration's (FAA) Toxicology Laboratory performs toxicological analyses on pilots fatally injured in plane crashes and cannabis is the most common illicit drug finding (9).

Pharmacognosy

Cannabis was used for at least 5,000 years for recreational and medicinal purposes but it was not until 1964 that the active component, THC, was first identified and isolated (10). The cannabis plant contains more than 550 compounds, of which 120 are phytocannabinoids that modulate the endogenous cannabinoid system (11). While THC is the main psychoactive component in cannabis, effects differ depending on the plant's chemical composition with additional cannabinoids and other chemicals (12). The presence of minor

cannabinoids and metabolites in biological specimens, in addition to THC and its metabolites, may improve cannabis exposure interpretation for forensic toxicologists by identifying patterns of cannabis exposure and consumption.

Phytocannabinoids are grouped into two categories, cannabinoid acids and neutral cannabinoids, depending on the presence or absence of an acidic carboxyl functional group. Within the cannabis plant, cannabinoid acids accumulate and are synthesized to form other phytocannabinoids, and upon storage and smoking, neutral cannabinoids are formed following decarboxylation (13). Cannabigerolic acid (CBGA) is the direct precursor for tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA). These three acids, CBGA, THCA and CBD then form their neutral cannabinoids, cannabigerol (CBG), THC and cannabidiol (CBD), respectively, through decarboxylation(Figure 1) (14, 15). The most studied and researched cannabinoids found in the cannabis plant are the neutral THC and CBD (6). Other neutral cannabinoids of interest include cannabinol (CBN) and Δ^9-tetrahydrocannabivarin (THCV).

The most abundant phytocannabinoid in cannabis is THC, although through genetic breeding other cannabinoids can predominate. Over the last 25 years, cannabis potency or THC concentration increased significantly causing concern for negative adverse health outcomes (6, 16). High THC concentrations lead to a higher risk of developing psychosis, cannabis use disorder and higher rates of hospitalizations and emergency department visits (17-19). During storage and over time and with heat and light exposure, THC oxidizes to CBN in the cannabis plant (20). CBN and its metabolite 11-hydroxy-CBN have far less pharmacological activity than THC (21).

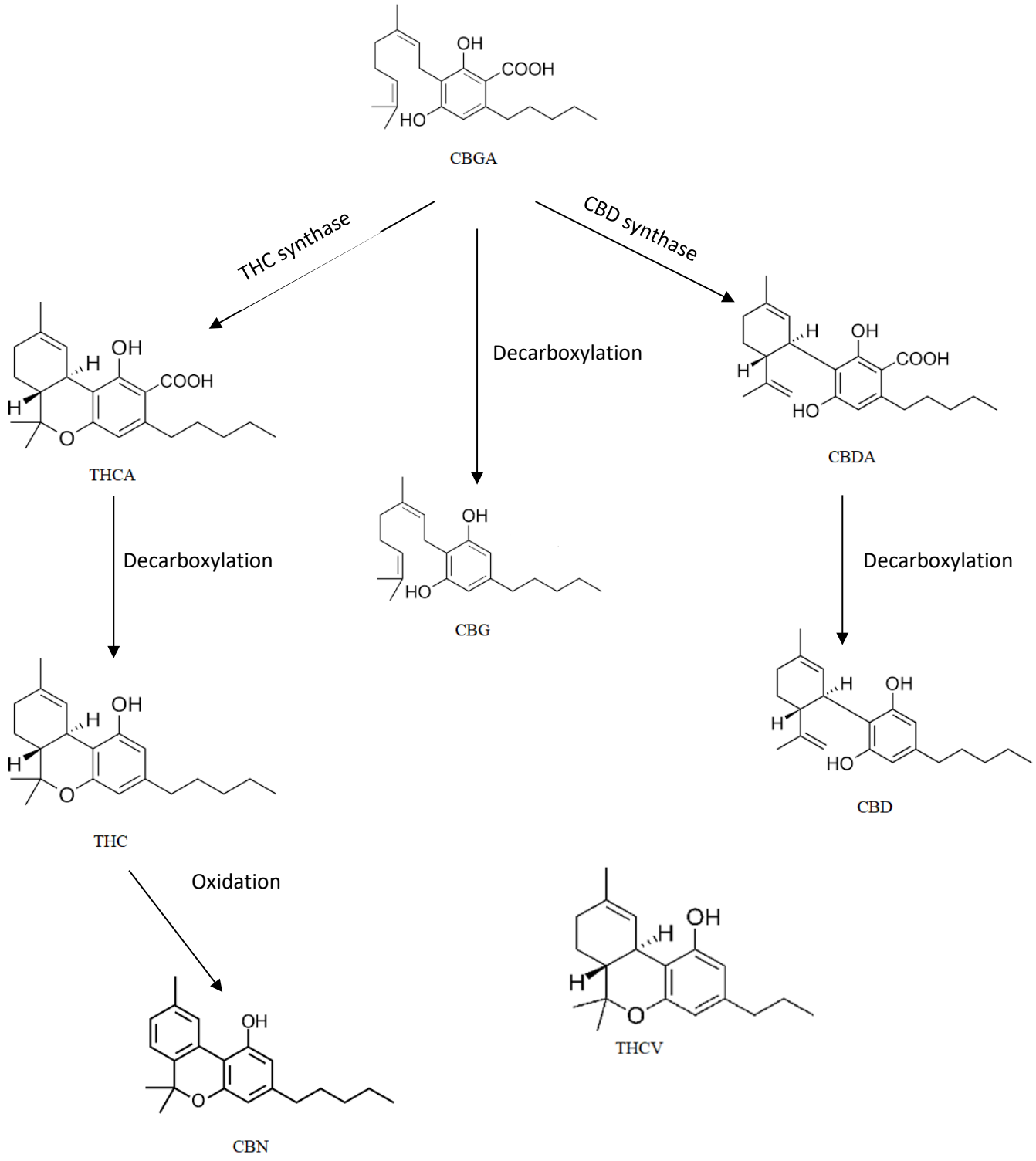

Figure 1. Phytocannabinoids biosynthesis pathways. CBGA: cannabigerolic acid; CBG: cannabigerol; THCA: Δ^9- tetrahydrocannabinolic acid; CBDA: cannabidiolic acid; THC: Δ^9-tetrahydrocannabinol; CBD: cannabidiol; CBN: cannabinol; THCV: Δ^9-tetrahydrocannabivarin

CBD is a non-psychomimetic cannabinoid that may reduce some of THC's psychoactive and physiological effects (22, 23). Therapeutic possibilities for CBD include treatment of anxiety, epilepsy, inflammation, vomiting and nausea, and psychosis (24). THC and CBD concentrations and their relative ratio are important factors in cannabis pharmacodynamics. Sativex is an oromucosal spray of 1:1 THC and CBD cannabis extracts. No significant differences in THC pharmacokinetics or pharmacodynamics were found when equivalent doses of oral THC and Sativex were administered to nine subjects (25, 26). However, greater cognitive impairment was documented when THC and CBD were present in higher THC:CBD ratios (27). From 2008 to 2017, the THC:CBD ratio across confiscated cannabis samples in the US increased dramatically from 23 to 104, raising public health concerns for the consumption of cannabis products with high THC and low CBD concentrations (6).

CBG was first isolated as a pure chemical substance in 1964 by Gaoni and Mechoulam (28). Despite its early identification in cannabinoid research, little exploration and characterization occurred with this cannabinoid over the next five decades.

However, interest in potential therapeutic benefits for cannabinoids other than THC and CBD brought CBG to the forefront since it is the common precursor in the cannabis plant. CBG has anti-inflammatory and antibacterial activity and may be a promising treatment in certain neurologic disorders, such as Parkinson's disease and multiple sclerosis (29). Furthermore, following smoked or vaporized cannabis, CBG and CBN were documented as markers of recent cannabis intake in blood of occasional and frequent cannabis users after controlled administration (30).

Forensic toxicologists often must determine whether cannabinoid positive cases are the result of cannabis intake or use of prescribed synthetic THC or Marinol®. Marinol or dronabinol is prescribed to treat anorexia-associated weight loss in AIDS patients, as well as nausea and vomiting associated with cancer chemotherapy (31). One phytocannabinoid, THCV, proved valuable in differentiating ingestion of cannabis (or a related product) and Marinol because the THCV carboxylic acid metabolite, THCVCOOH, is detected in human urine samples after smoking a cannabis cigarette. When subjects ingested Marinol, however, no THCVCOOH was detected in urine specimens (32, 33).

Endocannabinoid System

THC's effects are mediated primarily by two G-protein-coupled cannabinoid receptors (GPCR), cannabinoid 1 (CB_1) and cannabinoid 2 (CB_2), of the endocannabinoid system (34). In addition, other receptors such as transient receptor potential vanilloid (TRPV1), GPR55 and GPR18 bind cannabinoids (35). CB_1 receptors are located primarily in the central nervous system but also in peripheral tissues including skeletal muscle, liver, spleen and lung. In the brain, the highest levels of CB_1 receptors are found within the cortex, hippocampus, basal ganglia and cerebellum (36). Low to moderate CB_1 receptor densities are found in the hypothalamus and spinal cord with practically no receptors in the respiratory centers within the brainstem (37). CB_2 receptors are mainly expressed in peripheral cells of the immune system but are also expressed in the central nervous system albeit at much lower levels compared to CB_1 receptors (38).

The endogenous cannabinoid system plays an important role in memory, coordination, emotion, appetite, learning, reproduction, immune response and many other

critical functions, including promotion of homeostasis within the body. The most well-known and documented endogenous cannabinoid receptor ligands are 2-arachidonoyl glycerol (2-AG) and arachidonoylethanolamide (anandamide, AEA) (39, 40). Other endogenous substances, such as 2-arachidonylglycerol ether or O-arachidonoyl ethanolamine, were identified as CB receptor ligands but the biology of these substances is not as well developed. Unlike classical neurotransmitters that are synthesized in advance and stored in vesicles, precursors for both 2-AG and AEA are constituents of lipid membranes, and upon demand, endocannabinoids are released into the extracellular space or synapse (41). There are differences in the efficacy of 2-AG and AEA. AEA is a partial agonist at both receptors, while 2-AG is a full agonist at both receptors (42). Following uptake, three hydrolytic enzymes, monoacylglycerol lipase (MAGL) and alpha/beta domain hydrolases 6 and 12, are primarily responsible for 2-AG degradation (43). Anandamide deactivation occurs via the enzyme fatty acid amino hydrolase (FAAH) (44).

Pharmacokinetics

THC is rapidly absorbed after smoking, the primary route of cannabis administration. Plasma concentration profiles after smoking (19 mg THC) and intravenous (5 mg THC) were similar with slightly lower peak concentration for smoking compared to intravenous administration (45). One controlled administration study showed that THC appears in blood immediately after the first puff of a cannabis cigarette and concentrations peak prior to the last puff (46). Bioavailability was reported as 2 – 56% after smoking, thereby showing highly variable dose delivery due to a subject's smoking topography (47). At equivalent doses, oral administration shows lower peak THC concentrations and absorption is more delayed than the smoking route (30).

After THC inhalation by smoking, blood concentrations decrease rapidly as THC is distributed into highly perfused tissues, such as the lung, brain, heart, liver and kidney. THC is highly lipophilic with 95-99% bound to plasma proteins, primarily lipoproteins (47). Despite this high binding to plasma proteins, THC is extensively distributed, with a reported steady state volume of distribution ($V_{d(ss)}$) of approximately 3.4 L/kg in man (48). In one of the only studies of THC tissue distribution, the Large White pig was administered 200 mg/kg THC via intravenous injection into the jugular vein. Pigs were sacrificed 30 min after injection and had the highest THC concentrations in lung, heart, kidney and liver, with THC eliminated slowly over time from the brain and adipose tissue (49). THC remained measureable in adipose tissue 24 hours after death. THC accumulates in fat and can passively diffuse back into circulating blood over time following chronic administration (50).

Phase 1 metabolism occurs primarily by the cytochrome P450 enzyme system in the liver with more than 100 THC metabolites identified to date (48). THC hydroxylation produces the psychoactive metabolite, 11-OH-THC, as well as minor metabolites of the alpha and beta pathways, 8α-OH-THC and 8β-OH-THC (Figure 2) (47). Further oxidation of 11-OH-THC yields the non-psychoactive metabolite, THCCOOH. Phase 2 metabolism involves formation of glucuronide conjugates through UDP-glucuronosyltransferase, which facilitates urinary excretion by increasing water solubility. THC-glucuronide is an ether-linked glucuronide whereas THCCOOH-glucuronide is an ester glucuronide. 11-OH-THC-glucuronide can be ether or ester linked (Figure 3) (51).

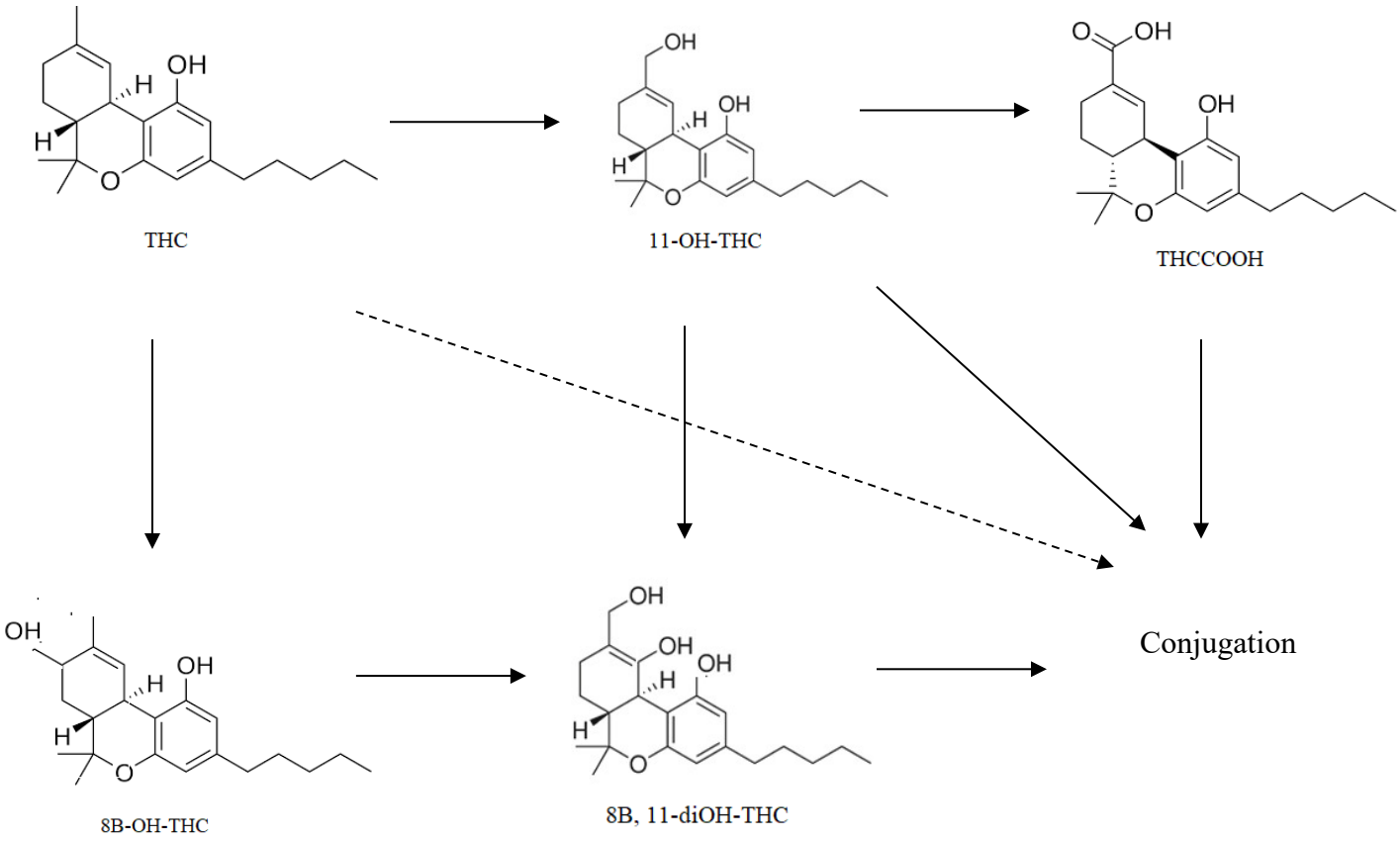

Figure 2. Phase I and Phase II metabolic pathways in man for Δ^9-tetrahydrocannabinol.

THC: Δ^9-tetrahydrocannabinol; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; 8β -OH-THC: 8β-hydroxy-THC; 8β,11-diOH-THC: 8β, 11-dihydroxy-THC.

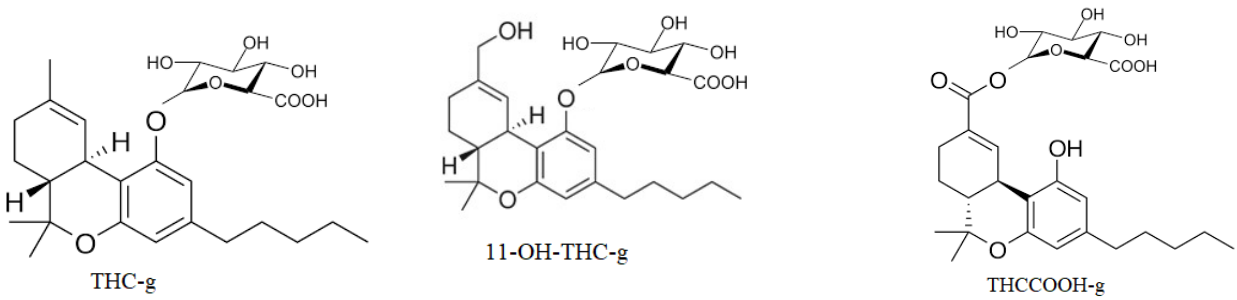

Figure 3. Molecular structure for glucuronide conjugates. THC-g: Δ^9-tetrahydrocannabinol glucuronide; 11-OH-THC-g: 11-hydroxy-THC glucuronide; THCCOOH-g: 11-nor-9-carboxy-THC glucuronide.

Tissues other than the liver, including lung, brain and intestine, also metabolize THC but to a much smaller extent than liver (52). Metabolism in rabbits is similar to that of man, with the two major metabolites identified in rabbit urine being 11-OH-THC and THCCOOH in both free and conjugated forms (53).

THC is excreted mostly as hydroxylated and carboxylated metabolites with about 65% eliminated in the feces and 20% in the urine (54). The acid-linked THCCOOH-glucuronide (THCCOOH-g) is the primary urinary metabolite observed in man whereas 11-OH-THC is the primary metabolite detected in feces (55). In recent years, analytical methodology is able to confirm and quantify THCCOOH-g directly in urine instead of requiring alkaline hydrolysis to cleave the glucuronide moiety, forming unconjugated THCCOOH (56).

Cannabis Effects

Cannabis use causes psychological effects on mood, perception, motor functions, cognition and memory (57). These effects are mainly dependent on dose, vehicle and route of administration, and user's experience. Physiologically, cannabis causes a rapid change in heart rate, peripheral vasodilation, increased appetite, conjunctival suffusion, and dry mouth and throat (12). More experienced cannabis smokers titrate their dose by varying inhalation depth and duration to achieve their desired high (58). Study participants obtained a euphoric or high feeling within 15 minutes of smoking a cannabis cigarette (59).

Acute cannabis intoxication produces dose-related neurocognitive and psychomotor impairments, as well as risk-taking behavior that can lead to impairment in performing complex tasks such as driving or flying (60). Skills that are necessary for driving, such as

reaction time, perception, short-term memory, attention, motor functions and tracking skills, are altered upon cannabis intake (61). Crash and driver culpability risks for THC positive cases increases with higher mean THC blood concentrations (62-64).

Driving under the influence of cannabis is a major public health and safety concern (65). On-road and simulator driving studies show that acute cannabis smoking affects cognitive functions, leading to decrements in driving skills (66). Drivers showed performance decrements with standard deviation of lateral position (or weaving), reaction time, time driven out of lane and standard deviation of headway after smoking cannabis (67).

Flying an aircraft is a complex task requiring a high level of cognitive function and psychomotor performance. Only a small number of studies examined the role of cannabis on pilot performance using simulated flight tests. Short-term memory, attention and concentration deficits were the most common adverse effects observed in flying studies.

Janowsky et al. found that pilots performing a specific flight sequence with an instrument flight simulator suffered a significant deterioration in flying performance within 30 minutes after smoking cannabis (68). An increase in navigational errors, altitude deviations and stalling and loss of control events were associated with pilots that consumed active cannabis rather than placebo. Acute effects persisted in pilots for up to six hours after administration.

Yesavage et al. evaluated pilots' skills and performance on a flight simulator for up to 24 hours after smoking a cannabis cigarette (69). Pilots exhibited difficulty aligning with and landing on the runway, an increased size of elevator changes, and an increase in degree of vertical and lateral deviation during approach to land. During touchdown, pilots showed a

significant increase in distance from center of runway. An interesting find in this study was that all pilots were unaware of any impairment with flying performance (69).

Cannabis Toxicity

Compared to other recreational drugs, THC toxicity is incredibly low with a median lethal dose of 800 mg/kg in rats, up to 3,000 mg/kg in dogs, and up to 9,000 mg/kg in monkeys (70). Sparse density of cannabinoid receptors in brainstem areas controlling vital cardiovascular or respiratory functions may explain why high THC doses are not lethal (71). However, with high THC concentration products and greater accessibility due to legalization, poison center calls and hospitalizations for unintentional cannabis intoxication are on the rise, particularly in children (72). In an observational study of 254 individuals acutely exposed to cannabis, tachycardia and neuroexcitation were the most common symptoms following inhalation exposures (73). Unintentional ingestion of cannabis edibles by children and adolescents led to sedation in 52.1% and 40.5% of the cases, respectively.

Acute cannabis use may lead to adverse cardiovascular events, including arrhythmias, fibrillation, myocardial infarctions and strokes (74-76). While there are several reports linking cannabis use to cardiovascular-related medical events, there are relatively few reports attributing death to cannabis itself (77). In six sudden and unexpected deaths reported by Bachs and Morland, THC was the only drug present in postmortem blood samples (78). The probable cause of death was an acute cardiovascular event associated with recent cannabis intake. Two young, healthy men died unexpectedly after smoking cannabis (79). Full death investigations involving autopsy, toxicology, histology, immunohistochemistry and genetic analyses were carried out to determine cause of death. Examinations revealed both decedents

suffered from cardiovascular complications and were positive for only THC and metabolites in blood and brain, leading the investigators to conclude that the fatal cardiovascular complications were evoked by smoking cannabis.

Cannabinoid Detection and Quantification in Biological Specimens

Numerous methodologies are developed for detection and quantification of cannabinoids in biological specimens including gas chromatography/mass spectrometry (GC-MS), two-dimensional GC-MS, gas chromatography tandem mass spectrometry (GC-MS/MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) (80-84). Mass spectrometry and tandem mass spectrometry improve specificity and sensitivity for confirmation of cannabinoids in forensic samples. Quantification is achieved with these instruments by utilizing selected ion monitoring and multiple reaction monitoring (MRM).

Cannabinoid analysis presents challenges due to THC's lipophilic nature and the low concentrations found in biological specimens. Complex biological matrices (e.g. blood, vitreous humor and tissues) are comprised of lipids and proteins that may interfere with analyses, therefore, sample preparation is an important aspect of cannabinoid quantification. Multi-step extraction techniques include liquid-liquid extraction (LLE), supported liquid extraction and solid-phase extraction (49, 56, 85-88). SPE is advantageous over other techniques due to its ability to extract analytes of a wide polarity range with high recoveries and good reproducibility. Furthermore, this highly selective technique provides sample cleanup for drug analysis in postmortem samples (89).

Many methods in the scientific literature for cannabinoid analysis are developed for antemortem whole blood, urine or oral fluid samples. Few methods test for cannabinoid

analysis in postmortem blood and tissue samples (49, 80, 84-86, 90). The combination of SPE and LC-MS/MS allows forensic toxicologists to perform direct quantification of THC, phase I and II metabolites and minor cannabinoids to provide insight into cannabis consumption prior to death.

Postmortem Cannabinoids

Limited research is available that assesses cannabinoid concentrations in postmortem specimens. Due to cannabis' low toxicity and the long-standing belief that cannabis does not directly cause death, some postmortem toxicology laboratories do not routinely include cannabinoid analysis as part of the screening process. Cannabinoid testing in postmortem cases are generally included in death investigations of drivers or pilots who may be responsible for a crash (90). Lack of data about postmortem cannabinoids distribution and postmortem redistribution poses serious challenges for interpreting the role that cannabinoids may play in death investigations.

Most postmortem work to date is performed on fluids rather than tissues and typically includes quantification of THC and two phase I metabolites, 11-OH-THC and THCCOOH. In one study, postmortem cannabinoid concentrations were compared in blood from individuals involved and not involved in fatal road traffic collisions (91). They found significantly higher blood THC concentrations (median = 4.2 ng/mL) for individuals in fatal road traffic collisions than those not involved in fatal traffic collisions (median = 2.6 ng/mL). Lemos and Ingle identified 30 postmortem cases positive for cannabinoids and observed median blood concentrations of 4.2, 10.5 and 37.5 ng/mL for THC, 11-OH-THC and THCCOOH, respectively (90). Twenty-five postmortem cases were evaluated for THC and

THCCOOH concentrations in central and peripheral blood in preserved (sodium fluoride, potassium oxalate) and unpreserved blood. THC concentrations ranged from 0 to 33 ng/mL in preserved central blood samples whereas THCCOOH concentrations ranged from 1.4 to 706 ng/mL (92). Slightly lower concentrations were observed in corresponding unpreserved blood samples with THC and THCCOOH concentrations ranging from 0 to 26 and 1.6 to 775 ng/mL, respectively.

Gronewold and Skopp performed the first study to investigate the distribution of cannabinoids in man by evaluating THC, 11-OH-THC, THCCOOH, THCCOOH-g, CBD and CBN in five postmortem cases (85). THC and its inactive metabolite, THCCOOH, were detected in blood of all five cases with higher concentrations noted for THCCOOH. In contrast, cannabinoids were hardly detectable in vitreous humor with THCCOOH-glucuronide detected in only one sample. All cannabinoids, except THC, were detected in high concentrations in bile. Muscle exhibited high THC concentrations and low CBD concentrations, but no other cannabinoids were detected in muscle. THC was detected in all lung samples but was low or not detectable in liver samples. Three of five brain samples contained low THC concentrations, whereas THCCOOH was the only metabolite detected in brain. Liver and kidney samples contained moderate amounts of THCCOOH-g.

Researchers at the FAA's Forensic Toxicology Laboratory performed two cannabinoid distribution studies in pilots fatally injured in aviation crashes. Kemp et al. evaluated blood, urine, liver, lung, heart, kidney, brain and muscle for the presence of THC and THCCOOH in 55 pilots (93). THC and THCCOOH were detected in most lung tissues with extremely high THC concentrations observed. Liver exhibited the highest THCCOOH concentrations of any tissue tested. Both THC and THCCOOH distributed well into kidney

with high concentrations observed. Saenz et al. expanded on the first study by incorporating 11-OH-THC, along with THC and THCCOOH in the analysis of postmortem fluids and tissues from 11 pilots (86). All three analytes were detected in highly perfused lungs with THC present in significant concentrations. Spleen and muscle samples proved to be acceptable matrices for detection of THC and THCCOOH. Analysis in brain revealed moderate concentrations of both psychoactive compounds, THC and 11-OH-THC, in over half of the cases. The highest THCCOOH concentrations were noted in bile.

In one of the only animal studies evaluating THC tissue distribution, researchers used the Large White pig as a model to assess cannabinoid metabolism (49). Eight pigs received an intravenous injection in the jugular vein of THC (200µg/kg) and two pigs were sacrificed at each time point of 30 min, 2, 6 and 24 h after injection. THC, 11-OH-THC and THCCOOH were measured in blood, vitreous humor, bile, fat and tissues. High THC concentrations were observed in lung, kidney, liver and heart at 30 min. As liver is the primary organ responsible for metabolism, THC was quickly eliminated from liver tissue with none detected 6 h after injection. 11-OH-THC was only detected in liver of pigs sacrificed at 30 min and 2 h. THCCOOH was not detected in any biological specimens except for bile. Different areas of the brain were examined and high THC concentrations were found in the cerebellum with low concentrations observed in the medulla oblongata. THC analysis confirmed prolonged retention of this highly lipophilic drug in fat tissue.

THC pharmacokinetics were evaluated in rabbits following single or multiple intravenous doses (94). Tissue analysis revealed high THC concentrations in fat, moderate heart THC concentrations and lower brain, lung and spleen concentrations. The authors noted that the lowest THC concentrations were found in brain, the major site of action for the drug.

Cannabis exerts its effects when THC or 11-OH-THC bind to cannabinoid receptors in various areas of the brain; therefore, an important consideration for postmortem analysis is detection of psychoactive constituents in the brain (36). Mura et al. reported THC, 11-OH-THC and THCCOOH results in blood and brain in 10 postmortem cases. THC brain concentrations were higher than blood concentrations in all cases and in three cases, THC was detected in the brain but was not detected in blood (95). In a recent study measuring THC concentrations in blood and brain after daily administration to squirrel monkeys, brain THC concentrations were twice as high as blood concentrations 24 hours after the last dose (96).

Postmortem Redistribution

In postmortem casework, forensic toxicologists must be aware of postmortem redistribution (PMR), a well-documented phenomenon that can make interpretation of results difficult (97-99). PMR refers to the diffusion of a drug along its concentration gradient from a tissue, such as lungs, liver or heart, with high drug concentrations into the surrounding area, such as blood, with lower concentrations. This may cause a falsely elevated drug concentration in a sample that is not reflective of the concentration at the time of death.

Factors which influence drug PMR include cell death, putrefaction, body position, and drug physicochemical characteristics (98). After death, cell membranes are eroded, allowing leakage of cellular contents into the extracellular space and allowing other molecules to enter cells (100). The putrefaction process is highly variable depending on environmental conditions and the state of the body. Bacteria present in the body at the time of death enter the blood and travel throughout the body, consuming and digesting tissue and

further metabolizing some drugs (97). Postmortem degradation by bacteria may be slowed when the ambient temperature is low (97).

Prouty and Anderson were the first to report the implications of blood collection site and postmortem interval to PMR (101). The study consisted of collecting blood from various locations including left and right subclavian vein, left and right heart chambers, and left and right femoral vein. Two hours later, pathologists collected another set of blood specimens to determine if drug concentration changed over time. Drug concentrations varied across blood samples collected from different sites, with the observation that femoral blood samples were generally lower in concentration than heart or subclavian blood samples. The authors also noted increased drug concentration in heart blood as the postmortem interval increased. For forensic toxicologists to render an accurate opinion in drug-related deaths, it is important to quantify drug concentrations in both central and peripheral sites.

Peripheral (P) blood, generally from the femoral vein, is less subjected to redistribution effects than cardiac blood because it is surrounded only by muscle and fat. Central (C) blood, generally heart blood or blood pooled from the chest cavity, is subjected to drug redistribution from thoracic organs that contain considerable concentrations of unabsorbed drugs (102). Since elevated concentrations are present in central blood after redistribution occurs, the C:P ratio of drug concentration is used as a tool to indicate if a drug undergoes PMR (103). A C:P ratio greater than one indicates that a drug is susceptible to concentrations changes after death; however, there are limitations to the C:P ratio as the definitive tool to assess a drug's ability to undergo PMR. For example, carisoprodol and salicylate reports show C:P ratios greater than one, but little PMR is noted with these drugs (103, 104).

Due to such limitations, McIntyre proposed using the liver tissue to peripheral blood ratio (L:P) as a marker for PMR (105). They compiled a list of C:P and L:P ratios for commonly encountered drugs in postmortem toxicology. For all 13 drugs, C:P ratios were between 1.0 and 3.0 while L:P ratios were between 1.6 and 97. Drugs, such as tricyclic antidepressants, known to undergo significant PMR exhibited an L:P ratio greater than 20. Other drugs that do not demonstrate PMR had an L:P ratio less than 5. Research supports the idea that the L:P ratio is advantageous and can be used in conjunction with the C:P ratio for interpreting a drug's redistribution potential.

For many years, the toxicology community believed that THC undergoes low to moderate PMR due to its chemical properties, despite a paucity of scientific data (106). Drugs susceptible to PMR include those that are lipophilic, and basic with a large volume of distribution (98). Although THC is not a basic compound, it is highly lipophilic with a relatively large volume of distribution. However, few studies evaluated cannabinoids PMR in humans or animals. One study compared THC results from a rat animal model to those from human cases (107). Rats exhibited a C:P ratio of 1.9 for heart blood concentrations to vena cava blood concentrations after 30 mg THC was administered orally. The reported central to peripheral ratios in two human cases included in the study were 0.4 and 2.5 (107). The most extensive study in animals by Brunet examined redistribution of THC in the Large White pigs (108). Fifteen pigs were administered THC via intravenous injection and biological specimens were collected at intervals up to 48 h after death. An increase in THC concentration in central blood with a corresponding decrease in concentration in peripheral blood, confirmed that THC is likely to undergo PMR in pigs (108).

Human studies evaluating postmortem redistribution in cannabinoid positive casework provide varying results. Holland et al. examined cannabinoids in 19 autopsy cases to determine the extent of PMR in humans (109). Cardiac (C) and iliac vein (P) blood samples were analyzed for the presence of THC and two metabolites, 11-OH-THC and THCCOOH. Median C:P ratios for all three analytes were less than two suggesting modest redistribution from tissues to central blood after death. All three analytes showed a slight increase in C:P ratios with an increase in postmortem interval. In another human study, Lemos and Ingle did not observe redistribution for cannabinoids in 30 postmortem cases (90). Mean (median) C:P ratios for THC, 11-OH-THC and THCCOOH were 0.62 (0.38), 0.90 (1.17) and 1.07 (0.89), respectively. They observed no relationship between C:P ratios and postmortem interval for THC or 11-OH-THC with a limited relationship noted for C:P ratios and postmortem interval for THCCOOH (90). Meneses and Hernandez found C:P ratio ranges from 0.12 – 5.70, 0.28 – 3.33 and 0.27 – 3.08 for THC, 11-OH-THC and THCCOOH, respectively, in 43 postmortem cases.

These three human studies assessing cannabinoid concentrations in postmortem central and peripheral samples showed considerable variation in identifying the extent, if any, that THC redistributes after death. Published cannabinoid concentrations in liver and peripheral blood samples would provide auxiliary data to aid interpretation of PMR; however, no such data are available.

Physiologically Based Pharmacokinetic Modeling

Physiologically based pharmacokinetic (PBPK) modeling uses a set of mathematical equations to describe a drug's absorption, distribution, metabolism and excretion processes in

the body based on relationships among physiological, biochemical, anatomical and physiochemical information (110). This type of modeling can be a vital part of drug discovery and development because it predicts drug concentrations in any organ or tissue of interest across different dosing regimens (111). The estimation of drug exposure at the site of action provided by a PBPK model is highly valuable as it may be difficult or impossible to measure the concentration in tissues experimentally (112). Benefits of developing a PBPK model include the ability to extrapolate across species, exposure routes, doses and duration (111).

A whole-body PBPK model consists of physiological compartments that are most relevant to the drug's absorption, distribution, metabolism and excretion properties. Typical tissue compartments include liver, kidneys, muscle and lungs, which are all linked to the arterial and blood compartments by blood flows, organ mass, tissue-partition coefficient and permeability (113). The building blocks of a PBPK model structure are species-specific physiological parameters, chemical-specific parameters and drug administration protocol (114). Physiological and anatomical parameters, such as body weight, cardiac output, organ weight or volume, blood flow rate and volume of blood in tissues, are dependent on the species evaluated by the model. The parameters specific to the compound utilized in the model include molecular weight, lipophilicity, and dissociation rate constants. Several drug properties, such as tissue-plasma partition coefficients, rate constants, permeability coefficients, and fraction of unbound drug, are dependent on properties of both the drug and species under consideration (112). The drug administration protocol of the PBPK model is tailored to the desired exposure route, dosing regimen and duration of administration.

Limited data are available for THC concentrations in tissues, thus a PBPK model that can predict THC concentrations in various tissues would aid in predicting THC distribution kinetics across species. Methaneethorn et al. developed the first PBPK model for describing THC distribution to seven tissue compartments in mice, rats and pigs (115). Predicted THC concentrations from the model were compared to data collected previously from three pharmacokinetic studies and found that the developed model adequately simulated THC tissue concentrations (49, 116, 117).

Further research yielded a PBPK model of THC in humans that was extrapolated from the PBPK model in mice, rats and pigs (118). Five THC pharmacokinetic studies following IV bolus, IV infusion, oral, smoking and vaping cannabis administration were used to assess model predictions (30, 46, 119-121). Simulated plasma concentration-time profiles from the model were comparable to observed concentrations from IV bolus, IV infusion and oral administration and from one of the three smoking administration studies (118). Observations from one smoking study showed a slower decrease in THC concentrations than predicted concentrations. Likewise, simulated concentrations for drug delivery by cannabis smoking and vaporization over-predicted THC concentrations compared to observation data (118). Differences from predicted and observed concentrations could result from the high inter-subject variability usually observed in THC pharmacokinetic studies. The observed concentrations for model comparison were average values from each of the studies. Furthermore, datasets utilized for model evaluation were obtained from several research groups with different study designs, participant demographics and pattern of cannabis use, and cigarette composition. All these factors could impact pharmacokinetics, thus causing variations between model predictions and observed concentrations.

Knowledge Gaps, Aims, and Hypotheses

Although cannabis use is prevalent in the US, limited data are available for detection and quantification of cannabinoids in postmortem forensic casework (80, 85, 86, 90-93, 122, 123). Most of these methods only include analysis for THC and its major metabolites, 11-OH-THC and THCCOOH, in blood and/or urine. Forensic interpretation of postmortem cases will improve with analysis of more cannabinoids, including major and minor components of the cannabis plant and Phase I and Phase II metabolites, and with analysis of more biological specimens, including a broad array of tissues. In Chapter 2, this dissertation focuses on an analytical method for determination of cannabinoids in postmortem samples.

Aim 1: Develop and validate an analytical method for the simultaneous identification and quantification of 12 cannabinoids in postmortem fluids and tissues.

Hypothesis 1: A sensitive and specific LC-MS/MS method can be validated for determination of cannabinoid concentrations in postmortem biological specimens.

Because few studies focus on cannabinoid determination in postmortem forensic casework, data are even more rare for cannabinoid distribution in tissues (85, 86, 93, 122, 124). Different cannabinoid analytes were quantified in tissues including THC and THCCOOH (93); others added 11-OH-THC (86, 124), and others CBD and CBN in brain (122). LC-MS/MS permitted analysis of all of these cannabinoids and THCCOOH-g in tissues (85). None of these research studies incorporated THC-g, 8β-diOH-THC, 8β-OH-

THC, CBG, THCV or THCVCOOH. In Chapter 3, this dissertation highlights cannabinoid distribution in postmortem fluids and tissues from authentic forensic cases.

Aim 2: Apply the validated method to forensic cases that are a part of the aviation incident investigation process.

Hypothesis 2: Analysis of pilots fatally injured in plane crashes will reveal high cannabinoid concentrations in highly perfused tissues after cannabis intake.

Despite extensive THC pharmacokinetic research, the disposition of THC into the body after cannabis administration is not fully detailed in literature. Relatively few controlled administration studies focused on THC distribution in tissues and none evaluated THC distribution after cannabis administration via the inhalation route (49, 94). In Chapter 4, this dissertation utilized an animal protocol approved by Oklahoma State University Institutional Animal Care and Use Committee (IACUC) to evaluate cannabinoid distribution (ACUP VM-18-12). Given the limited data available for THC distribution, a PBPK model can aid characterization of THC tissue kinetics. Currently, only one research study describes the development of a PBPK model for THC in mice, rats and pigs (115). In Chapter 4, this dissertation details the physiological, physiochemical and biochemical information required for a PBPK model.

Aim 3: Develop a PBPK model for THC in rabbits by the inhalation route of administration. Determine cannabinoid distribution in rabbits after controlled inhaled cannabis administration.

Hypothesis 3: The rabbit can appropriately model cannabinoid distribution after controlled cannabis administration. Cannabinoid concentrations in samples collected from rabbits immediately upon death will correlate to simulated concentrations from the PBPK model.

Postmortem redistribution, a well-known phenomenon in forensic toxicology, can result in significant changes in drug concentrations after death depending on blood collection site and postmortem interval. Despite this common knowledge, scarce and conflicting data are available describing PMR of cannabinoids in forensic casework (90, 109, 122). Two studies assess PMR for THC in pigs – one after intravenous injection (108) and one after pulmonary administration, which incorporated a temperature-dependent variable (125). In Chapter 5, this dissertation uses the approved IACUC protocol to evaluate cannabinoid concentration changes in rabbits after death.

Aim 4: Evaluate cannabinoid postmortem redistribution in rabbits following controlled cannabis administration.

Hypothesis 4: Cannabinoid concentrations in rabbits stored at two different temperature conditions and necropsied at multiple time intervals will reveal time- and temperature-dependent changes after death.

Summary

Accurate forensic interpretation of drug concentrations requires an understanding of a drug's pharmacokinetic properties before and after death. Lack of research characterizing cannabinoid distribution and postmortem redistribution after cannabis use limits a toxicologist's ability to provide appropriate interpretation in medicolegal investigations. A novel LC-MS/MS method was developed for postmortem analysis that includes more phytocannabinoids and THC metabolites than any other previously published method. The proof of method evaluated cannabinoid concentrations in a broad array of postmortem fluids and tissues, providing forensic toxicologists with extensive information about cannabinoid distribution in authentic forensic casework. The determination of cannabinoid concentrations in rabbits after controlled cannabis administration provides vital distribution data following an acute dose. A PBPK model was developed which furthers our understanding of cannabinoid distribution patterns. For the first time, postmortem redistribution was evaluated in rabbits after cannabis administration via the inhalation route and after storage of the carcasses at two different temperatures. The research described hereafter adds considerably to the body of knowledge regarding cannabinoid distribution and postmortem redistribution.

CHAPTER II

IDENTIFICATION AND QUANTIFICATION OF 12 CANNABINOIDS IN POSTMORTEM FLUIDS AND TISSUES BY LC-MS/MS

Abstract

Cannabis sativa is the most abused illicit drug worldwide and is often detected by forensic laboratories working with biological specimens from potentially impaired drivers or pilots. To address the problem of limited published data regarding cannabinoids quantification in postmortem specimens, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated to quantify Δ^9–tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), 8β,11-dihydroxy-THC (8β-diOH-THC), 8β-hydroxy-THC (8β-OH-THC), THC-glucuronide (THC-g), THCCOOH-glucuronide (THCCOOH-g), cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), Δ^9-tetrahydrocannabivarin (THCV), and 11-nor-9-carboxy-THCV (THCVCOOH) in postmortem matrices. Solid phase extraction concentrated analytes prior to analysis on a biphenyl column coupled to a mass spectrometer in electrospray positive ionization mode using multiple reaction monitoring. Linearity ranged from 0.25-50 ng/mL (THC-g), 0.5-100 ng/mL (CBN), 0.5-250 ng/mL (THC, 11-OH-THC, THCCOOH, CBD, and CBG), 1-100 ng/mL (8β-diOH-THC,

THCVCOOH, 8β-OH-THC, and THCV) and 1-250 ng/mL (THCCOOH-g). Within-run imprecision was <11.2% CV, between-run imprecision <18.1% CV, and bias within ±15.1% of target concentration in blood for all analytes at three concentrations across the linear range. No carryover or interferences from endogenous or exogenous substances were observed. All analytes were stable in blood at room temperature for 24 h, refrigerated (4ºC) for 96 h, and following three freeze/thaw cycles. Matrix effects greater than 25% were observed for most analytes in tissues. The proof of concept for method applicability involved measurement of cannabinoids in a pilot fatally injured in an aviation crash. This new analytical method is robust and sensitive, enabling collection of additional cannabinoid postmortem distribution data to improve interpretation of postmortem cannabinoid results.

1. Introduction

Other than alcohol, *Cannabis sativa* is the most widely abused drug in the world (1). According to the 2019 National Survey on Drug Use and Health, approximately 48.2 million (17.5%) Americans aged 12 and older consumed cannabis in the previous year (3). Its prevalence in the United States, along with recent legislative changes allowing use for recreational and medical purposes in many states, highlights the need for forensic toxicology laboratories to detect cannabinoids and their metabolites. Accurate cannabinoids identification and quantification is important for documenting impaired driving and/or crash causation.

The main psychoactive component of cannabis, Δ^9–tetrahydrocannabinol (THC), is rapidly metabolized in the liver to the active metabolite, 11-hydroxy-THC (11-OH-THC). Further oxidation produces the major non-psychoactive metabolite, 11-nor-9-

carboxy-THC (THCCOOH) (12). A minor metabolic pathway produces two metabolites, 8β,11-dihydroxy-THC (8β-diOH-THC) and 8β-hydroxy-THC (8β-OH-THC) (126). Phase II metabolism produces multiple glucuronide metabolites (12).

The cannabis plant contains over 550 chemical compounds, including at least 113 cannabinoids (127). Minor cannabinoids cannabidiol (CBD), cannabinol (CBN), and cannabigerol (CBG) were evaluated as markers of recent cannabis usage (30, 128, 129). Another cannabinoid, Δ^9-tetrahydrocannabivarin (THCV), and its metabolite 11-nor-9-carboxy-THCV (THCVCOOH), were identified as potential markers for ingestion of cannabis versus synthetic THC, dronabinol or Marinol® (8).

Forensic toxicologists are often asked to interpret cannabinoid concentrations in biological specimens. It is important that analytical procedures reliably identify and quantify cannabinoids and metabolites to provide data to aid the toxicologist in forming opinions. For years, gas chromatography-mass spectrometry (GC-MS) was the primary technique for the detection and quantification of THC, 11-OH-THC, and THCCOOH in forensic toxicology laboratories. Limitations of GC-MS analysis include the need for alkaline or enzymatic hydrolysis prior to extraction to liberate glucuronide functional group(s) from the cannabinoid and the need for chemical derivatization of hydroxyl and acid moieties (130-132). Hydrolysis and derivatization steps are time-consuming and expensive. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides a sensitive and reliable direct analysis of cannabinoids and cannabinoid glucuronides. Schwope et al. developed and validated the first LC-MS/MS method to simultaneously quantify THC, 11-OH-THC, THCCOOH, THC-glucuronide (THC-g), THCCOOH-glucuronide (THCCOOH-g), CBD, and CBN in blood using solid phase extraction (133)

(87). Scheidweiler et al. expanded this method to include three more cannabinoids – CBG, THCV, and THCVCOOH – in hopes of identifying a marker of recent cannabis use (83). While the Schwope and Scheidweiler methods are utilized in living subjects from clinical studies, there are few methods for the identification and quantification of cannabinoids in postmortem fluids and tissue samples. Saenz et al. employed SPE and LC-MS/MS to determine THC, 11-OH-THC, and THCCOOH blood and tissue concentrations in eleven pilots involved in aviation crashes (86). Gronewold and Skopp developed a liquid-liquid extraction LC-MS/MS method to investigate the distribution of THC, 11-OH-THC, THCCOOH, THCCOOH-glucuronide, CBD, and CBN in five postmortem cases (85).

The goal of this research is to develop and validate an LC-MS/MS method for the simultaneous detection and quantification of free and glucuronidated cannabinoids in postmortem fluids and tissues. While the data from one pilot has been included to demonstrate method applicability, the application of the method to multiple pilots and interpretation of the data will be the subject of a separate publication. To our knowledge, this is the first analytical method to include THC-g, CBG, THCV, THCVCOOH, 8β-diOH-THC, and 8β-OH-THC in postmortem forensic casework.

2. Materials and Methods

2.1 Reagents and supplies

THC, THCCOOH, 11-OH-THC, CBD, CBG, CBN, and THCCOOH-g methanolic standards were purchased from Cerilliant (Round Rock, TX). THC-g, 8β-diOH-THC, 8β-OH-THC, and THCVCOOH were obtained from ElSohly Laboratories, Inc. (Oxford, MS). Deuterated internal standards were acquired from Cerilliant and

ElSohly Laboratories. Ammonium hydroxide, glacial acetic acid, formic acid, LCMS-grade acetonitrile, hexanes, and water were from Fisher (Fisher Scientific, Pittsburgh, PA). Solid phase extraction (133) utilized Bond Elut Plexa PCX columns (3mL/30mg, Agilent, Wilmington, DE) and a positive pressure manifold was employed (United Chemical Technologies, Inc., Bristol, PA). Tissue samples required a Bead Mill Homogenizer (OMNI International, Inc., Kennesaw, GA) for solid tissue homogenization.

2.2 Calibrators, quality control (QC) and internal standards

Blank bovine blood was fortified with working stock solutions to prepare calibrators and quality control samples. A primary 1µg/mL THC-g stock solution and all other calibrator stocks at 2µg/mL were prepared in methanol. Appropriate dilutions were made in methanol to create a series of calibrator solutions. Twenty-five µL calibrator added to 0.5mL blank blood created blood calibrators at 0.25, 0.5, 1.25, 2.5, 5, 12.5, 25, and 50ng/mL for THC-glucuronide and 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, and 250ng/mL for all others. An internal standard solution containing 200ng/mL THC-d_3, THCCOOH-d_3, 11-OH-THC-d_3, CBD-d_3, CBG-d_9, CBN-d_3, THCCOOH-glucuronide-d_3, and 8β-diOH-THC-d_6 was prepared in methanol; 25µL fortified into each sample provided a 10ng/mL internal standard concentration.

QC samples were prepared with different reference standard lot numbers than the calibrators, when available. Low, medium, and high QC working solutions were prepared in methanol. Target concentrations were: THC-g 1, 10, 37.5ng/mL; 8β-diOH-THC, THCVCOOH, 8β-OH-THC, THCV, and CBN 2, 20, 75ng/mL; THCCOOH-g, 11-OH-THC, THCCOOH, CBD, CBG, and THC 2, 75, 200ng/mL.

2.3 Specimen pretreatment

Calibrators, controls, and blood and tissue specimens were subjected to protein precipitation with acetonitrile and pre-treated with ammonium hydroxide and glacial acetic acid prior to SPE. Blood (0.5mL) was pipetted into a disposable glass round bottom tube (VWR, 16x100 mm). The sample was fortified with 25µL internal standard. Ice-cold ACN (3mL) was added dropwise while vortexing. Tubes were capped and rotated for 15min, then centrifuged at 3000rpm for 10min. Supernatants were decanted into clean conical tubes and evaporated with nitrogen (40°C) to approximately 750µL. For urine, bile, and vitreous humor samples, 0.5mL sample was pipetted into a disposable glass round bottom tube. Prior to loading for SPE, 2.25mL 0.2% NH_4OH in deionized water (v/v) and 100µL glacial acetic acid was added to all samples with brief vortexing before the addition of the second reagent.

2.4 SPE

Extraction columns were conditioned with 0.5mL methanol, and samples loaded by gravity. Columns were washed with 2mL 79:20:1 de-ionized water:acetonitrile:glacial acetic acid (*v/v/v*) and dried under full vacuum for 10min. Columns were washed with an additional 200µL hexane under low vacuum, then dried under full vacuum for 3min. Analytes were eluted with two separate aliquots (0.5 and 1 mL) 1% glacial acetic acid in ACN (*v/v*) under gravity. Eluents were collected in 10mL conical tubes and dried under nitrogen at 40°C in a water bath. Samples were reconstituted in 100µL 0.1% formic acid in water:acetonitrile (60:40, *v/v*), vortexed and transferred to glass inserts in autosampler vials.

2.5 LC-MS/MS

The high performance liquid chromatography (HPLC) system consisted of a Shimadzu DGU-20A3 degasser, LC-20ADxr pumps, SIL-20ACxr autosampler, and a CTO-20 column oven (Shimadzu Corp, Columbia, MD). Chromatographic separation was accomplished with a Raptor Biphenyl column (2.7µm, 50 X 3.0mm) fitted with a Raptor Biphenyl guard cartridge (2.7µm, 5 X 3.0mm) (Restek Corp, Malvern PA). The autosampler temperature was 4°C, column oven 50°C, and injection volume 10µL. Gradient elution was performed at a flow rate 0.5mL/min. Initial gradient conditions were 60%A (0.1% formic acid in water (*v/v)*) and 40%B (0.1% formic acid in ACN (*v/v*)), then B increased to 65% at 10 min. From 10 to 10.5 min, 95%B maintained for 5min, followed by column re-equilibration at 60%A over 0.5min and hold for 2min. HPLC eluent was diverted to waste for the first 2 and final 9 min of analysis. An internal and external needle rinse and wash was performed on the autosampler at the 12min mark.

Tandem mass spectrometry analysis was performed on a Shimadzu LCMS-8040 with electrospray ionization (ESI) in positive ionization mode. MS/MS parameters (Table 1) were optimized via direct infusion of individual analytes at 100ng/mL in initial mobile phase. Optimized source parameters were as follows: nebulizing gas flow – 3L/min; drying gas flow – 15L/min; desolvation line temperature – 250°C; heat block temperature – 400°C. Argon collision gas was set at 230kPa. Data acquisition and processing was controlled by Lab Solutions software (version 5.65).

2.6 Method validation

Linearity, limit of detection (LOD), limit of quantification (LOQ), carryover, accuracy, within-run and between-run imprecision, matrix and drug interferences, dilution integrity, stability, and ion suppression/enhancement were evaluated during method validation following guidelines from the Scientific Working Group for Toxicology (134).

Preliminary experiments with eight calibrator concentrations revealed the most appropriate calibration model. Calibration curves with at least 7 concentrations were best fit by linear-least squares regression across the linear dynamic range for each analyte. Calibrators were required to quantify within ±15% of target and curve correlation coefficients required to be greater than or equal to 0.99. Linearity was determined by preparing and analyzing a calibration curve on five separate days.

LOD was defined as the lowest concentration with MRM transitions with a signal-to-noise of greater than 3, retention time within 2% average calibrator retention time and qualifier/quantifier transition peak ratios within ±20% mean calibrator transition peak ratios. LOQ was defined as the lowest concentration quantified with adequate precision (%CV <20%) and accuracy (±20% of target concentration), a signal-to-noise ratio of at least 10, retention time within 2% average calibrator retention time. LOD and LOQ were evaluated in triplicate over three runs using fortified blood samples.

Carryover was evaluated for all analytes by injecting extracted blank blood samples after the highest calibrator of five calibration curves. To document carryover absence, blank blood samples should not meet LOD criteria.

Table 1. LC-MS/MS parameters for identification and quantification of 12 cannabinoids in postmortem specimens.

Analyte	Retention Time (min)	Dwell Time (ms)	Precursor (m/z)	Product ion (m/z)	Q1 Pre Bias (V)	Collision Energy (V)	Q3 Pre Bias (V)
8β-diOH-THC	2.1	37	347.0	311.1	-17	-16	-20
				329.1	-17	-12	-22
8β-diOH-d6	2.1	37	353.0	317.2	-17	-16	-20
				335.2	-17	-11	-22
THCCOOH-g	2.3	37	521.1	299.3	-26	-34	-20
				327.2	-26	-20	-22
THCCOOH-g-d3	2.3	37	523.9	348.2	-26	-16	-23
				330.2	-26	-24	-22
THCVCOOH	2.7	37	317.2	299.0	-16	-16	-20
				271.2	-16	-19	-28
THC-g	3.2	47	491.1	315.3	-25	-19	-20
				193.0	-25	-36	-19
8β-OH-THC	3.5	37	331.0	271.1	-25	-19	-28
				201.1	-17	-24	-21
11-OH-THC	4.0	37	331.2	200.9	-17	-26	-20
				193.2	-17	-27	-19
11-OH-THC-d3	4.0	37	334.1	316.2	-17	-15	-21
				196.1	-25	-25	-20
THCCOOH	4.2	37	345.2	193.0	-18	-30	-20
				299.2	-18	-21	-30
THCCOOH-d3	4.2	37	348.1	330.1	-18	-16	-15
				302.3	-18	-21	-20

Table 1 continued. LC-MS/MS parameters for identification and quantification of 12 cannabinoids in postmortem specimens.

Analyte	Retention Time (min)	Dwell Time (ms)	Precursor (m/z)	Product ion (m/z)	Q1 Pre Bias (V)	Collision Energy (V)	Q3 Pre Bias (V)
THCV	5.7	37	287.0	164.9	-14	-23	-28
				122.9	-21	-32	-21
CBD	6.4	37	315.1	259.1	-16	-17	-27
				193.0	-16	-19	-19
CBD-d3	6.4	37	318.1	262.1	-24	-19	-27
				196.1	-24	-21	-20
CBG	6.5	37	317.0	193.0	-16	-14	-19
				123.0	-30	-35	-20
CBG-d9	6.4	37	326.0	202.2	-16	-16	-20
				122.9	-34	-38	-20
CBN	7.3	47	311.1	241.1	-16	-20	-25
				223.1	-16	-22	-23
CBN-d3	7.3	47	314.2	223.2	-16	-21	-22
				241.3	-16	-23	-23
THC	7.5	47	315.1	192.9	-16	-20	-20
				123.0	-16	-35	-20
THC-d3	7.5	47	318.1	196.0	-24	-25	-19
				123.0	-24	-36	-20

8β-diOH: 8-Beta-diHydroxy-THC; THCCOOH-g: 11-nor-9-carboxy-THC-glucuronide; THCVCOOH: 11-nor-9-carboxy-THCV; THC-g: THC-glucuronide; 8β-OH: 8-Beta-Hydroxy-THC; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; THCV: Δ^9-tetrahydrocannabivarin; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; THC: Δ^9-tetrahydrocannabinol

Imprecision and accuracy were determined from three replicates at three different concentrations across the linear dynamic range for each cannabinoid over five different runs. Blood low, medium, and high quality control target concentrations were: THC-g 1, 10, 37.5ng/mL; 8β-diOH-THC, THCVCOOH, 8β-OH-THC, THCV, and CBN 2, 20, 75ng/mL; THCCOOH-g, 11-OH-THC, THCCOOH, CBD, CBG, and THC 2, 75, 200ng/mL. Accuracy (bias) was calculated by comparing the difference of the overall mean result to target concentration for all analytes and was expressed as the percent of target concentration. Maximum acceptable bias was ±20% of target. Between- and within-run imprecision was calculated across multiple analytes at three different control concentrations using a One-Way Analysis of Variation (ANOVA) approach and expressed as the percent coefficient of variation (%CV). The %CV could not exceed 20% at each concentration.

Interferences may come from endogenous compounds in the matrix, presence of other drugs, or internal standards. Analyte peak identification criteria for all interference studies were retention time within ±2% average calibrator retention times and qualifier/quantifier transition peak area ratios within ±20% of mean calibrator transition peak ratios. Matrix interferences were evaluated by combining 10 different blank sources into 2 matrix pools. Matrices assessed were blood, urine, vitreous humor, liver, lung, kidney, spleen, muscle, brain, and heart. The pools were extracted and analyzed to demonstrate the absence of matrix interferences.

To evaluate other commonly encountered drugs, 4 mixes were prepared at 1µg/mL drug in methanol. The mixes included: Opiates - hydrocodone, oxycodone, morphine, oxymorphone, codeine, hydromorphone; Drugs of Abuse - phencyclidine,

methamphetamine, amphetamine, JWH-073, JWH-018, AB-PINACA; Benzodiazepines-diazepam, nordiazepam, lorazepam, oxazepam, temazepam, midazolam, alprazolam, α-hydroxyalprazolam; Over-the-Counter Medications - diphenhydramine, chlorpheniramine, acetaminophen, ibuprofen, naproxen, ephedrine, dextromethorphan, doxylamine.

Deuterated internal standards may contain a small amount of the native drug analyte as an impurity. To evaluate, blank blood was fortified with 10ng/mL deuterated internal standard mix, extracted and analyzed to monitor signal. No interferences were noted if the peak did not meet identification criteria at the LOD. Hydrolysis of glucuronides and conversion of cannabinoids during sample processing were evaluated by fortifying a blank blood sample with each cannabinoid individually at 500ng/mL.

Dilution integrity was explored by fortifying a blood sample with 50ng/mL THC-g and 100ng/mL of other cannabinoids. Two different dilutions, 1:2 and 1:5, were evaluated by extracting a smaller sample volume, 0.25mL (n=5) and 0.10mL (n=5), respectively. Dilution integrity was upheld if samples quantified within ±20% of expected concentration.

Short-term analyte stability was evaluated by fortifying blank blood with analytes at low and high QC concentrations and analyzed under three different conditions (n=3): room temperature, refrigeration (4°C) and freeze/thaw cycles (-20°C). Room temperature samples were analyzed after 24h and refrigerated samples were analyzed after 24, 48, 72 and 96h in storage. All freeze/thaw samples were removed from the freezer each day, allowed to thaw, and three aliquots were extracted, while remaining samples were

returned to the freezer. Analytes were deemed stable if observed concentrations were within 20% of target.

Processed sample stability was measured by extracting low and high QC samples (n=3), combining reconstituted samples, dividing them into different autosampler vials, and immediately analyzing them on the instrument. Ratios of peak area of analyte to internal standard were calculated and triplicates were averaged for each concentration to establish time zero response. Vials were stored on the autosampler (4°C) and re-injected at 24, 48 and 72h and the calculated ratios of peak areas of analyte to internal standard were compared to time zero. Analytes were considered stable if the response was within ±20% of time zero response average.

Ionization suppression/enhancement was assessed by post-extraction addition. Two sample sets were prepared, with set one neat standards prepared at two concentrations –low (5ng/mL) and high (75ng/mL). Neat standards were injected six times to establish a mean peak area for each concentration. Set two consisted of two blank matrix pools used from the matrix interference studies. Matrices evaluated for ion suppression/enhancement were: blood, urine, vitreous humor, bile, liver, lung, kidney, spleen, muscle, brain, and heart. Two pools were extracted in duplicate and after extraction, the sample was reconstituted with low or high neat standard mix. Ion suppression or enhancement was calculated by dividing mean analyte peak area in set 2 by mean analyte peak area in set 1, subtracting 1, and multiplying by 100 to convert to a percentage. Any positive value is considered enhancement and any negative value is considered suppression.

Matrix effects were further evaluated in tissues with three replicates fortified at low, mid, and high QC concentrations to establish if a quantitative value could be accurately and precisely determined in matrices other than blood. Non-blood matrices evaluated were liver, lung, kidney, spleen, muscle, brain, and heart. Each tissue (~5g) was mixed with deionized water (~15g) to produce a 1:4 tissue homogenate and 0.5g was taken through the extraction process. Accuracy and imprecision were calculated for each matrix type at the QC concentrations. Acceptable criteria were ±30% of target and 20% CV for accuracy and imprecision, respectively.

2.7 Application to an authentic case

As proof of method, postmortem fluids and tissues from a fatally injured pilot received by the Federal Aviation Administration's (FAA) Forensic Sciences Section during 2019 was analyzed using the described LC-MS/MS method.

3. Results

3.1 Method development and validation

The best fit calibration model was a linear least-squares regression with $1/x^2$ weighting. All correlation coefficients exceeded 0.99. Table 2 details LOD, LOQ, linearity, and calibration results. LOD was administratively set as the lowest non-zero calibrator for each analyte (Figure 4). There was no carryover observed with any analytes.

Accuracy and imprecision were evaluated at three QC concentrations (n=5): 2, 20 75ng/mL for 8β-diOH-THC, THCVCOOH, 8β-OH-THC, THCV, CBN; 2, 75, 200ng/mL for THCCOOH-g, 11-OH-THC, THCCOOH, CBD, CBG, THC; 1, 10, 37.5ng/mL for

THC-g. Bias was less than ±20% of target. The ANOVA approach defined by SGWTOX guidelines determined overall within- and between-run imprecision. All CV values were less than 20% (Table 3).

There were no interfering peaks in 10 different biological matrices sources. None of 28 drugs evaluated produced peaks that met LOD identification criteria. There was no interference noted with deuterated internal standards fortified at 10ng/mL and standards did not produce interferences with other standards. There was no evidence of CBD to THC conversion during the analysis for a 500ng/mL CBD sample. When glucuronide standards were fortified at 500ng/mL in blood, a small amount of THCCOOH was detected with the THCCOOH-g standard. The THCCOOH-g standard contained a trace amount of THCCOOH. A 20ng/mL THCCOOH-g standard contained an analytically insignificant 0.5% THCCOOH.

Dilution integrity was assessed by extracting blood samples (100ng/mL) with volumes of 0.25mL and 0.10mL, instead of the typical assay volume of 0.5mL. Concentrations of replicates (n=5) for both reduced volumes were within ±20% of target for all cannabinoids except THCCOOH-g.

All analytes at low and high QC concentrations in blood extracts were stable at room temperature for 24h, 4°C for 96h, and after three freeze/thaw cycles. Extracted samples at low and high QC concentrations were re-injected after 24, 48, and 72h in the cooled (4°C) autosampler to test for processed sample stability. Three analytes, 8β-OH-THC, THC-g, and THCCOOH-g, were least stable, showing greater than 20% change in both low and high concentrations after only 24h. CBD, CBG, 11-OH-THC, THCV, and

Table 2. Calibration Results (n=5) for 12 Cannabinoids in Blood by LC-MS/MS.

Analyte	Internal Standard	LOD/LOQ (ng/mL)	Linear range (ng/mL)	R^2 (mean)
8β-diOH-THC	8β-diOH-d6	1	1 - 100	0.998
THCCOOH-g	THCCOOH-g-d3	1	1 - 250	0.990
THCVCOOH	THCCOOH-d3	1	1 – 100	0.997
THC-g	THCCOOH-d3	0.25	0.25 – 50	0.993
8β-OH-THC	11-OH-THC-d3	1	1 – 100	0.995
11-OH-THC	11-OH-THC-d3	0.5	0.5 – 250	0.994
THCCOOH	THCCOOH-d3	0.5	0.5 – 250	0.994
THCV	CBD-d3	1	1 – 100	0.996
CBD	CBD-d3	0.5	0.5 – 250	0.997
CBG	CBG-d9	0.5	0.5 – 250	0.998
CBN	CBN-d3	0.5	0.5 – 100	0.999
THC	THC-d3	0.5	0.5 – 250	0.996

8β-diOH: 8-Beta-diHydroxy-THC; THCCOOH-g: 11-nor-9-carboxy-THC-glucuronide; THCVCOOH: 11-nor-9-carboxy-THCV; THC-g: THC-glucuronide; 8β-OH: 8-Beta-Hydroxy-THC; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; THCV: Δ^9-tetrahydrocannabivarin; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; THC: Δ^9-tetrahydrocannabinol

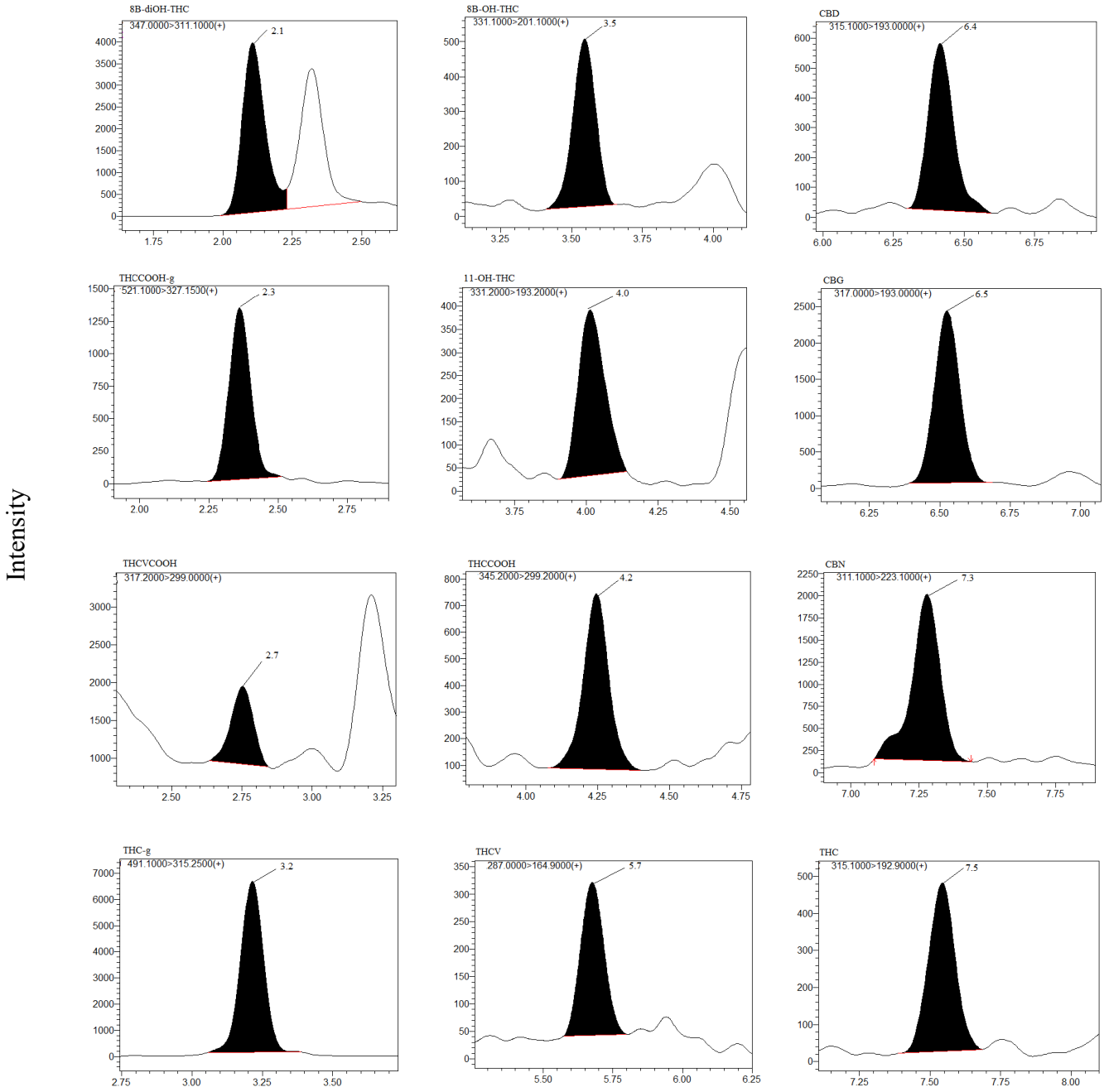

Figure 4. MRM ion chromatograms of cannabinoid analytes at the limits of quantification. 8β-diOH: 8-Beta-diHydroxy-THC; THCCOOH-g: 11-nor-9-carboxy-THC-glucuronide; THCVCOOH: 11-nor-9-carboxy-THCV; THC-g: THC-glucuronide; 8β-OH: 8-Beta-Hydroxy-THC; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; THCV: Δ^9-tetrahydrocannabivarin; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; THC: Δ^9-tetrahydrocannabinol

Table 3. Bias and Imprecision (Within-run and Between-run) of 12 Cannabinoids in Blood (n=15).

	Low QC			Mid QC			High QC		
	Bias (%)	Imprecision (%CV)		Bias (%)	Imprecision (%CV)		Bias (%)	Imprecision (%CV)	
Analyte		Within-run	Between-run		Within-run	Between-run		Within-run	Between-run
8β-diOH-THC[a]	3.2	8.7	11.7	-1.8	7.9	11.3	-7.3	4.8	9.0
THCCOOH-g[b]	-13.0	4.9	8.5	-12.6	2.9	8.1	-4.5	4.4	5.3
THCVCOOH[a]	1.6	10.6	10.3	10.0	7.2	7.5	13.8	4.7	4.2
THC-g[c]	7.8	11.2	11.7	1.7	8.3	9.1	-11.0	8.3	10.2
8β-OH-THC[a]	2.0	7.7	10.8	-2.1	7.6	11.4	3.4	5.2	9.5
11-OH-THC[b]	13.0	7.0	9.8	6.5	7.0	10.5	-5.3	3.6	9.1
THCCOOH[b]	11.5	5.6	5.9	0.7	4.7	8.8	-2.2	5.2	6.7
THCV[a]	10.5	6.9	9.9	2.2	6.3	9.9	-1.4	5.0	9.7
CBD[b]	10.4	5.9	9.3	-0.3	5.7	7.7	-15.1	7.9	18.1
CBG[b]	0.0	4.3	6.7	-8.3	3.6	3.9	1.2	4.8	6.7
CBN[a]	2.0	3.8	8.8	-7.4	3.7	4.4	-11.5	3.8	4.1
THC[b]	13.6	4.3	7.0	0.4	4.9	7.4	1.2	5.3	4.6

a Low-, mid-, and high-quality control concentrations for 8β-diOH-THC, THCVCOOH, 8β-OH-THC, THCV, and CBN were 2, 20, and 75 ng/mL, respectively

b Low-, mid-, and high-quality control concentrations for THCCOOH-g, 11-OH-THC, THCCOOH, CBD, CBG, and THC were 2, 75, and 200 ng/mL, respectively

c Low-, mid-, and high-quality control concentrations for THC-g was 1, 10, 37.5 ng/mL, respectively.

8β-diOH: 8-Beta-diHydroxy-THC; THCCOOH-g: 11-nor-9-carboxy-THC-glucuronide; THCVCOOH: 11-nor-9-carboxy-THCV; THC-g: THC-glucuronide; 8β-OH: 8-Beta-Hydroxy-THC; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; THCV: Δ^9-tetrahydrocannabivarin; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; THC: Δ^9-tetrahydrocannabinol

THCVCOOH were stable on the instrument for at least 48h. CBN, THC, THCCOOH, and 8β-diOH-THC were stable for at least 72h after extraction.

Matrix effects were evaluated at low and high concentrations in different matrices – blood, vitreous humor, urine, liver, lung, kidney, spleen, muscle, brain, and heart. The post-extraction addition method determined ionization suppression (negative value) or ionization enhancement (positive value). Deuterated internal standards were included to assess the impact of ionization suppression/enhancement on internal standards. Matrix effects in blood, urine, and vitreous humor were within ±25% for all analytes except 8β-diOH-THC and THCVCOOH.

Due to extensive matrix effects seen in tissues, additional experiments were undertaken to prove that effects do not adversely affect the method's ability to detect or quantify analytes in these matrices. Ten negative human tissue samples were pooled and homogenized. Tissue homogenates (n=3) were fortified with various concentrations to evaluate accuracy and imprecision for each analyte. Criteria for analyte detection in tissues is a peak with a signal-to-noise of at least 3, retention time within 2% average retention time for calibrators, Gaussian peak shape, and qualifier/quantifier transition peak ratios ±20% mean calibrator transition peak ratios. Accuracy criterion was widened to ±30% due to the complex nature of tissue analysis and significant matrix effects identified with extracting tissue matrices. Imprecision criteria was 20%CV for each concentration. When detection, accuracy, and imprecision criteria were met, a quantitative range was established for the analyte in the tissue type. For any analytes that met detection criteria, but failed to meet accuracy or imprecision criteria, the analyte is designated as detected. Table 4 summarizes matrix effects and quantitative ranges for

tissues. Two analytes, 8β-diOH-THC and THCVCOOH, are excluded from the table because these analytes exhibited significant matrix effects in all tissues and they failed to meet detection, accuracy, and imprecision criteria at low and mid quality control concentrations.

3.2 Application to an authentic specimen

Application of the present method to a pilot fatally injured in an aviation crash revealed the presence of all cannabinoids, except 8β-diOH-THC, 8β-OH-THC, THCV, and CBN, in postmortem fluids and tissues (see Table 5). THC, 11-OH THC, and THCCOOH were all detected in blood and brain samples. THC-g was detected in urine, bile, liver, and kidney samples, while THCCOOH-g was present in each specimen type that was tested. CBG and CBD were both detected in bile. THCVCOOH was detected in urine and bile. Results from the method application to 10 fatally injured pilots, including the example in Table 5, will be presented in a future publication.

4. Discussion

Most methods described in the literature for determination of postmortem cannabinoids were developed using GC-MS and typically limited to THC, 11-OH-THC, and THCCOOH in blood and/or urine (84, 90, 109, 135). This LC-MS/MS method simultaneously detects THC and its major and minor metabolites, 11-OH-THC, THCCOOH, 8β-diOH-THC, and 8β-OH-THC, and glucuronide metabolites in postmortem biological specimens. In addition, other cannabinoids, CBD, CBG, CBN, and THCV, are included. While there are several studies in the scientific literature evaluating

Table 4. Matrix Effects (%) and Quantitative Range (ng/g homogenate) for Tissues of 10 Cannabinoids.

Cannabinoid	Liver			Lung			Kidney			Spleen			Muscle			Brain			Heart		
	ME (%)		Range	ME (%)		Range	ME (%)		Range	ME (%)		Range	ME (%)		Range	ME (%)		Range	ME (%)		Range
	Low	High		Low	High		Low	High		Low	High		Low	High		Low	High		Low	High	
THC	-59	-68	0.5 -250	-4	-15	0.5 -250	-41	-31	0.5 -250	-22	-18	0.5 -250	-30	-8	0.5 -250	-24	-11	0.5 -250	-66	-66	0.5 -250
11-OH-THC	-35	-40	0.5 - 250	42	27	5 - 250	21	22	5 - 250	26	31	5 - 250	17	19	0.5 - 250	15	16	0.5 - 250	-21	-30	5 - 250
THCCOOH	-54	-56	5 - 250	-4	-29	5 - 250	-22	-14	5 - 250	6	-2	5 - 250	-30	-22	0.5 - 250	-1	-23	5 - 250	-62	-58	5 - 250
8β-OH	2	-9	1 - 100	140	71	1 - 100	38	57	1 - 100	95	48	1 - 100	98	49	1 - 100	45	35	1 - 100	3	-8	1 - 100
THC-g	-29	-29	0.25 - 50	-22	-19	Detected	-21	-14	Detected	-18	-13	Detected	-34	-23	Detected	-29	-23	Detected	-45	-38	Detected
THCCOOH-g	-10	-7	1 -250	-2	2	1 -250	1	0	1 -250	4	3	1 -250	-7	2	1 -250	-17	-9	1 -250	-14	-9	5 -250
CBD	-78	-78	5 - 250	-39	-41	5 - 250	-42	-39	5 - 250	-29	-26	Detected	-54	-41	Detected	-41	-46	5 -250	-77	-74	Detected
CBG	-75	-79	0.5 -250	-54	-43	0.5 -250	-44	-41	5 -250	-32	-27	0.5 -250	-58	-40	0.5 -250	-45	-45	0.5 -250	-77	-75	0.5 -250
CBN	-50	-80	X	-37	-45	0.5 -100	-47	-39	10 - 100	-30	-23	10 - 100	-64	-39	10 - 100	23	-45	10 - 100	-81	-77	10 - 100
THCV	-75	-77	Detected	-43	-42	Detected	-45	-39	Detected	-33	-28	Detected	-59	-43	Detected	-47	-46	Detected	-79	-75	Detected

THC: Δ^9-tetrahydrocannabinol; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; 8β-OH: 8-Beta-Hydroxy-THC; THC-g: THC-glucuronide; THCCOOH-g: 11-nor-9-carboxy-THC-glucuronide; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; THCV: Δ^9-tetrahydrocannabivarin; X: No quantitative range established

Table 5. Cannabinoids and Cannabinoid Glucuronides Quantified in Postmortem Specimens Collected from a Pilot Fatally Injured in an Aviation Crash

Analyte	Blood (ng/mL)	Urine (ng/mL)	Bile (ng/mL)	Liver (ng/g)	Lung (ng/g)	Kidney (ng/g)	Spleen (ng/g)	Muscle (ng/g)	Brain (ng/g)	Heart (ng/g)
THC	< 0.5	ND[a]	11.5	4.1	19.9	5.0	24.3	32.2	1.0	7.3
11-OH-THC	1.2	ND	83.6	10.5	< 5.0	ND	< 5.0	2.8	2.4	< 5.0
THCCOOH	35.1	60.8	340	236	25.2	182	18.6	18.5	5.4	36.0
THC-g	ND	9.9	2.8	0.7	ND	POS[b]	ND	ND	ND	ND
THCCOOH-g	65.1	359	POS	474	34.0	140	22.0	10.6	2.0	20.0
CBD	ND	ND	11.5	ND	ND	ND	ND	ND	ND	ND
CBG	ND	ND	8.6	ND	ND	ND	ND	ND	ND	ND
THCVCOOH	ND	2.3	2.4	ND	ND	ND	ND	ND	ND	ND

[a] ND = Not Detected

[b] POS = Positive

THC: Δ^9-tetrahydrocannabinol; 11-OH-THC: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; 8β-OH: 8-Beta-Hydroxy-THC; THC-g: THC-glucuronide; THCCOOH-g: 11-nor-9-carboxy-THC-glucuronide; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; THCVCOOH: 11-nor-9-carboxy-Δ^9-tetrahydrocannabivarin

postmortem cannabinoid concentrations in blood specimens, little research exists regarding cannabinoids concentrations in tissue samples.

The method was sensitive, specific, accurate and precise for all cannabinoids in blood. Blood is the preferred specimen of choice in forensic toxicological analyses since it provides interpretive value from a pharmacological perspective and there are large amounts of data referencing blood drug concentrations (136). However, blood may not be available in all situations. At the FAA, the forensic toxicology laboratory relies solely on tissues in about 30-40% of cases due to the nature of aviation crashes. It is vital that analytical methods are available to accurately quantify cannabinoids in postmortem tissues to understand distribution and to provide additional information on postmortem cannabinoid results. Tissues are heterogenous in nature containing large numbers of cells, membranes, electrolytes, and enzymes. They are complex and diverse matrices that require sample preparation and pretreatment before LC-MS/MS analysis. When utilizing LC-MS/MS for tissue analysis, it is not uncommon to observe ionization enhancement or suppression (137). While several LC-MS/MS methods evaluated matrix effects for cannabinoids in blood or urine, there are currently no published methods that thoroughly evaluated matrix effects in postmortem tissues samples. Although many analytes exhibited significant matrix effects in the tissues tested, the method described was able to quantify seven cannabinoids accurately and precisely in tissues.

The main limitation of the method is the substantial matrix effects identified for cannabinoids in the tissue matrices. The complex nature of tissues did not allow for many changes to the extraction or instrumentation to improve matrix suppression. Including deuterated internal standards wherever possible compensated for the variability in sample

extraction and LC-MS/MS analysis due to its nearly identical properties to the unlabeled standard. In addition, the bias criteria was expanded to 30% for tissue samples, enabling cannabinoid quantification in tissues.

Limited data are available for cannabinoids concentrations in postmortem fluids and tissues. In one death investigation case, Kudo et al. examined blood, urine, and tissue samples for THC and THCCOOH by GC-MS (80). THC was positive at low concentrations in blood and liver with the highest concentration noted in adipose tissue; THC was not detected in the urine. The lack of THC in urine is expected since THC is primarily excreted as a glucuronide conjugate. LC-MS/MS permits the simultaneous determination of more polar cannabinoids, glucuronide conjugates, in postmortem urine. The pilot case evaluated with the validated method confirms the presence of THC-g and absence of the parent compound in urine. Furthermore, Kudo et al. was not successful in identifying THCCOOH in any tissue samples tested due to interfering peaks. SPE and LC-MS/MS utilized by the current method improves sample preparation and eliminates interfering peaks.

Gronewold and Skopp were the first to include THCCOOH-g, CBD, and CBN with THC, 11-OH-THC, and THCCOOH for a preliminary investigation into distribution of cannabinoids in man (85). In five cases, kidney, liver, and bile had high THCCOOH and THCCOOH-g concentrations, consistent with observations from the pilot samples analyzed with this validated method. CBD and CBN were prevalent in Gronewold and Skopp cases. While the pilot case presented is negative for CBN, other cannabinoids tested, CBD and CBG, were positive proving that the validated method is capable of detecting minor cannabinoids in postmortem forensic casework.

When authentic pilot samples were analyzed, it was apparent that the retention time of THCCOOH-g (RT=2.46 min) was slightly different from the commercially available reference standard (RT=2.34 min) observed in method development and validation. Hubbard et al. also noted a retention time shift for THCCOOH-g between human blood samples and calibrators (138). A recent research study showed that THCCOOH-g undergoes acyl glucuronide migration with eight different isomers identified, confirming that commercially prepared reference substances are a different isomer than what is present in human biological specimens (139). Enzymatic hydrolysis was performed on the authentic sample, resulting in the formation of the expected THCCOOH peak, thus corroborating that the peak detected is an isomer of THCCOOH-g. The quantitative values for THCCOOH-g in pilots was determined with the calibration curve prepared from the commercially available standard.

5. Conclusion

A robust and sensitive method for the simultaneous quantification of a broad array of cannabinoids, phase I metabolites, and phase II glucuronide metabolites in postmortem samples was developed utilizing SPE and LC-MS/MS. This is the first quantitative analytical method for THC-g, 8β-diOH-THC, 8β-OH-THC, CBG, THCV, and THCVCOOH in postmortem fluids and tissues. This method adds significantly to the body of knowledge available and enables collection of additional postmortem cannabinoid data to improve interpretation of postmortem cannabinoid results. Further application of the method to authentic forensic casework will describe postmortem cannabinoid distribution in more detail.

CHAPTER III

CANNABINOID DISTRIBUTION IN FATALLY-INJURED PILOTS' POSTMORTEM FLUIDS AND TISSUES

Abstract

The primary psychoactive component of cannabis, Δ^9-tetrahydrocannabinol (THC) can impair cognitive function and psychomotor performance, particularly for complex tasks like piloting an aircraft. The Federal Aviation Administration's (FAA) Forensic Sciences Section at the Civil Aerospace Medical Institute (Oklahoma City, OK) performs toxicological analyses on pilots fatally injured in general aviation crashes, permitting the measurement of cannabinoids in a broad array of postmortem biological specimens. Cannabinoid concentrations in postmortem fluids and tissues from 10 pilots involved in airplane plane crashes are presented. Mean±SEM THC blood concentration was 4.3±1.5 ng/mL. Phase I metabolites, 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (THCCOOH) and phase II glucuronide metabolites, THCCOOH-glucuronide (THCCOOH-g), had mean±SEM blood concentrations of 1.3±0.2, 17.9±6.5vand 47.3± 13.3ng/mL, respectively. Urine analyses revealed positive results for THCCOOH, THC-glucuronide, THCCOOH-g and 11-nor-9-carboxy-Δ^9-tetrahydrocannabivarin (THCVCOOH). THC was readily distributed to lung, brain, kidney, spleen and heart.

The psychoactive metabolite, 11-OH-THC, was identified in liver and brain with mean±SEM concentrations 7.1±1.6 and 3.1±0.7 ng/g, respectively. Substantial THCCOOH and THCCOOH-g concentrations were observed in liver, lung, brain, kidney, spleen and heart. These data improve our understanding of postmortem cannabinoids distribution to support toxicology interpretation of cannabinoid postmortem concentrations in forensic investigations.

Introduction

The National Survey on Drug Use and Health reported that 31.6 million Americans aged 12 and over are current cannabis (*Cannabis sativa*) users, with 3.5 million Americans initiating cannabis use in 2019 (3). Cannabis has wide-ranging central nervous system and physiological actions, and legalization in the United States has led to adverse public health and safety effects, including increased emergency department visits and impaired driving cases related to cannabis use (19).

Δ^9-tetrahydrocannabinol (THC), the primary psychoactive component of cannabis, is responsible for the psychological effects noted with cannabis use. Cannabis impairs cognitive functions and psychomotor performance which impact both driving or flying ability (60). Driving simulators and on-the-road driving tests show that smoking cannabis leads to impairment in reaction times, divided-attention tasks and critical-tracking tests (66). Flying simulator studies demonstrate that cannabis caused gross impairment of flying skills and performance (68, 140, 141). Major flying mishaps, including navigational errors, major altitude deviations, stalling and loss of control events and minor heading errors increased after cannabis use.

Many postmortem toxicology laboratories do not routinely perform cannabinoid testing, with the notable exceptions of laboratories that support causation determination in motor vehicle or plane crashes. The most likely explanation for the scarcity of postmortem testing for cannabinoids is due to the long-standing belief that cannabis does not directly cause death. This led to a lack of data regarding postmortem cannabinoids distribution. Most research on postmortem samples is performed on fluids and typically limited to THC and the inactive metabolite, 11-nor-9-carboxy-THC (THCCOOH) (90, 135, 142, 143). A recent report expanded on postmortem analysis to include THC, 11-hydroxy-THC (11-OH-THC), THCCOOH, cannabidiol (CBD) and cannabinol (CBN) blood concentrations from two postmortem groups - fatal road traffic collision (RTC) victims and non-traffic related coroners' cases (91). They found 29 and 21 cases positive for cannabinoids in the non-RTC and RTC group, respectively, which was higher than positive alcohol (>80 mg/dL) cases for both groups.

Forensic toxicologists are often called upon to provide opinions regarding relationships between cannabinoid concentrations in biological specimens and cannabis effects. While blood is the specimen of choice for correlating concentrations to effects, this specimen may not be available for analysis in all postmortem cases due to trauma or decomposition. Therefore, it is vital that forensic toxicologists understand cannabinoid distribution in postmortem tissues to improve interpretation of cannabinoid concentrations in alternative matrices. However, limited research is available detailing cannabinoid concentrations in postmortem biological specimens. Meneses and Hernandez compared brain cannabinoid concentrations to blood cannabinoid concentrations after death and observed higher 11-OH-THC concentrations in brain than blood (122). An

animal model for THC distribution revealed fast elimination in the liver, while the slowest elimination was observed in adipose tissue (49).

The Federal Aviation Administration's (FAA) Forensic Sciences Section at the Civil Aerospace Medical Institute (Oklahoma City, OK) performs toxicological analyses on pilots fatally-injured in general aviation crashes. This provides a unique opportunity to study cannabinoid concentrations in a broad array of postmortem fluids and tissues. Kemp et al. characterized THC and THCCOOH distribution in blood, urine and tissues from 55 pilots. They found high THC concentrations in lung and considerable THCCOOH concentrations in urine, liver and kidney (93). More recently, Saenz et al. performed a comprehensive distribution study for THC, 11-OH-THC and THCCOOH in 11 fatally-injured pilots. They noted that spleen and muscle were viable tissues for THC analysis, whereas brain was a great specimen for detection of the psychoactive metabolite, 11-OH-THC (86).

The research described here aims to characterize cannabinoids, their phase I metabolites, and phase II glucuronide conjugates in postmortem samples by quantifying THC, 11-OH-THC, THCCOOH, THC-glucuronide (THC-g), THCCOOH-glucuronide (THCCOOH-g), 8β-di-hydroxy-THC (8β-diOH-THC), 8β-hydroxy-THC (8β-OH-THC), CBD, CBN, cannabigerol (CBG), Δ^9-tetrahydrocannabivarin (THCV) and 11-nor-9-carboxy-THCV (THCVCOOH) in fluids and tissues from 10 anonymized pilots fatally-injured in plane crashes. Cannabinoid concentrations were identified and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). To our knowledge, this is the first comprehensive evaluation of THC-g, 8β-diOH-THC, 8β-OH-THC, CBG, THCV and THCVCOOH in postmortem samples.

Materials and Methods

Pilot specimen collection

Biological specimens from pilots fatally-injured in plane crashes are submitted to the FAA's Forensic Sciences Section in a specialized collection kit via commercial carrier. Blood samples are collected in glass, gray top Vacutainer® tubes (sodium fluoride and potassium oxalate) or glass, green top Vacutainer® tubes (sodium heparin). Tissues are submitted individually in zip lock bags. All fluids and tissues are stored frozen (-20°C) until analysis. The toxicology database was searched for positive cannabinoid cases from 2019. Ten positive cannabinoid cases were selected to undergo cannabinoid analysis of blood, vitreous humor, urine, bile, liver, lung, kidney, spleen, muscle, brain and heart.

Cannabinoid analysis

Cannabinoids in human fluids and tissues were quantified by a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method (144). Briefly, protein precipitation was performed by adding ice-cold acetonitrile to 0.5 mL blood or 0.5 g tissue homogenate (1:4 *w:w)*. Urine, vitreous humor and bile (0.5 mL) were not subjected to protein precipitation. All samples were pre-treated with 0.2% ammonium hydroxide and glacial acetic acid prior to solid-phase extraction (Agilent Plexa PCX, 30mg/3mL). Eluents were evaporated to dryness with nitrogen, then reconstituted in mobile phase and injected onto an LCMS 8040 (Shimadzu Corporation). Linear ranges were 0.25–50 ng/mL for THC-g; 0.5–100 ng/mL for CBN and THCV; 0.5–250 ng/mL for THC, 11-OH-THC, THCCOOH, CBD and CBG; 1–100 ng/mL for 8β-diOH-THC, THCVCOOH

and 8β-OH-THC; and 1–250 ng/mL for THCCOOH-g. Interassay imprecisions were ≤18.1% CV and bias was between -15.1% and 13.6% of target concentrations.

Results

Concentrations of 12 cannabinoids were determined in postmortem fluids and tissues from 10 pilots received by the FAA's Forensic Sciences Section (Table 6). All cannabinoids, except 8β-diOH-THC, were identified in at least one biological matrix from the 10 pilots. Table 7 summarizes cannabinoid concentrations in blood, vitreous humor, urine, bile, liver, lung, kidney, spleen, muscle, brain and heart. Substantial variability was noted for the different cannabinoids' concentrations in different biological matrices and between the pilots' specimens.

THC, THCCOOH and THCCOOH-g were the most prevalent cannabinoids detected in the tested biological matrices. Figures 5-7 display the relationship between the pilots' blood cannabinoid concentrations and their tissue concentrations. In the figures, each biological matrix has a different symbol and each pilot has a different color. Regression analyses were performed for blood and biological matrix concentrations. The correlation observed between THC blood and any tissue concentrations (Figure 5) was not statistically significant ($p>0.05$). Linear regression analysis between THC blood and tissues revealed R2 values: lung, 0.07; brain, 0.22; kidney, 0.38; spleen, 0.01; heart, 0.04. There was not enough liver and muscle samples positive for THC to perform regression analysis. There is a statistically significant relationship between THCCOOH blood concentrations and THCCOOH concentrations in liver ($p<0.001$), brain ($p<0.001$), kidney ($p<0.001$), spleen ($p<0.001$) and heart ($p=0.008$) (Figure 6). The greatest

Table 6. Cannabinoid concentrations in fluids (ng/mL) and tissues (ng/g) in 10 pilots fatally-injured in plane crashes.

	Specimen	THC	11-OH-THC	THC-COOH	THC-g	THC-COOH-g	8B-OH-THC	THCV	THCV-COOH	CBD	CBG	CBN
Case 1	Blood	1.0		5.9		21.7						
	Urine			67.5	1.8	56.9			4.1			
	Bile	8.4		367	2.3	POS			3.3	7.6	16.1	51.2
	Liver		4.4	90.5		140						
	Lung	55.8		8.5		7.1						
	Kidney	2.8		111		78.6						
	Spleen	3.7		9.7		10.7						
	Muscle			4.2								
	Brain	2.4	2.0	3.2								
	Heart											
Case 2	Blood			2.2		22.9						
	Vitreous Humor											
	Urine			12.3	0.3	72.1			1.0			
	Bile	1.0	2.6	93		POS						
	Liver			22.0		106						
	Lung					10.9						
	Kidney			45.1		46.8						
	Spleen					5.5						
	Muscle	2.4				2.6						
	Brain											
	Heart					9.5						

	Specimen	THC	11-OH-THC	THC-COOH	THC-g	THC-COOH-g	8B-OH-THC	THCV	THCV-COOH	CBD	CBG	CBN
Case 3	Blood	1.1		6.1		7.1						
	Vitreous Humor											
	Urine			3	0.4	9.4						
	Liver	6.0										
	Lung			47.8		36.2						
	Kidney			4		5.0						
	Spleen			6.0		7.7						
	Muscle											
	Brain	6.8		7.5								
	Heart											
Case 4	Blood	7.7	1.8	72.6		160.0			3.3			
	Vitreous Humor			1.9		2.9						
	Bile	118.0	0.9	32.3		POS		1.2	1.9		1.5	
	Liver			458	1.5	150.0						
	Lung	21.6		44.7		143						
	Kidney	11.9		926.0	4.2	600.0						
	Spleen			65.1		86.8						
	Muscle											
	Brain	23.3	6.0	23.7		12.1						
	Heart	3.2		27.5		59.0					2.9	

	Specimen	THC	11-OH-THC	THC-COOH	THC-g	THC-COOH-g	8B-OH-THC	THCV	THCV-COOH	CBD	CBG	CBN
	Blood	2.1		13.6		39.9						
	Vitreous Humor			0.6								
	Urine		0.5	208	3.4	178						
	Bile	4.0	36.7	414	1.5	POS					7.8	
	Liver			39.8		177						
Case 5	Lung	11.8		16.7		18.4						
	Kidney			101		139						
	Spleen	3.3		13.2		14.3						
	Muscle											
	Brain	4.1	2.1	7.1		3.1						
	Heart	10.8		6.7		3.8						
	Blood	1.0		4.0		15.6						1.1
	Vitreous Humor											
	Urine			1.5	0.3	26.0						
	Bile	1.0	2.7	133	1.2	POS						
	Liver					36.9						
Case 6	Lung			2.0		7.7						
	Kidney			34		37.0						
	Spleen			2.1		5.0						
	Muscle											
	Brain											
	Heart	10.5		3.8		4.3						

	Specimen	THC	11-OH-THC	THC-COOH	THC-g	THC-COOH-g	8B-OH-THC	THCV	THCV-COOH	CBD	CBG	CBN
Case 7	Blood	6.5	1.5	18.7		67.3						
	Vitreous Humor					0.5						
	Bile	8.5	35.3	154	2.0	POS	15.6			1.1	1.7	
	Liver		9.8	208	5.2	407						
	Lung	3.5		15.0		36.8						
	Kidney	6.2		108		196						
	Spleen	5.4		11.2		12.4						
	Muscle	14.8										
	Brain	2.9		5.5								
	Heart	76.4		13.8		12.4						
Case 8	Heart Blood	1.1		5.5		37.4						
	Femoral Blood	1.6		5.1		29.4						
	Vitreous Humor											
	Urine			6.4	0.6	138						
	Bile			108	0.5	POS					1.4	
	Liver			23		95.3						
	Lung			3.5		12.9						
	Kidney			40		92.9						
	Spleen	2.3		3.5		5.0						
	Muscle										7.5	
	Brain											
	Heart	21.2		2.9		5.2					3.2	

	Specimen	THC	11-OH-THC	THC-COOH	THC-g	THC-COOH-g	8B-OH-THC	THCV	THCV-COOH	CBD	CBG	CBN
	Blood		1.2	35.1		65.1						
	Vitreous Humor											
	Urine			60.8	9.9	359			2.3			
	Bile	11.5	83.6	340	2.8	POS			2.4	11.5	8.6	
	Liver	4.1	10.5	236		474						
Case 9	Lung	19.9	2.1	25.2		34.0						
	Kidney	5.0		182								
	Spleen	24.3	1.9	18.6		22.0						
	Muscle	32.2	2.8	18.5		10.6						
	Brain	1.0	2.4	5.4								
	Heart	7.3	2.9	36.0		20.0						
	Blood	13.7	0.5	15.2		35.7						
	Vitreous Humor			0.6		1.0						
	Urine			81.7	7.6	443			2.6			
	Bile	15.4	30.6	198		POS			24.1	2.6	3.1	24.1
	Liver		3.5	170		438.0			1.5			
Case 10	Lung	29.8		26.0		36.4	3.2					
	Kidney	5.1		182		151.0			1.1			
	Spleen	6.3	1.8	20.7		25.4						
	Muscle											
	Brain	4.9	2.9	10.0								
	Heart	4.5		21.1		27.6						

THC: Δ^9-tetrahydrocannabinol; 11-OH: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; THC-g: THC glucuronide; THCCOOH-g: THCCOOH glucuronide; 8β-OH: 8β-hydroxy-THC; THCV: Δ^9-tetrahydrocannabivarin; THCVCOOH: 11-nor-9-carboxy-THCV; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; NA: no specimens available; POS: positive

Table 7. Statistical summary of cannabinoid concentrations in postmortem fluids (ng/mL) and tissues (ng/g) from 10 fatally-injured pilots in plane crashes.

Specimen		THC	11-OH	THC-COOH	THC-g	THC-COOH-g	8β-OH	THCV	THCV-COOH	CBD	CBG	CBN
Blood												
	Total	8	4	10		10			1			1
	Mean	4.3	1.3	17.9		47.3						
	SEM	1.5	0.2	6.5		13.3						
	Median	1.6	1.4	9.9		36.6						
	Range	1.0-13.7	0.5-1.8	2.2-72.6		7.1-160			3.3			1.1
Vitreous Humor												
	Total			3		3						
	Mean			1		1.5						
	SEM			0.4		0.6						
	Median			0.6		1						
	Range			0.6-1.9		0.5-2.9						
Urine												
	Total		1	8	8	8			4			
	Mean			55.2	3	160			2.5			
	SEM			23.1	1.2	53			0.6			
	Median			36.6	1.2	105			2.5			
	Range		0.5	1.5-208	0.3-9.9	9.4-443			1.0-4.1			
Bile												
	Total	8	7	9	6	10	1	1	4	4	7	2
	Mean	21	27.5	204	1.7				7.9	5.7	5.7	
	SEM	13.1	10.3	42.7	0.3				4.7	2.1	1.9	
	Median	8.5	30.6	154	1.8				2.9	5.1	3.1	
	Range	1.0-118	0.9-83.6	32.3-414	0.5-2.8	POS	15.6	1.2	1.9-24.1	1.1-11.5	1.4-16.1	24.1-51.2

Specimen		THC	11-OH	THC-COOH	THC-g	THC-COOH-g	8β-OH	THCV	THCV-COOH	CBD	CBG	CBN
Lung												
	Total	6	1	9		10	1					
	Mean	23.7		21		34.3						
	SEM	6.7		5.2		12.1						
	Median	20.8		16.7		26.2						
	Range	3.5-55.8	2.1	2.0-47.8		7.1-143	3.2					
Liver												
	Total	2	4	8	2	9						
	Mean		7.1	156		225						
	SEM		1.6	49.1		52.4						
	Median		7.1	130		150						
	Range	4.1-6.0	3.5-10.5	22.0-458	1.5 - 5.2	36.9-474						
Brain												
	Total	7	5	7		2						
	Mean	6.5	3.1	8.9								
	SEM	2.7	0.7	2.4								
	Median	4.1	2.4	7.1								
	Range	1.0-23.3	2.0-6.0	3.2-23.7		3.1-12.1						
Kidney												
	Total	5		10		9			1			
	Mean	6.2		173		150						
	SEM	1.4		81.4		56.4						
	Median	5.1		105		92.9						
	Range	2.8-11.9		4.2-926		5.0-600			1.1			

Specimen		THC	11-OH	THC-COOH	THC-g	THC-COOH-g	8β-OH	THCV	THCV-COOH	CBD	CBG	CBN
Spleen												
	Total	6	2	9		10						
	Mean	7.6		16.7		19.5						
	SEM	3.1		6		7.4						
	Median	4.6		11.2		11.6						
	Range	2.3-24.3	1.8-1.9	2.1-65.1		5.0-86.8						
Heart												
	Total	7	1	7		8					2	
	Mean	19.1		16		17.7						
	SEM	9.1		4.4		6.2						
	Median	10.5		13.8		11						
	Range	3.2-76.4	2.9	2.9-36.0		3.8-59.0					2.9-3.2	
Muscle												
	Total	3	1	2		2					1	
	Mean	16.5										
	SEM	7.1										
	Median	14.8										
	Range	2.4-32.2	2.8	4.2-18.5		2.6-10.6					7.5	

THC: Δ^9-tetrahydrocannabinol; 11-OH: 11-hydroxy-THC; THCCOOH: 11-nor-9-carboxy-THC; THC-g: THC glucuronide; THCCOOH-g: THCCOOH glucuronide; 8β-OH: 8β-hydroxy-THC; THCV: Δ^9-tetrahydrocannabivarin; THCVCOOH: 11-nor-9-carboxy-THCV; CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; POS: positive

correlation was noted between blood concentrations and spleen (r=0.96), kidney (r=0.95) and liver (r=0.95) for THCCOOH. THCCOOH-g also exhibited statistically significant relationship between blood and lung ($p<0.001$), brain ($p=0.002$), kidney ($p<0.001$), spleen ($p<0.001$) and heart ($p<0.001$) concentrations (Figure 7). The greatest correlation was observed between blood concentrations and lung (r=0.91), kidney (r=0.91) and spleen (r=0.93) for THCCOOH-g.

Discussion

The major strength of the current study was that cannabinoid distribution was evaluated in a broad array of postmortem biological specimens. Distribution was performed in heart, kidney, spleen and muscle, tissues that are not typically included in postmortem toxicology analyses. Furthermore, methodology was expanded to include THC-g, 8β-diOH-THC, 8β-OH-THC, CBG, THCV and THCVCOOH, providing much needed data for postmortem forensic interpretation. Limitations with this study include a small sample size, due to the number of cannabinoid positive pilots received in 2019. Additionally, the FAA laboratory lacks information about plane crashes including a pilot's prior cannabis use, postmortem interval and blood site collection, which can hinder interpretive value in certain situations. Finally, the traumatic nature and post-crash fires associated with many plane crashes may impact postmortem drug results.

Overall, blood THC concentrations were appreciably higher than blood 11-OH-THC concentrations but lower than blood THCCOOH and THCCOOH-g concentrations. This finding was consistent with studies that showed blood 11-OH-THC concentrations are approximately 10% of blood THC concentrations at Cmax after smoking, and blood

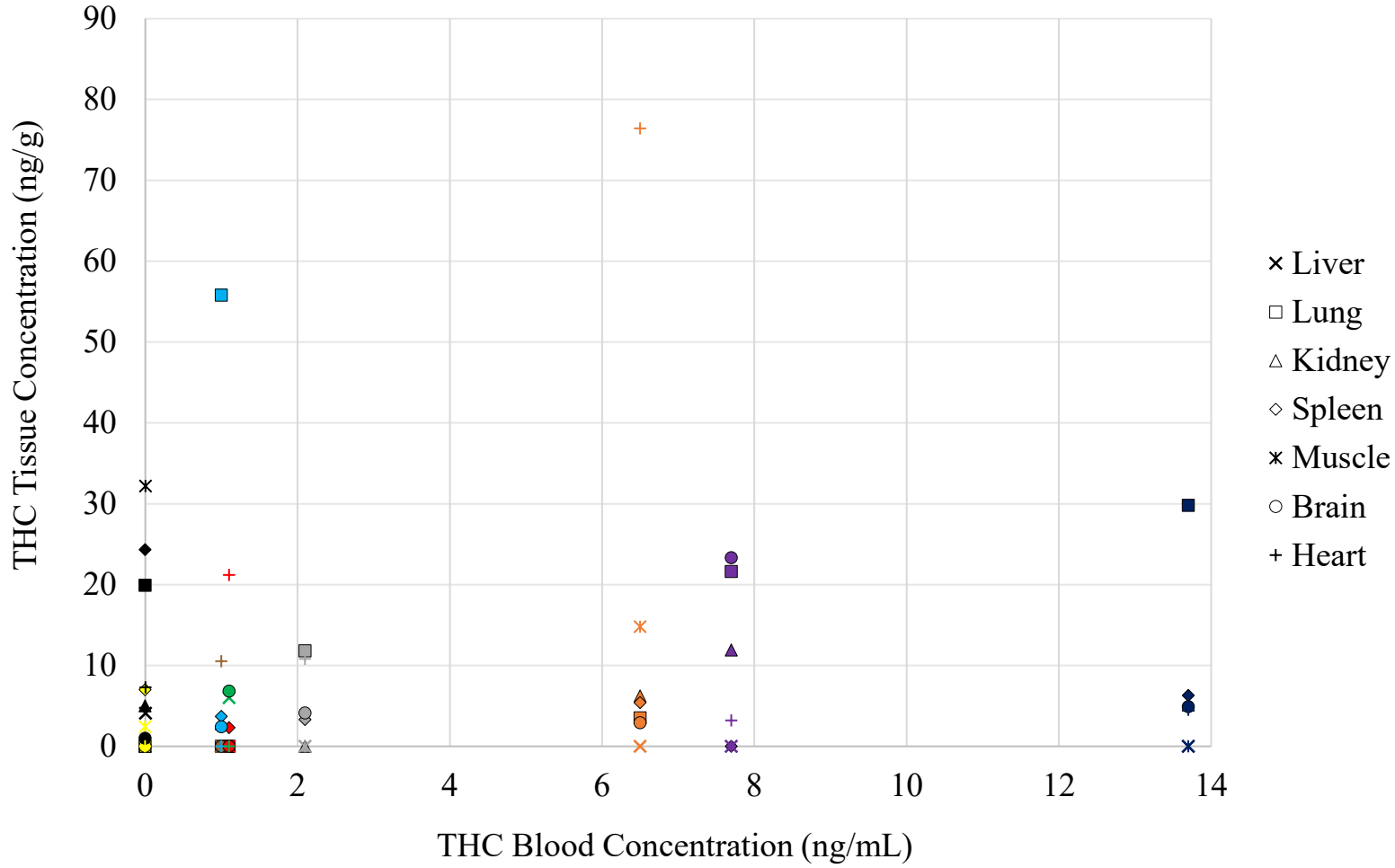

Figure 5. Δ^9-tetrahydrocannabinol (THC) concentrations in blood (ng/mL) and tissues (ng/g) for 10 pilots fatally-injured in plane crashes. No statistically significant ($p>0.05$) relationship exists between THC blood and tissue concentrations.

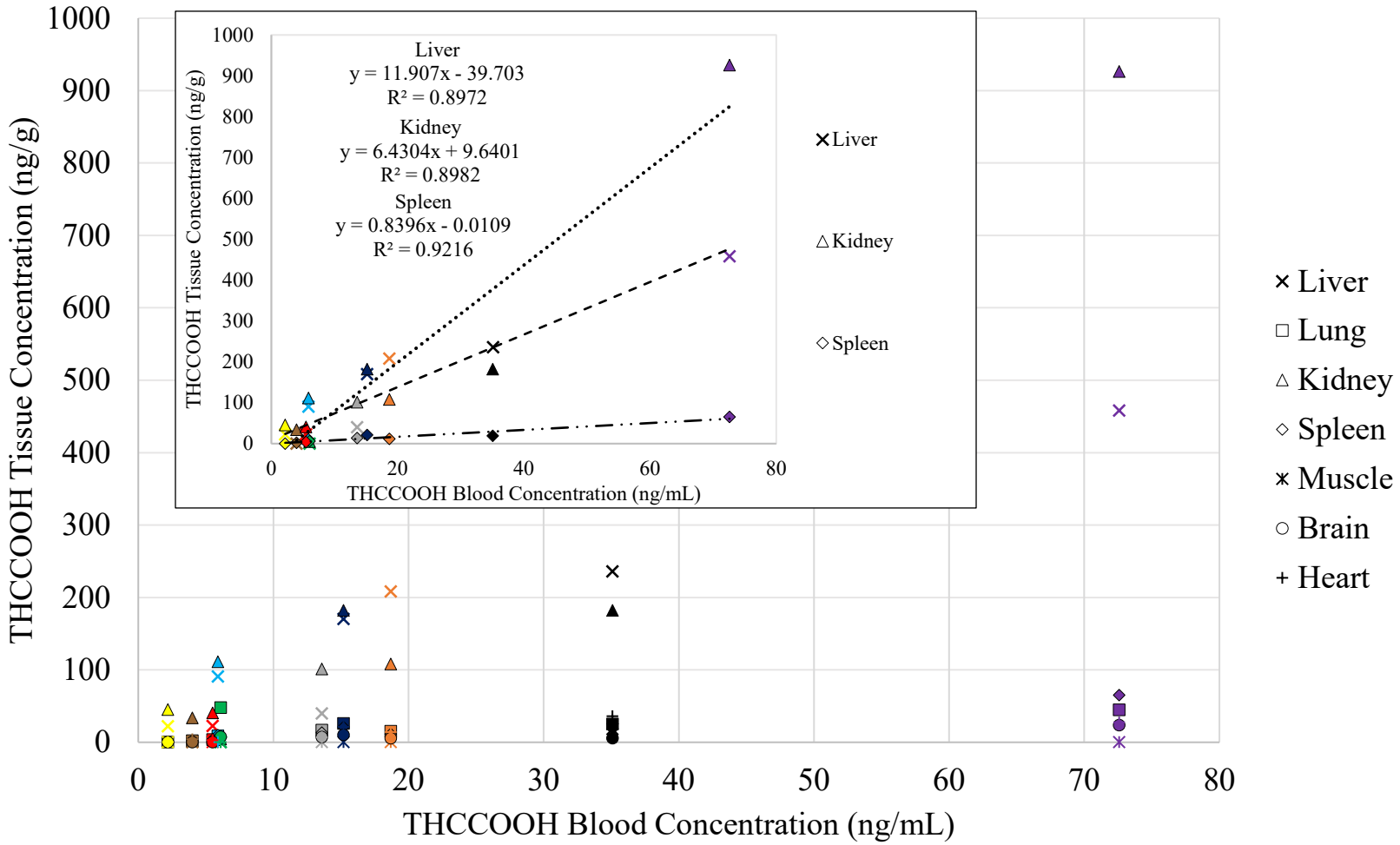

Figure 6. 11-nor-9-carboxy-THC (THCCOOH) concentrations in blood (ng/mL) and tissues (ng/g) for 10 pilots fatally-injured in plane crashes. A statistically significant relationship ($p<0.05$) exists between blood and liver, kidney, spleen and brain THCCOOH concentrations.

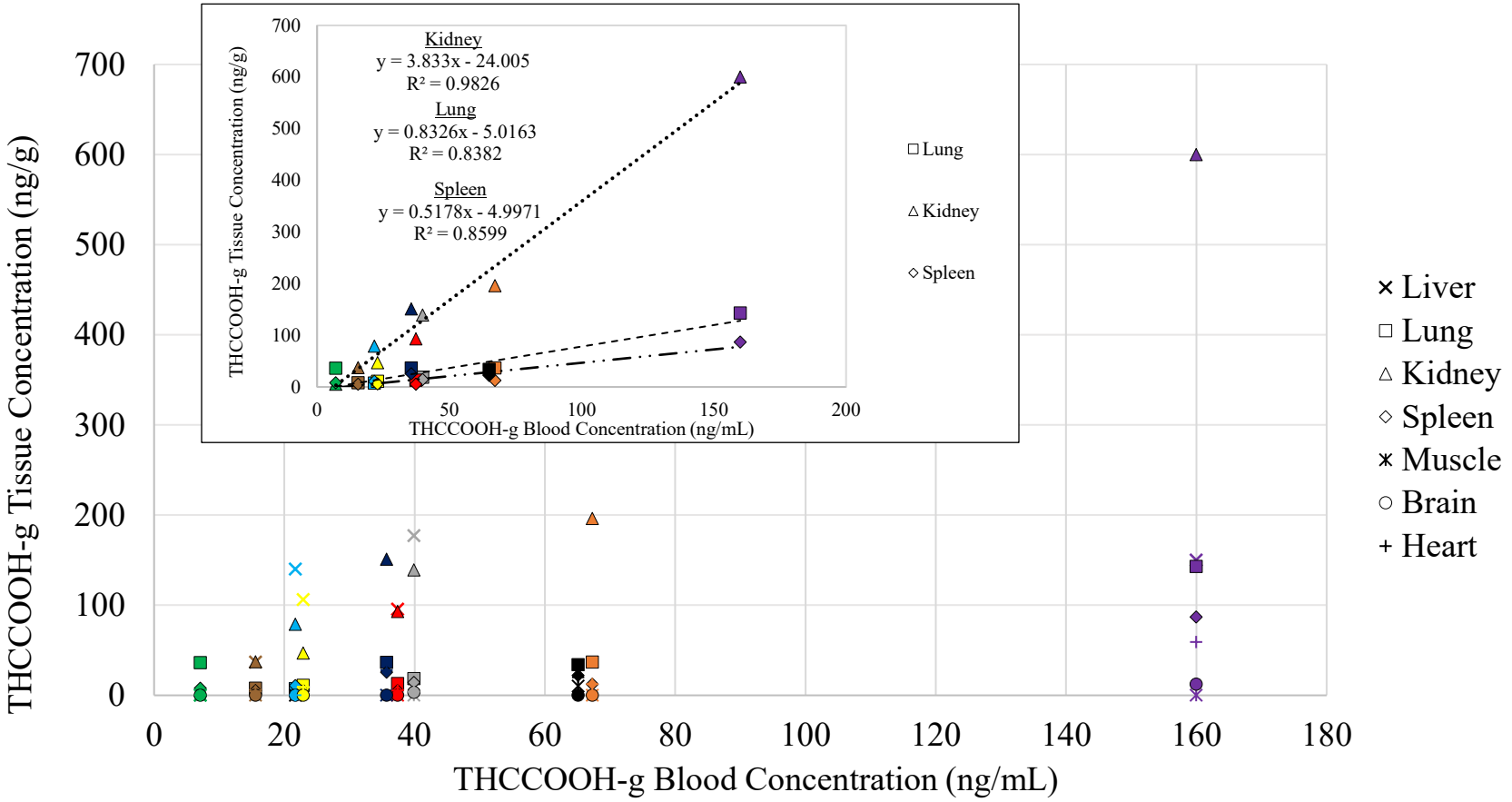

Figure 7. 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol glucuronide (THCCOOH-g) concentrations in blood (ng/mL) and tissues (ng/g) for 10 pilots fatally-injured in plane crashes. A statistically significant relationship ($p<0.05$) exists between blood and lung, kidney, spleen, brain and heart

THCCOOH and THCCOOH-g concentrations are near equivalent to blood THC concentrations about 30 min after inhalation (18). Unsurprisingly, high THC concentrations in blood were associated with high 11-OH-THC, THCCOOH and THCCOOH-g concentrations in blood, as well as high THC concentrations in kidney, brain and bile.

Blood is normally the specimen of choice in forensic toxicology, especially for crash investigations, to correlate blood concentrations of psychoactive compounds with psychomotor effects. After inhalation, THC is rapidly absorbed in the blood from the lungs with peak blood concentrations observed in humans prior to the last cigarette puff (145). Although controlled cannabis administration studies provide information about the drug's pharmacokinetics and pharmacodynamics, including potential markers of exposure, interpretation of postmortem THC blood concentrations may be challenging due to blood collection site variability and postmortem redistribution (PMR). During an autopsy, a sample may be collected from the pool of blood within the thoracic cavity instead of direct collection from the heart. Blood cavity samples are considered non-homogenous due to possible contamination from multiple sources, including liver, lungs and bladder; therefore, interpretive value of cavity blood is low (136). Even with a blood sample collected directly from the intact heart, caution in interpretation is necessary due to the well-known phenomenon of PMR, the diffusion of drugs from tissues with higher concentrations into the surrounding blood (99). Although the FAA requests collection site listed on blood specimen tubes, not all pathologists provide this information. Only one pilot blood sample within the study was identified as "heart blood"; all other samples were simply noted as "blood" which may explain the variability noted in THC blood

concentrations. In the current study, THC blood concentrations ranged from 1.0-13.7 ng/mL with a median concentration of 1.6 ng/mL. Other postmortem studies showed more variability in blood concentrations. Concentrations from 18 fatal road collision cases ranged from 0.7 to 69.5 ng/mL with a median concentration of 4.2 ng/mL (91). In another, researchers determined THC concentrations in 18 blood samples with preservative ranging from 2.0 – 74.0 ng/mL with a median concentration of 11 ng/mL and THC concentrations in 21 blood samples without preservative ranging from 3.0 to 61.0 ng/mL with a median concentration of 13 ng/mL.

Urine analysis is commonly employed in forensic toxicology to identify past cannabis exposure as the sample contains high cannabinoid concentrations and long windows of detection for many cannabinoid metabolites (146). Previous methods utilized hydrolysis prior to mass spectrometry to quantify total cannabinoids in urine; however, with the availability of glucuronide reference standards, analysis can be performed without hydrolysis to directly measure phase two glucuronide metabolites (56, 147). The current method includes the detection of two glucuronide conjugates, THC-g and THCCOOH-g, but does not include the 11-OH-THC-glucuronide, as there is no commercially available reference standard for method development and validation. Direct determination of glucuronides negates any issues observed with glucuronide stability or poor hydrolysis efficiency.

No parent THC was detected in any pilot's urine sample and only one urine sample was positive for 11-OH-THC. The lack of positive THC and 11-OH-THC in urine is expected since THC and 11-OH-THC are primarily excreted as glucuronide conjugates in urine. The 11-OH-THC findings are inconsistent with those by Saenz et al. that

showed all available postmortem urine samples positive for 11-OH-THC. An explanation for this is that the Saenz study employed hydrolysis measuring total 11-OH-THC concentration in urine, whereas the current analysis measures only free 11-OH-THC (86). All urine samples were positive for THCCOOH and THCCOOH-g with higher concentrations observed for the latter confirming reports that THCCOOH-g is the predominant cannabinoid in urine after cannabis administration (146, 148).

One research group assessed urinary THC-g disposition from frequent and occasional cannabis users after smoking cannabis. In some frequent smokers, THC-g was detected in urine samples at the last time collected (30 h), negating the value of THC-g as a marker of recent usage in frequent users. Some occasional smokers had negative THC-g interspersed with positive THC-g in urine samples collected over 20 h. The researchers concluded that if there is strong indication of occasional cannabis consumption, then THC-g can be used as an inclusionary marker of recent use (146). In the current study, THC-g was detected in seven of eight pilot urine samples. Due to the lack of information about the deceased pilot's prior cannabis use, it is not possible to estimate time of last use based on urinary THC-g disposition. Research in urine samples identified THCVCOOH as a marker for ingestion of a cannabis-related product containing THCV (32). Four of the eight pilot urine samples tested positive for THCVCOOH suggesting that these pilots consumed a cannabis-containing product prior to flying.

Vitreous humor is an alternative matrix for the determination of drugs in medicolegal death investigations. Drugs and metabolites undergo passive diffusion across the blood-vitreous barrier to enter the aqueous environment. Vitreous humor is composed of 98-99% water and has a much lower protein content than whole blood, thus

concentrations for highly protein-bound drugs tend to be much lower in vitreous fluid (149). No vitreous humor samples from the deceased pilots in this study were positive for THC. THCCOOH and THCCOOH-g were positive at low concentrations in three and two cases, respectively. This agrees with several other reports indicating that cannabinoids do not readily transfer into aqueous matrices (142, 150, 151). Although vitreous humor may be a good alternative matrix for detection of some drugs of abuse, it is not ideal for determining cannabis exposure.

Liver is the primary organ responsible for metabolism, hence cannabinoid metabolites are expected to be present in substantial concentrations as compared to that of the parent compound. THC was detected in only two cases at low concentrations (4.1 and 6.0 ng/g) whereas 11-OH-THC was detected in four (3.5 – 10.5 ng/g). Liver had the highest 11-OH-THC concentration determined in this study, which is consistent with results from a tissue distribution study of THC in the pig (49). The psychoactive metabolite, 11-OH-THC, is further oxidized to form the inactive metabolite, THCCOOH. High concentrations of THCCOOH in liver were observed in this study. Phase II metabolism produces glucuronide conjugates. Nine pilots had considerably high THCCOOH-g concentrations in their livers. One study regarding the distribution of cannabinoids in man found a similar trend with liver exhibiting high THCCOOH-g concentrations (85).

To the authors' knowledge, this study is the first comprehensive assessment of THC-g distribution in postmortem samples. Phase II metabolism, which occurs in the liver, produces the polar, water-soluble glucuronide conjugate improving renal and biliary excretion of THC-g (152). In the deceased pilots, THC-g was detected in urine,

bile, liver and kidneys, all specimens involved in the body's drug elimination processes. The presence of THC-g in kidney and liver was associated with the cases with the highest blood THC concentrations.

Cannabinoid concentrations in lungs are typically higher compared to other tissues since inhalation is the most common route of administration. Mean THC concentrations were 18 times greater than mean THCCOOH concentrations in lungs in a distribution study from 55 pilots (93). These findings are contrary to the present study. Mean THCCOOH concentrations in both studies were similar, however, THC concentrations in the current study were much lower than those found in Kemp et al (93). Information regarding cannabis ingestion or time of use for pilots in aviation crashes is rarely, if ever available; therefore, it is possible that the Kemp study included more pilots with recent cannabis use prior to the crash resulting in higher THC lung concentrations. Although lung concentrations were lower in the current study than in other published reports, analysis in lung is useful for cannabis exposure as smoking is the most common route of administration and THC accumulates in this tissue.

THC is highly lipophilic and rapidly crosses the blood-brain barrier, distributing the drug from the lungs to the brain after inhalation (47). THC and its active metabolite, 11-OH-THC, produce effects when they bind to cannabinoid receptors within the brain. Consequently, brain is an important matrix for cannabinoid analysis. THC and 11-OH-THC were detected in brain samples of 6 and 5 cases, respectively. Mura et al. analyzed 12 paired brain and blood samples for THC, 11-OH-THC and THCCOOH and found that THC brain concentrations were greater than the corresponding blood concentrations (95). This is slightly different from the current study findings in that not all THC brain

concentrations were greater than corresponding blood concentrations. One pilot was negative for THC in both blood and brain; five pilots exhibited higher brain THC concentrations than blood while four pilots revealed lower brain THC concentrations than blood. Varying data could be the result of differences with the pilot's time of last cannabis use, route of administration, or time between death and sample collection. A pharmacokinetic study for THC concentrations in blood and brain tissue following pulmonary, oral and subcutaneous administration in rats revealed brain concentrations several times higher than serum concentrations after oral administration (153). Pulmonary administration yielded maximum brain concentrations 15 minutes after dosing but concentrations were about three times lower than serum concentrations. A study considering PMR of THC in the pigs found that brain concentrations increased as the postmortem interval increased (108). Without information regarding prior cannabis use and the postmortem interval for these pilots, it is hard to identify a clear reasoning for the varying THC results observed in brain and blood samples.

When considering effects of THC on psychomotor performance, it is important to evaluate 11-OH-THC, an equipotent metabolite of THC, in the brain. Hlozek et al. found higher 11-OH-THC concentrations in brain than serum samples after pulmonary, oral and subcutaneous administration to rats and proposed that the high concentrations observed after oral administration added to the behavioral effects seen in their study (153). A recent study evaluating cannabinoid concentrations in blood and brain samples from medical examiner cases found 11-OH-THC concentrations two times higher than THC brain concentrations in three cases (122). One deceased pilot in the current study was negative for THC in blood and brain samples but was positive for 11-OH-THC in the

brain, suggesting that some psychomotor effects could be present despite a negative THC result.

While the Gronewold et al. study detected THC in all muscle samples and the Saenz et al. study detected THC in 10 of 11 muscle samples, the current study detected THC in only 30% of muscle samples (85, 86). In a study evaluating THC concentrations in the pig following a single intravenous dose, muscle concentrations decreased with increasing postmortem interval (PMI) and THC was no longer detected in muscle after 48 h (108). Aviation crashes may occur in remote locations or may have extensive wreckage to process leading to a significant amount of time between death and autopsy. PMI is unknown in these cases, but the low cannabinoid concentrations in muscle samples could be a result of a lengthy PMI.

Drug elimination is primarily carried out by the kidneys after biotransformation occurs in the liver. Kidneys filter polar drugs and metabolites to complete the elimination process; therefore, the kidneys can contain high concentrations of cannabinoid glucuronide metabolites and could be a valuable specimen for the detection of cannabinoid metabolites and glucuronide conjugates. In the 10 deceased pilots, the primary urinary metabolites, THCCOOH and THCCOOH-g, were present in all kidney samples at high concentrations. Only one other study is available that evaluated distribution of THCOOH-g in kidneys; researchers found results comparable to the current study with the large THCCOOH-g concentrations observed in kidneys (85). Analysis in kidneys proves beneficial for past cannabis exposure with high THCCOOH and THCCOOH-g concentrations found in this tissue.

Scarce information is available regarding cannabinoid concentrations in spleen tissue with only two papers reporting THC or THCCOOH in human spleen (80, 86). The spleen is a highly perfused tissue and acts as a filter for blood by recycling aging red blood cells, making it a suitable organ for the determination of highly lipophilic compounds like THC (154). THC, THCCOOH and THCCOOH-g showed positivity rates of 60%, 90% and 100%, respectively, in pilot spleen samples further confirming that spleen is a suitable specimen for cannabinoid analysis. For the current study, the two cases with the highest THC concentration in spleen were the only two cases in which 11-OH-THC was detected in spleen.

Analysis of heart tissue appears to be valuable for the detection of cannabinoids as THC, THCCOOH and THCCOOH-g were found in 7, 7 and 8 of 10 cases, respectively. In most of the cases that were positive for THC and THCCOOH, THC concentrations were higher than THCCOOH in heart. This may be the result of normal blood flow through the circulatory system. Deoxygenated blood flows through the heart into the lungs where oxygen and inhaled THC can move into the blood. The oxygenated and THC-rich blood is then returned to the heart via the pulmonary veins, exposing the left-sided heart tissue to high concentrations of THC. Another explanation for high THC concentrations in heart tissue could be due to proximity of the heart to the lungs allowing simple diffusion of compounds from the lung into the heart tissue.

Limited data are available regarding cannabinoid concentrations in bile or its significance in interpretation. Many drugs and metabolites, particularly those that are lipophilic with a large molecular weight, are eliminated through biliary excretion (155). All cannabinoids, except 8β-diOH-THC, were positive in at least one bile sample.

Analysis of samples from the ten deceased pilots revealed that concentrations of THC, 11-OH-THC, THCCOOH and THC-g were higher in bile than any of the other specimen types that were analyzed. THCCOOH-g was detected in all bile samples; however, matrix effects produced poor recovery of the deuterated internal standard, THCCOOH-g-d_3. This metabolite was therefore reported qualitatively in bile after meeting identification criteria. Other studies determining cannabinoid concentrations in bile showed similar results to the current study. One analyzed free and glucuronidated cannabinoids in bile samples from 10 cases found high concentrations of THCCOOH-g, moderate concentrations of THCCOOH and THC-g and lower concentrations of THC, 11-OH-THC, CBD and CBN (143). Gronewold and Skopp observed high concentrations of CBD and CBN in bile, as well as the highest 11-OH-THC concentration (85). The present study revealed that bile produced the most positive samples for minor cannabinoids - CBD, CBG, CBN, THCV and THCVCOOH. Testing bile in postmortem forensic casework would provide insight into prior cannabis use as this specimen revealed the presence of multiple cannabinoids and metabolites.

Several investigators attempted to identify compounds of interest, or "markers", to provide forensic toxicologists with patterns of cannabis use and exposure. Markers could give crash investigators substantial interpretive information that is generally not available in postmortem cases. Since THC was detected in blood for up to 30 days after abstinence, minor cannabinoids and metabolites were evaluated as markers of recent cannabis intake (156). Newmeyer et al. showed that CBG and CBN in blood are markers for recent cannabis use after inhalation (30). Although minor cannabinoids were included in the present study to potentially identify recent usage, it is unclear if postmortem

samples will yield insight into prior cannabis use. CBN was only detected in one blood sample and CBG was found in bile, heart and muscle of seven cases. The presence of CBN in a blood sample indicates recent inhaled cannabis by the pilot prior to flying, but further investigation regarding the tissue distribution of minor cannabinoids is needed before any interpretive value can be made regarding cannabis exposure or intake.

Many human pharmacokinetic studies were performed over the years to identify absorption patterns, metabolic profiles and elimination pathways after controlled cannabis administration. In this study, THC, THCCOOH, and THCCOOH-g tissue concentrations in each case were compared to blood concentrations to determine if distribution patterns could be identified (Figures 5-7). No relationship was observed with THC concentrations between blood and tissues, whereas THCCOOH and THCCOOH-g both showed significant relationship between blood and tissues. The lack of THC correlation is somewhat expected considering postmortem cases often lack information regarding route of administration, dose or potency, time of last use, and past user experience, all of which impact the absorption, distribution, metabolism and excretion of cannabinoids.

Conclusion

Cannabinoids and cannabinoid glucuronides were quantified in postmortem fluid and tissues from 10 fatally-injured pilots involved in aviation crashes. This is the first comprehensive distribution study for THC-g, CBG, THCV and THCVCOOH. The data add to the growing body of knowledge describing postmortem cannabinoids distribution.

Blood samples tested from 10 deceased pilots in this study showed individual variability for THC concentrations. THCCOOH and THCCOOH-g were prevalent in all postmortem fluids and tissues except for vitreous humor and muscle. Several tissues proved useful for detecting cannabis exposure in postmortem cases. Heart tissue revealed high THC concentrations in 7 of 10 cases suggesting it could be a viable specimen for postmortem cannabinoid analysis. High concentrations of THCCOOH and THCCOOH-g were found in liver and kidney, tissues responsible for drug biotransformation and elimination. The presence of psychoactive analytes, THC and 11-OH-THC, in brain confirmed that these cannabinoids are readily distributed to this lipophilic tissue and that brain should be analyzed in cases attempting to determine crash causation. In addition, the data presented proves that urine, bile, liver and kidney are good specimens for the detection of the THC glucuronide conjugate, THC-g. A metabolite of THCV, THCVCOOH, was identified in bile and urine samples indicating that some pilots ingested a cannabis product rather than Marinol® prior to flying. Further research is necessary before it is known whether postmortem cannabinoid analysis can aid in interpretation of recent cannabis use and exposure or concurrent psychomotor impairment.

CHAPTER IV

APPLICATION OF PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELING FOR Δ^9-TETRAHYDROCANNABINOL DISTRIBUTION IN RABBITS FOLLOWING CONTROLLED CANNABIS ADMINISTRATION

Abstract

Cannabis sativa is widely used for both medical and recreational purposes and Δ^9-tetrahydrocannabinol (THC) is the most frequently identified illicit drug in investigations of driving under the influence of drugs and plane crashes. Despite the prevalence of THC, its tissue distribution is incompletely characterized. The objective of this study was to construct a physiologically based pharmacokinetic (PBPK) model to describe blood and tissue THC concentrations in rabbits following the administration of smoked cannabis by inhalation. Predicted values were compared with results from rabbits exposed to cannabis smoke under controlled conditions. High THC concentrations were observed in lungs, moderate concentrations were seen in the brain, heart, and kidneys, and low concentrations were noted in the liver. Theoretical and experimental partition coefficients were similar and produced similar results. The results showed similar THC concentrations in blood and tissues between the predicted and experimental data. This PBPK model can be used to predict THC concentrations in multiple tissues of rabbits

after inhalation of cannabis smoke and might also be extended to other species of interest, including humans.

Introduction

For thousands of years, *Cannabis sativa* has been used for medical and recreational purposes (157). The cannabis plant is complex, containing 565 different chemical compounds of which 120 are classified as cannabinoids (158). Numerous conditions or diseases, such as anorexia, multiple sclerosis, Parkinson's disease, epilepsy, and pain are treated with cannabinoids (133, 159-161). An increase in therapeutic uses, as well as increasing legalization, creates a need to fully understand cannabinoid pharmacokinetics for interpreting drug concentrations in forensic casework.

Smoking, the primary route of cannabis administration, provides a rapid and efficient delivery of the main psychoactive component, Δ^9-Tetrahydrocannabinol (THC) (162). THC is highly lipophilic with a large volume of distribution (V_D = 10 L/kg) and is 97-99% protein bound in plasma; hence, it readily distributes to highly perfused tissues (163). Metabolism of THC occurs by the hepatic cytochrome P450 enzyme system producing the equipotent metabolite, 11-hydroxy-THC (11-OH-THC) (54). Further oxidation produces the inactive metabolite, 11-nor-9-carboxy-THC (THCCOOH). Glucuronide conjugates of THC, 11-OH-THC and THCCOOH are formed with Phase II metabolism and facilitate renal excretion. Approximately 20% of cannabis is excreted in the urine while more than 65% is eliminated in the feces (164).

Several pharmacokinetic studies were conducted in animals following intravenous THC administration (94, 116, 165, 166). While these studies provide valuable data regarding THC pharmacokinetics, most did not concentrate on describing THC kinetics

in tissues. One of the most extensive studies focusing on THC distribution in tissues was performed in Large White pigs (49). Thirty minutes after intravenous injection in the jugular vein, high THC concentrations were noted in lung, kidney, heart, and liver. Elimination kinetic profiles for kidney, heart, spleen, and lung were analogous the blood concentration profile. Six hours after administration, THC concentrations were not detectable in liver. Over the 24 h period, THC brain concentrations decreased slower than blood.

Given the limited data describing THC distribution, a physiologically-based pharmacokinetic (PBPK) model would allow predictions and cross-species comparisons of THC tissue toxicokinetics. A whole-body PBPK model consists of physiological compartments that are most relevant to a drug's absorption, distribution, metabolism, and excretion properties (113). PBPK modeling is a computational tool that predicts concentrations in various tissues based on species-specific physiological information, chemical-specific parameters, and drug administration protocol (110). Physiological and anatomical parameters, such as body weight, cardiac output, organ weight or volume, blood flow rate, and volumes of blood in tissues, are dependent on the species evaluated by the model. The parameters specific to the compound utilized in the model include molecular weight, lipophilicity, and dissociation rate constants. Several drug properties, such as tissue-plasma partition coefficients, rate constants, permeability coefficients, and fraction of unbound drug, are dependent on properties of both the drug and species under consideration (112). The drug administration protocol of the PBPK model is tailored to the desired exposure route, dosing regimen, and duration of administration. Benefits of

developing a PBPK model include the ability to extrapolate across species, exposure routes, doses, and duration (114).

To date, only one PBPK model describing THC distribution in an animal model is available (115). Methaneethorn et al. developed a PBPK model in mice, rats, and pigs following intravenous THC administration. The researchers compared predicted THC concentrations from their model to data collected previously from three pharmacokinetic studies and found that the developed model adequately simulated THC tissue concentrations.

In the current study, we developed a PBPK model in rabbits after inhaled THC administration, approximating the most common route of administration by humans. We determined cannabinoid concentrations in postmortem fluids and tissues from rabbits following controlled cannabis administration in order to assess cannabinoid distribution. The experimentally observed THC concentrations were compared to those predicted from the model.

Materials and Methods

Animals

Three female and two male adult New Zealand white rabbits (Charles River Laboratories, Canada) weighing approximately 2.5 kg were used in this study. All animal work was conducted at Oklahoma State University (OSU) and was approved by the OSU Institutional Animal Care and Use Committee (IACUC). Rabbits were housed individually in cages with food (LabDiet 5321, St. Louis, MO) and water available *ad libitum* prior to experimental testing.

Cannabis

Cannabis cigarettes were obtained through the National Institute on Drug Abuse Drug Supply Program. Cigarettes contained 6.8% THC, 0.20% cannabidiol (CBD), and 0.41% cannabinol (CBN) and were stored at -20°C. About 24 h before use, cigarettes were removed from the freezer then placed under 75% humidity at room temperature.

Experimental design

Rabbits were sedated with xylazine (2 mg/kg) administered by intramuscular injection into the cranial thigh before being placed into an exposure chamber. Sense of smell of a rabbit is very good; thus, xylazine was administered to combat any response to breathing patterns during drug exposure. Furthermore, two rabbits were housed together in the exposure chamber separated by a wire rack. Sedation prevented any interaction between rabbits housed in the same chamber. Cannabis smoke was generated using a microprocessor-controlled cigarette smoking machine (model TE-10, Teague Enterprises, Davis, CA). Mainstream and sidestream smoke were transported to a mixing and diluting chamber before being introduced into two exposure chambers. Four rabbits were exposed to smoke from 6 cannabis cigarettes over about a 40 min period. Two cigarettes were burned simultaneously with the constant puff machine setting for approximately 13 min, then removed and 2 more cigarettes loaded in the machine, until all 6 cigarettes were burned. Once the cannabis exposure was complete, the rabbits remained in the chamber for 5 minutes for venting of smoke. Rabbits were anesthetized with xylazine (5 mg/kg) and ketamine (35 mg/kg) administered by intramuscular injection into the cranial thigh and then euthanized by cervical dislocation, with the time of death occurring at approximately 65 min after beginning the cannabis administration or 10 minutes after leaving the smoking chamber.

Sample collection

At least 0.5 mL of heart blood was collected from the left ventricle. The heart was removed and rinsed with water. All lung lobes were excised and rinsed with water. Cavity blood was obtained from the pooled blood in the chest cavity after removing the heart and lungs. Urine was collected and its total volume in the urinary bladder was measured. A peripheral blood sample was obtained by exposing the caudal vena cava at its bifurcation into the external iliac veins. Blood from the femoral, iliac and caudal veins was milked to the iliac bifurcation where 0.5 mL was collected. Bile was collected before removal of the entire left medial lobe of the liver. Spleen, right kidney and approximately 5 g of skeletal muscle from the psoas major were excised. Vitreous humor was obtained from one eye followed by removal of the entire brain. Blood samples were stored in 10 mL grey top vacutainers (MedEx Supply, Passaic, NJ) containing potassium oxalate and sodium fluoride, whereas bile, urine and vitreous humor were stored in 15 mL polypropylene conical tubes (Beckton Dickson, Franklin Lakes, NJ) and tissues were stored in 30 mL polypropylene tubes (OMNI International, Kennesaw, GA). All collected samples were stored at -20°C until analysis.

Determination of cannabinoids concentration

Cannabinoids were quantified via a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method (144). Briefly, protein precipitation was performed by adding ice-cold acetonitrile to 0.5 mL of blood or 0.5 g of tissue homogenate (1:4 *w:w)*. Urine, vitreous humor, and bile (0.5 mL) were not subjected to protein precipitation. All samples were pre-treated with 0.2% ammonium hydroxide and glacial acetic acid prior to solid-phase extraction (Agilent Plexa PCX, 30mg/3mL). Eluents were

evaporated to dryness with nitrogen, then reconstituted in mobile phase and injected onto an LCMS 8040 (Shimadzu Corp). Limit of quantification was 0.25 ng/mL for THC-glucuronide (THC-g); 0.5 ng/mL for THC, THCCOOH, cannabidiol (CBD), cannabinol (CBN) and cannabigerol (CBG); 1 ng/mL for THCCOOH-glucuronide (THCCOOH-g). Bias determinations were between -15.1% and 13.6% of target concentrations; imprecision calculations were ≤18.1% CV for all analytes.

PBPK model structure

A nine-compartment PBPK model was structured to include lung, muscle, heart, brain, spleen, kidney, liver, fat and the rest of the body. The schematic diagram is shown in Figure 8. THC elimination was assumed to occur through hepatic and renal routes. A perfusion-limited and well-stirred model was assumed for all compartments. Lungs were included as the site of administration. The brain is the target site for the psychoactive effects of THC. Liver is the primary metabolizing organ and kidney is the main excretion organ, thus both organs are modeled as individual compartments. Heart, spleen, and muscle were also included individually to evaluate THC disposition in these organs. Fat was included as part of the model since THC accumulates in this tissue over long periods of time; however, no THC concentrations were measured in rabbit fat tissues in this study.

PBPK model parameters

Physiological parameters, including organ blood flow (Q) and organ volumes (V), were determined by published data from Davies and Morris (167). Physiochemical and biochemical parameters, such as partition coefficient, metabolic rate constant, and urinary elimination rate constant, were acquired from literature (117, 163, 168, 169).

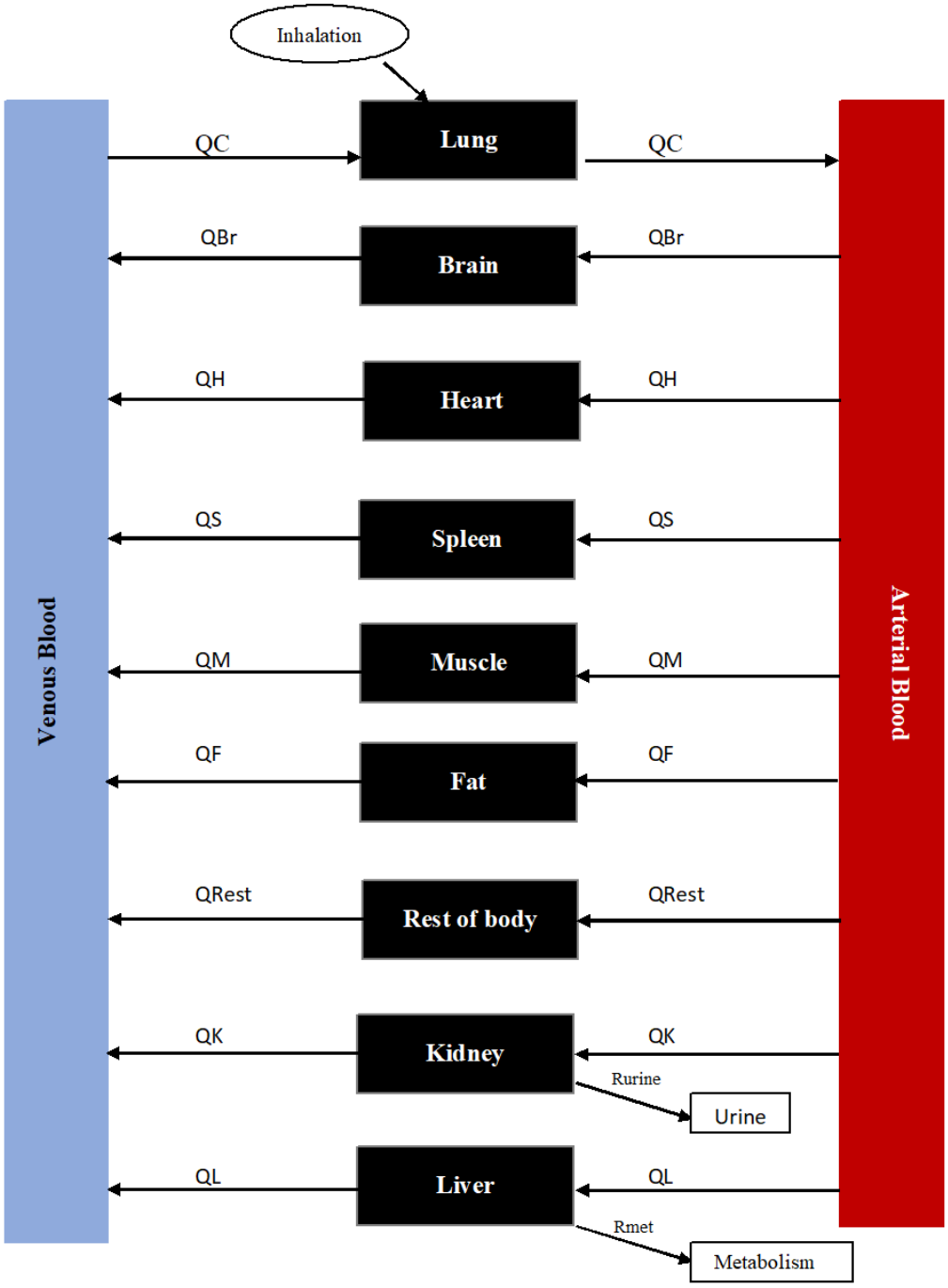

Figure 8. Schematic of the physiologically-based pharmacokinetic model of Δ^9-tetrahydrocannabinol in rabbits through the inhalation route of administration. QC: Cardiac output; QM: blood flow to muscle; QH: blood flow to heart; QBr: blood flow to brain; QS: blood flow to spleen; QF: blood flow to fat; QRest: blood flow to rest of body; QK: blood flow to kidney; QL: blood flow to liver; Rurine: urinary excretion of THC; Rmet: metabolism of THC.

Physiological and biochemical parameters used in the PBPK model development are summarized in Table 8. Additionally, tissue:blood partition coefficients were calculated by the Rodgers and Rowland method, obtained from the literature, and measured experimentally from data obtained in all five rabbits (Table 9). Berkeley Madonna software (Version 8.3.18; University of California at Berkeley, CA, USA) was used to develop the PBPK model. Equations utilized to develop the PBPK model are detailed in Tables 10-13.

Total THC dose administered to rabbits was calculated using a mass balance approach (Table 14) to determine concentration (ppm) necessary for the PBPK model. After determining total dose to rabbits, the concentration inhaled (CI) in this study was calculated using the following equations:

$$CI\ (mg/L) = \frac{\text{Total THC dose (mg)}}{\text{QP (L/h) x T (h)}} \qquad [1]$$

$$CONC\ (ppm) = \frac{24450 * CI\ (mg/L)}{MW} \qquad [2]$$

where QP is respiratory minute volume for rabbits, T is the total time of exposure, and MW is THC molecular weight. Equation 2 converts concentration inhaled from mg/L to ppm concentration for the model (170). The exposure concentration (CONC) of THC to the rabbits was calculated as 0.107 ppm.

Table 8. Physiological and biochemical parameters for PBPK model of THC in rabbits.

Parameter	Abbreviation	Value	References
Body Weight (kg)	BW	2.5	(167)
Respiratory minute volume (L/h/kg)	QPC	24.4	(171)
Cardiac output (L/h/kg)	QCC	12.7	(167)
Tissue volume (fraction of body weight, unitless)			
Blood	VbloodC	0.059	(167)
Liver	VLC	0.0308	(167)
Kidney	VKC	0.0052	(167)
Muscle	VMC	0.54	(167)
Brain	VBrC	0.0034	Experimentally measured
Lung	VLuC	0.0037	Experimentally measured
Spleen	VSC	0.0007	Experimentally measured
Heart	VHC	0.0024	Experimentally measured
Fat	VFC	0.08	(167)
Rest of the body	VRC	0.338	Calculated
Blood flow (fraction of cardiac output, unitless)			
Liver	QLC	0.334	(167)
Kidney	QKC	0.151	(167)
Muscle	QMC	0.292	(167)
Brain	QBrC	0.014	(172)
Spleen	QSC	0.001	(167)
Heart	QHC	0.006	(167)
Fat	QFC	0.060	(173)
Rest of the body	QRC	0.3338	Calculated
Hepatic metabolic clearance rate (L/h/kg)	KmC	3.0	(168)
Percentage of plasma protein binding	PB	0.97	(174)
Urinary elimination rate constant (L/h/kg)	KurineC	0.48	(169)
Percentage of THC bound to plasma protein	PPB	0.97	(163)
THC molecular weight (g/mol)	MW	314.45	(163)

Table 9. Tissue:blood partition coefficient (unitless) for THC

Parameter	Abbreviation	Published Value	Measured Value	Predicted Value[a]	Reference[b]
Brain	PBr	2.7	9.0	12.8	(117)
Liver	PL	12.1	3.5	14.6	(117)
Kidney	PK	6.5	8.1	6.9	(117)
Spleen	PS	3.5	5.4	2.7	(117)
Muscle	PM	3.0	5.4	5.1	(117)
Heart	PH	6.7	9.4	13.7	(117)
Lung	PLu	55.2	75.0[c]	8.7	(117)

[a] Predicted value determined by Rowland and Rodgers method (175)
[b] Reference for published values
[c] Adjusted value used in final model

PBPK model evaluation

The developed PBPK model was used to simulate THC concentrations in blood and tissues. The model was evaluated based on comparison of predicted versus measured THC concentrations in biological specimens from five rabbits euthanized approximately 65 minutes after the start of THC administration. Residuals were calculated as the difference between the measured THC concentrations and the predicted THC concentration in blood and tissues.

Table 10. Equations for physiological parameters of PBPK model

	Equation	Units
Cardiac output and blood flows to tissues		
Cardiac output	QC=QCC*BW	L/h
Respiratory ventilation	QP=QPC*BW	L/h
Blood flow to liver	QL=QLC*BW	L/h
Blood flow to kidney	QK=QKC*BW	L/h
Blood flow to muscle	QM=QMC*BW	L/h
Blood flow to brain	QBr=QBrC*BW	L/h
Blood flow to heart	QH=QHC*BW	L/h
Blood flow to spleen	QS=QSC*BW	L/h
Blood flow to fat	QF=QFC*BW	L/h
Blood flow to rest of body	Qrest=QC-QL-QK-QM-QF-QH-QS	L/h
Tissue Volumes		
Liver	VL=VLC*BW	L
Kidney	VK=VKC*BW	L
Muscle	VM=VMC*BW	L
Brain	VF=VFC*BW	L
Lung	VLu=VLuC*BW	L
Heart	VH=VHC*BW	L
Spleen	VS=VSC*BW	L
Blood	VBlood=VBloodC*BW	L
Fraction of blood as arterial	VartC=0.26*VBloodC	L
Arterial blood	Vart=VartC*BW	L
Fraction of blood as venous	VVenC=0.74*VBloodC	L
Venous blood	Vven=VvenC*BW	L
Rest of body	Vrest=BW-VL-VK-VM-VF-VLu-VH-VS-VBlood	L

Table 11. Equations for inhalation and dosing used in the PBPK model

	Equation	Units
Exposure concentration	0.107	ppm
Conversion of exposure concentration from ppm to mg/L	CIX=CONC*MW/24450	mg/L
Inhalation dosing	CI=CIX*AIR[a]	mg/L
Amount inhaled	AINH=QP*CI	mg
Initial Amount inhaled	init AINH=0	mg

[a] AIR: modification repeated square wave function for coding if repeated exposure is desired

Table 12. Equations for metabolic and urinary elimination used in the PBPK model

	Equation	Units
Metabolism of THC in liver compartment		
Rate of metabolism	Rmet=Km*CL*VL	mg/h
Metabolic elimination rate	Km=KmC*BW	h^{-1}
Rate of urinary elimination	Rurine=Kurine*CVK	mg/h
Urinary elimination rate	Kurine=KurineC*BW	h^{-1}

Table 13. Equations for compartments used in the PBPK model

Compartments	Equation	Units
Blood compartment		
Rate of change of THC in venous blood	RV=QL*CVL+QK*CVK+QM*CVM+ QH*CVH+QS*CVS-QC*CV	mg/h
Amount of THC in venous blood	AV	mg
Initial amount of THC in venous blood	Init AV=0	mg
Concentration of THC in venous blood	CV=AV/Vven	mg/L
Concentration of unbound THC in venous blood	CVfree=CV*(1-PPB)	mg/L
Rate of change of THC in arterial blood	RA=QC*CVLu-QC*CAfree	mg/h
Amount of THC in arterial blood	AA	mg
Initial amount of THC in arterial blood	Init AA=0	mg
Concentration of THC in arterial blood	CA=AA/Vart	mg/L
Concentration of unbound THC in arterial blood	CAfree=CA*(1-PPB)	mg/L
Lung compartment		
Rate of change of THC in lung	RALu=QC*(CV-CVLu)+CI*QP	mg/h
Amount of THC in lung	ALu	mg
Initial amount of THC in lung	Init ALu=0	mg
Concentration of THC in lung	CLu=ALu/VLu	mg/L
Concentration of THC in vein of lung	CVLu=CLu/PLu	mg/L
Liver compartment		
Rate of change of THC in liver	RL=QL*(CAfree-CVL)-Rmet	mg/h
Amount of THC in liver	AL	mg
Initial amount of THC in liver	Init AL=0	mg
Concentration of THC in liver	CL=AL/VL	mg/L
Concentration of THC in vein of liver	CVL=CL/PL	mg/L

Compartments	Equation	Units
Kidney compartment		
Rate of change of THC in kidney	RK=QK*(CAfree-CVK)-Rurine	mg/h
Amount of THC in kidney	AK	mg
Initial amount of THC in kidney	Init AK=0	mg
Concentration of THC in kidney	CK=AK/VK	mg/L
Concentration of THC in vein of kidney	CVK=CK/PK	mg/L
Muscle compartment		
Rate of change of THC in muscle	RM=QM*(CAfree-CVM)	mg/h
Amount of THC in muscle	AM	mg
Initial amount of THC in muscle	Init AM=0	mg
Concentration of THC in muscle	CM=AM/VM	mg/L
Concentration of THC in vein of muscle	CVM=CM/PM	mg/L
Brain compartment		
Rate of change of THC in brain	RBr=QBr*(CAfree-CVBr)	mg/h
Amount of THC in brain	ABr	mg
Initial amount of THC in brain	Init ABr=0	mg
Concentration of THC in brain	CBr=ABr/VBr	mg/L
Concentration of THC in vein of brain	CVBr=CBr/PBr	mg/L
Heart compartment		
Rate of change of THC in heart	RH=QH*(CAfree-CVH)	mg/h
Amount of THC in heart	AH	mg
Initial amount of THC in heart	Init AH=0	mg
Concentration of THC in heart	CH=AH/VH	mg/L
Concentration of THC in vein of heart	CVH=CH/PH	mg/L

Compartments	Equation	Units
Spleen compartment		
Rate of change of THC in spleen	RS=QS*(CAfree-CVS)	mg/h
Amount of THC in spleen	AS	mg
Initial amount of THC in spleen	Init AS=0	mg
Concentration of THC in spleen	CS=AS/VS	mg/L
Concentration of THC in vein of spleen	CVS=CS/PS	mg/L
Fat compartment		
Rate of change of THC in fat	RF=QF*(CAfree-CVF)	mg/h
Amount of THC in fat	AF	mg
Initial amount of THC in fat	Init AF=0	mg
Concentration of THC in fat	CF=AF/VF	mg/L
Concentration of THC in vein of fat	CVF=CF/PF	mg/L
Rest of body compartment		
Rate of change of THC in rest of body	Rrest=Qrest*(CAfree-CVrest)	mg/h
Amount of THC in rest of body	Arest	mg
Initial amount of THC in rest of body	Init Arest=0	mg
Concentration of THC in rest of body	Crest=Arest/Vrest	mg/L
Concentration of THC in vein of rest of body	CVrest=Crest/Prest	mg/L

Table 14. Mean±SD THC dose administered to five rabbits

	THC		THCCOOH			THCCOOH-g			THC-g		
Sample	Concentration (ng/mL, ng/g)	Amount (ng)	Concentration (ng/mL, ng/g)	Amount (ng)	Equivalent THC (ng)	Concentration (ng/mL, ng/g)	Amount (ng)	Equivalent THC (ng)	Concentration (ng/mL, ng/g)	Amount (ng)	Equivalent THC (ng)
Peripheral Blood	2.1±0.9	351±146	0.7±0.4	123±71.6	112±65.4	1.6±1.0	263±171	159±103	0.08±0.07	13.8±10.8	8.9±6.9
Vitreous	0.8±1.0	0.8±1.2	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Bile	1.0±1.4	1.0±1.4	0±0	0.2±0.4	0.2±0.4	0±0	0±0	0±0	19.8±17.8	19.8±17.8	12.7±11.4
Urine	0±0	0±0	0±0	0±0	0±0	1.7±2.1	31±40.8	18.7±24.6	16.7±11	248±222	159±142
Liver	6.8±2.9	525±220	6.2±3.6	476±275	435±251	18.4±10.4	1420±803	858±485	2.1±0.9	160±73	102±46.8
Lung	391±165	3607±1471	1.7±1.4	12.2±12.4	11.2±11.3	0±0	0±0	0±0	0±0	0±0	0±0
Kidney	17.3±12.2	286±200	1.0±1.0	5.8±11.6	5.3±10.6	26.5±19.1	434±316	262±191	5.0±4.8	82.1±78.6	52.6±50.4
Spleen	13.9±10.3	21.4±12	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Muscle	12.1±12.6	16300±16952	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Brain	19.0±8.1	160±67.2	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Heart	19.9±12.5	119±80.5	0±0	0.3±0.6	1.6±3.2	0±0	0±0	0±0	0±0	0±0	0±0
Total THC Amount (ng)		21372±17917			565±322			1298±793			333±173
Percent Dose Recovered (%)		85.4±9.3			3.3±3.5			9.2±5.9			2.1±0.9

THC: Δ^9-tetrahydrocannabinol; THCCOOH: 11-nor-9-carboxy-THC; THCCOOH-g: THCCOOH glucuronide; THC-g: THC glucuronide; ng: nanogram; mL: milliliter; g: gram; mcg: microgram

Results

Cannabinoid Concentrations

Cannabinoids and Phase I and Phase II metabolites were quantified in blood, vitreous humor, urine, and bile samples (Table 15). No 11-OH-THC was detected in any samples throughout the study. THC was detected in all heart, cavity, and peripheral blood samples. Mean±SD THC concentrations in heart blood were 4.3±1.7 ng/mL while mean±SD THC concentrations in peripheral blood were 2.1±0.9 ng/mL. No THC was detected in urine samples and only two vitreous humor samples were quantified for THC at low concentrations, 0.5 and 2.5 ng/mL. A sufficient volume (≥0.5 mL) of bile was available to permit cannabinoid analysis in four of the five rabbits, and THC could be quantified in three out of four of these samples. THCCOOH was quantified in heart, cavity, and peripheral blood samples in four rabbits with mean (range) concentrations of 1.1 (0.5-1.5) ng/mL, 1.2 (0.5-1.5) ng/mL and 0.9 (0.5-1.1) ng/mL, respectively. THC-g and THCCOOH-g were quantified in blood, urine, and bile with considerable THC-g concentrations noted in urine and bile. Due to a lack of internal standard recovery for THCCOOH-g in bile, THCCOOH-g was not quantified. The only minor phytocannabinoid detected in any blood sample was CBN and when positive, was found at a concentration approximately 10% of THC concentration in the corresponding blood sample.

THC was quantified in all lung, liver, brain, heart, kidney, and spleen samples and in all but one rabbit muscle sample. As expected from inhalational administration, lung tissue exhibited the highest THC concentrations of all the tested tissues, in all five rabbits, with a mean (range) concentration of 391 (103-387) ng/g. Figure 9 shows the

Table 15. Cannabinoid Concentrations in Blood, Urine, Vitreous Humor, and Bile (ng/mL)

Rabbit	Specimen	THC	THCCOOH	THCg	THCCOOHg	CBN
1	Heart Blood	5.0	0.5	0.3	1.5	0.5
	Cavity Blood	6.4	0.5	0.3	3.0	0.7
	Peripheral Blood	1.6	0.5	-	1.2	-
	Urine	-	-	8.9	-	-
	Vitreous	0.5	-	-	-	-
	Bile	-	-	48.6	POS	-
2	Heart Blood	4.1	1.1	-	1.6	-
	Cavity Blood	4.1	1.5	-	3.3	-
	Peripheral Blood	3.0	1.1	-	2.3	-
	Urine	-	-	4.2	-	-
	Vitreous	-	-	-	-	-
	Bile	1.3	-	14.3	POS	1.8
3	Heart Blood	6.5	1.5	0.3	1.4	0.6
	Cavity Blood	16.1	1.5	-	2.5	1.7
	Peripheral Blood	2.9	1.0	0.3	1.0	-
	Urine	-	-	25.3	1.2	-
	Vitreous	2.5	-	-	-	-
	Bile	3.5	1.1	16.4	POS	3.4
4	Heart Blood	1.4	-	-	-	-
	Cavity Blood	0.7	-	-	-	-
	Peripheral Blood	0.7	-	-	-	-
	Urine	-	-	11.2	-	-
	Vitreous	-	-	-	-	-
	Bile	N/A	N/A	N/A	N/A	N/A
5	Heart Blood	4.5	1.3	-	3.8	0.5
	Cavity Blood	4.1	1.3	-	6.9	0.6
	Peripheral Blood	2.4	1.1	-	3.2	-
	Urine	-	-	33.6	5.8	-
	Vitreous	-	-	-	-	-
	Bile	2.7	-	30.9	POS	2.2

THC: Δ^9- tetrahydrocannabinol; THCCOOH: 11-nor-9-carboxy-THC; THC-g:THC glucuronide; THCCOOH-g: THCCOOH glucuronide; CBN: Cannabinol; POS: positive

mean±SEM THC concentration for tissues, excluding lung. Liver, the primary site of THC metabolism, exhibited the lowest mean THC value while heart showed the highest mean THC value.

In addition to high THC concentrations in the lungs, minor phytocannabinoids present in cannabis material were quantified in lung samples. CBN was the most abundant of these cannabinoids with concentrations approximately 18% of lung THC concentration. Lung CBN concentrations ranged from 21.6 to 116 ng/g with mean concentration of 69.9 ng/g. Other cannabinoids, CBD and CBG, were found in similar concentrations to each other, with CBD concentrations ranging from 5.8 to 26.7 ng/g and CBG concentrations ranging from 4.4 to 26.9 ng/g in lung. Mean CBD and CBG concentrations were 16.6 and 15.2 ng/g, respectively. Low concentrations for THCV in lung was observed with mean (range) concentration of 5.6 (0-9.5) ng/g.

Metabolites were prevalent in liver and kidney, tissues that are responsible for biotransformation and drug elimination. The phase I metabolite, THCCOOH, was quantified in four of the five liver samples with concentrations ranging from 4.6 to 9.9 ng/g and mean concentration of 6.2 ng/g. Phase II metabolites, THC-g and THCCOOH-g, were quantified in all five kidney samples with mean (range) concentration of 5.0 (1.5-14.4) ng/g and 26.5 (2.1-59.6) ng/g, respectively. Liver THC-g and THCCOOH-g concentrations were consistently less than those determined in the kidney. Mean concentration for THC-g in liver was 2.0 ng/g with concentrations ranging from 0 to 3.1 ng/g. Mean concentration for THCCOOH-g in liver was 18.4 ng/g with concentrations ranging from 4.5 to 36.9 ng/g.

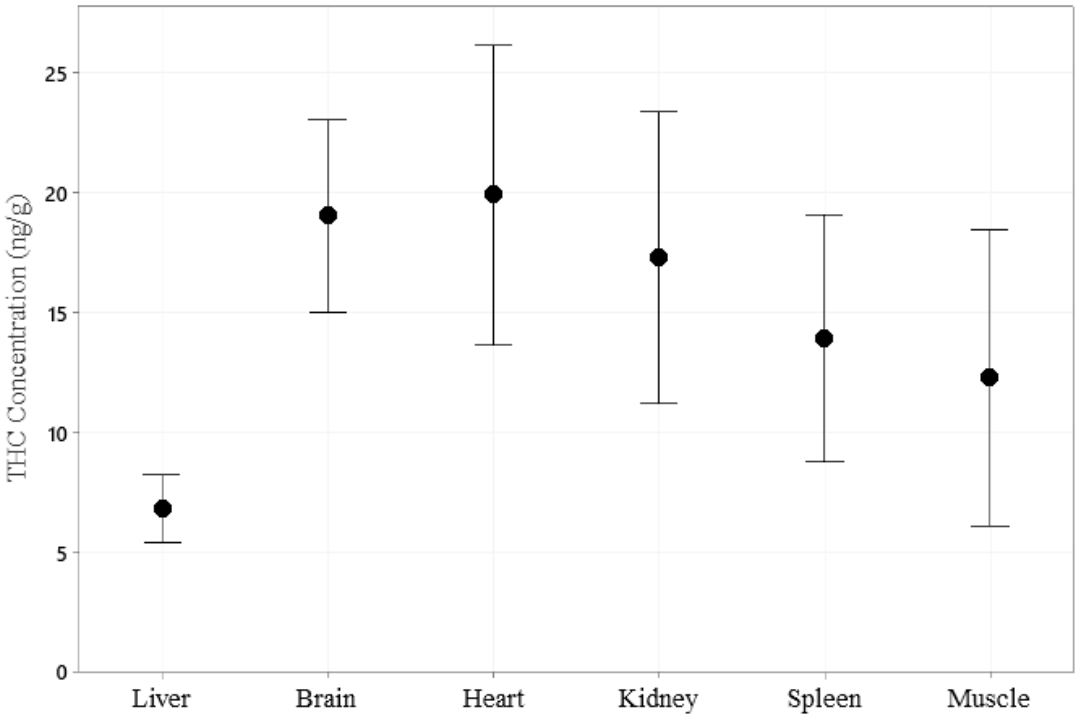

Figure 9. Mean±standard error mean Δ^9-tetrahydrocannabinol (THC) concentrations in rabbit tissues approximately 65 minutes after start of dosing.

PBPK Model

Tissue:blood partition coefficients (PC) are available in the scientific literature, predicted using a biologically-based algorithm, or calculated as the ratio of the concentration of an analyte in a tissue to the concentration in blood. Mean THC concentrations for liver, kidney, spleen, muscle, brain and heart for the five rabbits were divided by the mean THC concentration in peripheral blood to obtain a measured tissue:blood PC (Table 9 – measured values).

Model simulations were performed in liver, brain, kidney, spleen, muscle, and heart using measured, predicted, and published PC values listed in Table 9 (Figure 10). Small differences between all three simulations were observed for spleen, muscle, kidney and heart. Simulated THC concentrations for liver with the predicted and published data were very similar, whereas the simulated THC concentrations for liver with the observed PC value produced slightly lower THC concentrations. Predicted THC concentrations using published PC value for brain produced lower THC concentrations than THC concentrations using the measured and predicted PC values for brain. Model simulations were performed in lung and blood using measured PC values for all tissues and optimized lung PC value of 75 (Figure 11).

Residuals were calculated between the average THC concentration and its predicted concentration at 1.1 h for blood and tissues. Tissue residuals were determined as 9.7, 9.0, 7.7, 6.7, 5.8, 2.3 and 0.6 ng/mL for muscle, heart, spleen, kidney, lung, brain and liver, respectively. Blood was the only sample type in which the measured value was below the predicted value giving a residual of -1.0 ng/mL.

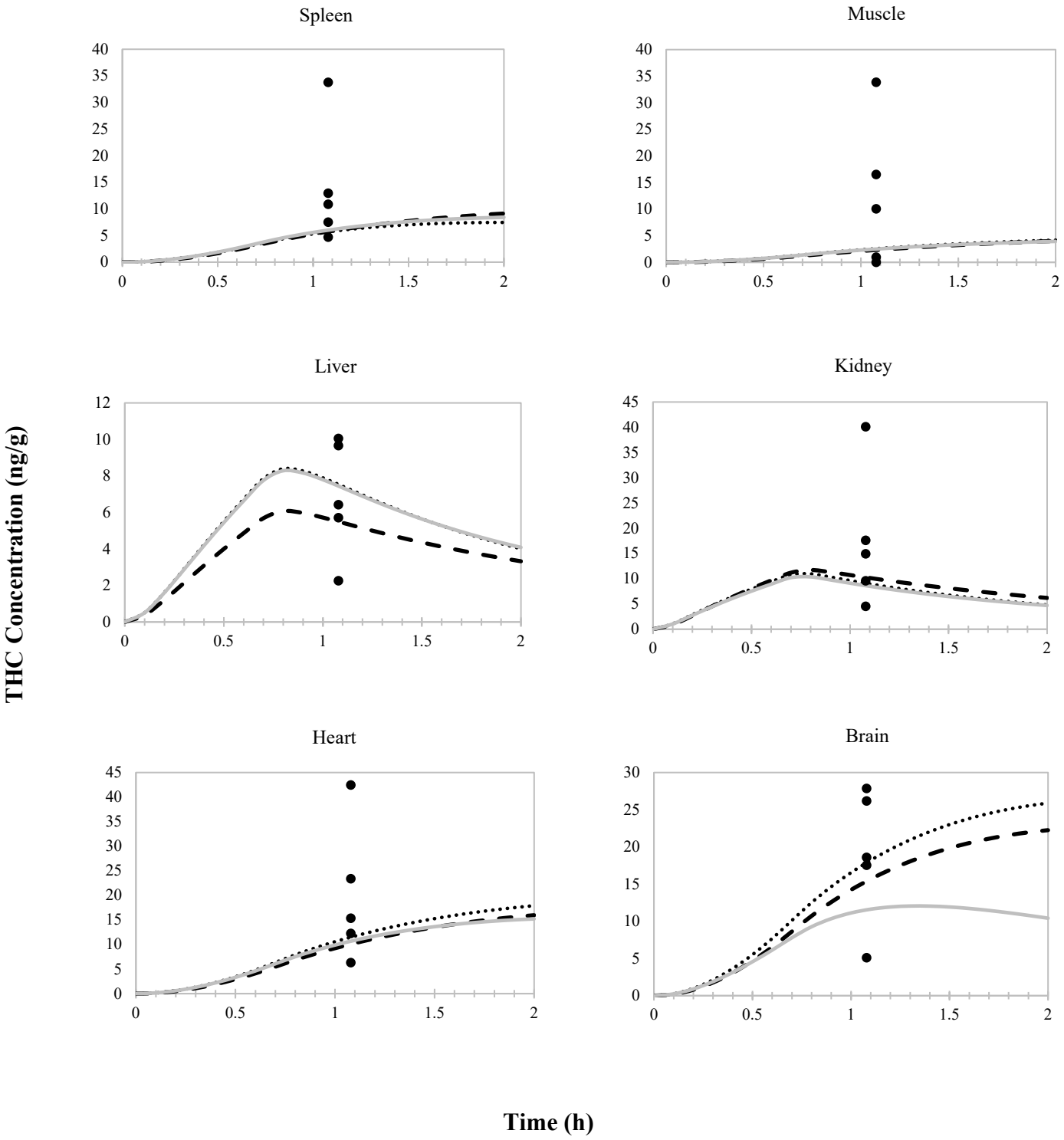

Figure 10. Simulated THC concentration results in tissues from the PBPK model using blood:tissue partition coefficient values from measured data (– – –), predicted data (·····), and published data (——). Observed (●) THC concentrations in tissues from five rabbits necropsied immediately upon death.

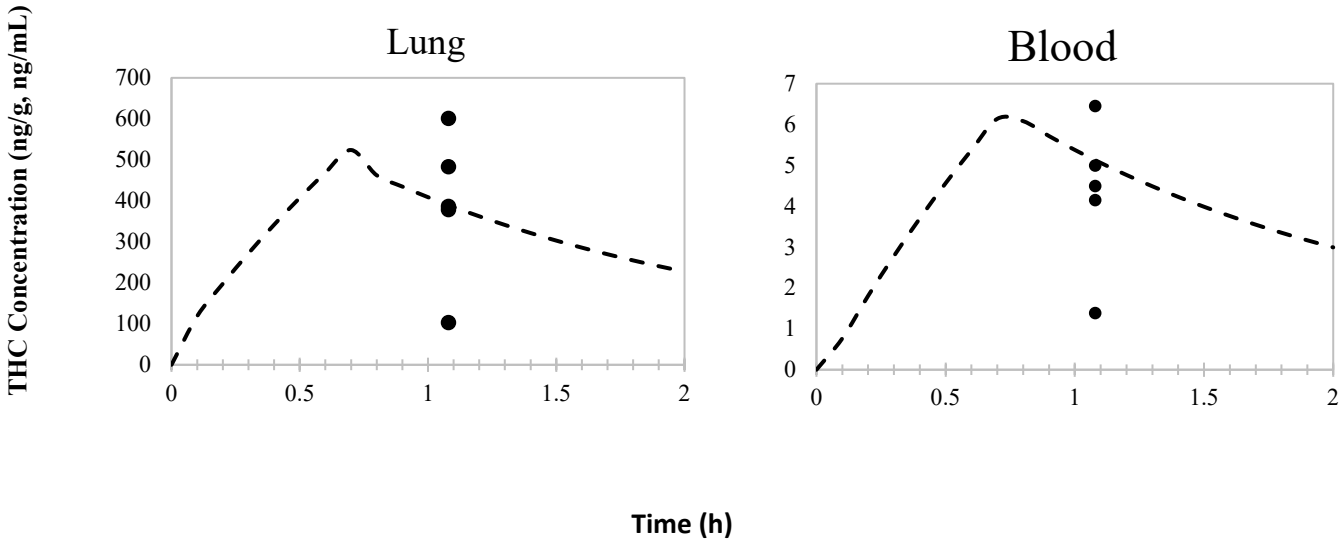

Figure 11. Simulated THC concentrations (– –) in lungs and blood from the PBPK model using measured blood:tissue partition coefficient values. Observed THC concentrations (●) in lungs and heart blood from five rabbits necropsied immediately upon death.

Discussion

Many distribution studies in animals use mice or rats; however, we utilized the rabbit with our experimental protocol for several reasons (176). First, the rabbit is larger than rats or mice, allowing for collection of blood to exceed the minimum amount (0.5mL) necessary for analysis. Furthermore, rabbits have a gallbladder, unlike rats, for the collection of a bile sample (177). Bile revealed high cannabinoid concentrations in human studies, thus obtaining a bile sample and determining cannabinoid concentrations in this sample type was important (85, 143). In addition, THC metabolic pathways in the rabbit are similar to humans providing a comparison between rabbits and humans for cannabinoid metabolite results obtained in the current study (178). Finally, the experimental set-up was an ideal situation that allowed two rabbits in each exposure chamber during cannabis administration. The rabbit proved to be a good animal model for determining cannabinoid distribution after inhaled cannabis administration.

Interestingly, THC concentrations were higher in heart blood than in peripheral blood, even though samples were collected immediately after death. Therefore, it is likely that THC heart blood concentrations exceeded peripheral blood concentrations ante mortem as well. Given that THC concentrations were higher in the lung than in any other tissues measured, it is likely that heart blood THC concentrations reflected absorption of THC, as the pulmonary vein drains into the left side of the heart. A similar observation was reported by Brunet et al. after intravenous administration of THC to pigs via the jugular vein (108). Higher THC concentrations were noted in cardiac blood than inferior vena cava 2 hours after administration. The authors also postulate that higher THC

concentration in cardiac blood is due to diffusion of THC from lung tissue into cardiac blood from pulmonary blood.

Cannabinoid excretion into urine is well documented and characterized. While most of an inhaled cannabis dose in humans is excreted in the feces, about 20% of the dose is excreted in the urine as acidic metabolites (54). Many of the primary acidic metabolites are the glucuronide conjugate form, which increases water solubility and facilitates excretion (47). For many years, cannabinoid quantification in urine has involved alkaline or enzymatic hydrolysis to measure unconjugated THC, 11-OH-THC, and THCCOOH concentrations. With the availability of commercial conjugated standards for THC and THCCOOH, methods were developed and utilized for direct quantification of glucuronide conjugates in biological specimens (56). Direct quantification allows for the determination of free and glucuronidated THC and THCCOOH in urine. In the current study, only conjugated metabolites, THC-g and THCCOOH-g were detected in rabbit urine samples, confirming that primary metabolites in urine after acute cannabis administration are glucuronidated metabolites.

Bile was used as an alternative matrix to urine for drug analysis in postmortem cases because many drugs concentrate in bile. Biliary excretion most commonly occurs for polar drugs with high molecular weights, like the Phase II glucuronide metabolites. Scant research is available regarding cannabinoid concentrations determined in bile or its significance in interpretation. A limited investigation of the distribution of THC, 11-OH-THC, THCCOOH, THCOOH-g, CBD, and CBN in man revealed substantial concentrations in bile for all cannabinoids, except THC (85). Fabritus analyzed THC, 11-OH-THC, THCCOOH, THC-g, THCCOOH-g, CBD, and CBN in 10 bile human samples

that were positive for cannabinoids in blood and urine. Extremely high concentrations of the glucuronidated conjugates were found in bile as compared to free THC and THCCOOH (143). In the current study, rabbit bile samples exhibited the highest THC-g concentrations of any specimen tested. Besides blood and lung, bile was the only specimen with quantifiable results for CBN. Furthermore, THC was detected in 3 out of 4 available bile samples at higher concentrations than heart blood. This study showed that bile is an excellent specimen for identifying cannabinoid exposure with its extremely high concentrations of THC-g and THC after acute inhaled administration.

Tissue analysis is an important aspect of drug distribution, showing which tissue is most likely to store THC after administration. Concentrations of THC in rabbit lungs were 10 to 100 times higher than all other organs tested. This result is expected as lungs are the organ responsible for absorption of THC after inhalation. Following inhalation, THC enters the systemic circulation by the pulmonary veins with distribution dependent on several chemical properties. Drug distribution is also dependent upon the drug's physiochemical properties including volume of distribution, lipophilicity and protein binding. THC is highly lipophilic with a large volume of distribution despite the fact that it is 95 to 99% bound to plasma proteins (52). Based on its properties, THC is initially taken up by highly perfused tissues, such as the lungs, heart and brain. In addition to high THC concentrations observed in the lungs, this study revealed high THC concentrations in heart, brain and kidney.

Metabolism of THC occurs primarily in the liver catalyzed by the cytochrome (CYP) P450 enzyme system with involvement of CYP 2C9, 2C19, and 3A4 (48). Hydroxylation of THC leads to the production of the psychoactive metabolite, 11-OH-

THC. Further oxidation of 11-OH-THC produces the inactive metabolite, THCCOOH (179). The major end products of THC metabolism in most species is THCCOOH and its glucuronide conjugate (47). Primary THC metabolites in rabbits are identified as 11-OH-THC and THCCOOH (53, 180). In the present study, no 11-OH-THC was detected in any samples, most likely due to the low THC concentrations observed and the rapid formation of THCCOOH from any 11-OH-THC present in the liver. Liver exhibited the lowest THC but the highest THCCOOH concentrations of any rabbit tissue samples tested, most likely a result of THC metabolism in this organ. Phase II metabolites, THCCOOH-g and THC-g were also found in liver samples with substantial THCCOOH-g concentrations observed.

THC was readily distributed into rabbit kidneys as concentrations were about 3.7 times higher than THC heart blood concentrations. Both glucuronide conjugates, THC-g and THCCOOH-g, were detected in all five rabbit kidneys with higher concentrations found for THCCOOH-g. To our knowledge, this is the first report of cannabinoid glucuronides in liver and kidney after controlled administration. Results indicate that analysis in both liver and kidney can detect glucuronidated metabolites following an acute dose.

Heart tissue is a viable specimen for cannabinoid analysis as THC was detected in rabbit myocardium at concentrations approximately 4.5 times higher than those found in heart blood. High concentrations are probably a result of normal blood circulation within the cardiopulmonary system. Other drugs, including tricyclic antidepressants, narcotic analgesics, and antihistamines were reported to store in high concentrations within the myocardium (181).

There is limited research regarding cannabinoid distribution into the spleen. THC concentrations in rabbit spleen were similar to those found in kidney with THC concentrations ranging from 4.7 to 33.8 ng/g. However, the main difference in cannabinoid distribution between spleen and kidney was the lack of metabolites detected in any spleen samples. The exact reason for this phenomenon is unknown but is likely due to the chemical nature of THC and metabolites and the role and physical nature of the spleen. Spleen has been identified as one of the most perfused organs in the body (154). Highly lipophilic drugs like THC are good candidates for distribution into the highly perfused tissues whereas more polar compounds like THCCOOH are less likely distributed to these tissues.

Forensic toxicologists are often asked to make interpretations regarding drug concentrations found in biological specimens. To make an appropriate interpretation, information regarding drug exposure, time of use, and frequency of use are rarely available in postmortem cases. Therefore, researchers have tried to distinguish patterns of cannabis exposure in humans by identifying markers of recent cannabis use in biological specimens. A whole blood and plasma cannabinoid pharmacokinetics study showed that THC-g, CBD, and CBN exhibited short detection times, making them possible markers of recent use (129). A more recent study by Newmeyer et al. revealed that CBG and CBN were better markers of recent use after inhalation (30). A limitation with the current rabbit study was that minor cannabinoids, CBD, CBG, and THCV, were not present outside lungs samples and CBN was only detected in three blood samples at low concentrations. This prevented any clear conclusion about using minor cannabinoids as markers for cannabis ingestion.

In the present study, a PBPK model for THC disposition in rabbits was established for inhalation administration and its predictive capacity was tested based on data from five rabbits administered cannabis. Due to the lipophilic nature of THC, the developed model assumed blood flow-limited transport into tissues. A main strength of this research is the ability to compare simulated THC concentrations from the PBPK model with observed THC concentrations in various biological specimens. Despite some deviations between observed and predicted concentrations, THC distribution into tissues was appropriately characterized by the proposed model.

An important parameter in perfusion-limited PBPK models is the tissue:blood partition coefficient for each tissue, which represents the drug concentration in tissue relative to the drug concentration in blood. Despite the importance of tissue specific partition coefficients, in vivo experiments to measure tissue and blood concentrations over time are expensive and time-consuming (182). Several *in silico* methods to predict partition coefficients using tissue composition and the drug's physiochemical characteristics are available (175, 183, 184). These methods incorporate tissue composition and a drug's physiochemical properties to account for distribution of the drug between water, proteins, lipids and phospholipids. The first predictive method for partition coefficients was described by Poulin and Theil for neutral compounds with an emphasis on tissue binding to neutral lipids and drug lipophilicity (183). Rodgers and Rowland extended the Poulin and Thiel method by integrating pH, dissolution of drug into water, and binding of ionized base to acidic phospholipids as extra components of tissue binding (175). The Rodgers and Rowland method was utilized in this study for the predicted tissue:blood partition coefficients as it is a well-established model for acid and

base drugs (Table 9). From THC concentrations obtained in tissues and peripheral blood from the five rabbits in the study, a measured partition coefficient was calculated for tissues except for lung. No measured partition coefficient was determined for lung as this tissue is the site of administration and would be expected to have an extremely high tissue:blood partition coefficient that would not correctly predict concentrations within other compartments of the model. By adjusting the lung partition coefficient from the published value of 55.2 to 75.0, the simulation better correlated with the measured data. All tissue:blood partition coefficients utilized for model development and evaluation are listed in Table 9.

Results of the model indicate that variations in some partition coefficients have little impact on model simulations. For spleen, muscle, kidney, and heart, all three published, predicted, and measured tisse:blood partition coefficients provided similar concentration time-profiles for simulated data (Figure 10). On the other hand, liver concentrations using the measured partition coefficient were slightly lower than both predicted and published partition coefficient values. Published partition coefficients were determined after intravenous administration to rats (117). Both route of administration and differences in tissue composition between rat and rabbit could result in higher partition coefficient values, thus causing higher predicted concentrations from published values as compared with measured values. Brain concentrations also exhibited differences between published and measured partition coefficient values. The potential reason for discrepancies could be because of slight differences in tissue composition or blood flow between rabbits and rats.

In the current study, we were able to compare experimentally determined blood and tissue concentrations obtained with theoretical concentrations predicted from the PBPK model (Figure 10, Figure 11). Although inter-subject variability was seen with THC concentrations from the five rabbits, the average observed values were comparable to the simulated concentration for blood and tissues. Calculated residuals showed small differences between the measured and simulated concentrations at 1.1 h after the start of smoking, validating that the PBPK model is appropriate for THC in rabbits.

Conclusion

This study assessed cannabinoid distribution in rabbits following controlled cannabis administration via a smoking machine. Using rabbit physiological parameters, THC chemical properties, and the cannabis administration protocol, a PBPK model was developed to predict THC concentrations in blood and tissues. To our knowledge. this is the first PBPK model for THC in an animal with inhalation as the route of administration. Furthermore, this is the first research study to compare THC concentrations observed in rabbits with predicted THC concentrations from a PBPK model. Within 1 h after cannabis administration, THC was readily distributed from the lungs to the brain, heart, and kidney. Low THC concentrations were exhibited in liver due to the extensive metabolism of THC. Mean THC concentrations in blood and tissues from the five rabbits were comparable to concentrations simulated from the PBPK model. Further research with the development of a PBPK model for THC in humans would expand our understanding of THC pharmacokinetics and provide forensic toxicologists with much needed information regarding THC concentrations in tissues.

CHAPTER V

TIME- AND TEMPERATURE-DEPENDENT POSTMORTEM CONCENTRATION CHANGES OF CANNABINOIDS IN RABBITS FOLLOWING CONTROLLED INHALED CANNABIS ADMINISTRATION

Abstract

Postmortem redistribution (PMR), a well-known phenomenon in forensic toxicology, can result in significant changes in drug concentrations after death, depending on chemical characteristics of the drug, blood collection site, and postmortem interval (PMI). Limited data are available regarding the importance of PMR of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive component in *Cannabis sativa*. New Zealand white rabbits were selected to test whether PMR occurred after controlled cannabis inhalation via a smoking machine and exposure chamber. A necropsy was performed on five rabbits immediately after euthanasia while others were stored at room temperature (21°C) or refrigerated conditions (4°C) until necropsy at various time points after death – 2, 6, 16, 24, or 36 h. Cannabinoid concentrations were quantified in blood, vitreous humor, urine, bile and tissue samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Heart blood concentrations significantly increased at PMI 2 h in rabbits stored at the refrigerated temperature, whereas peripheral blood

concentrations significantly increased at PMI 16 h in rabbits stored at the refrigerated temperature. Central:peripheral and liver:peripheral ratios for THC ranged from 0.13 to 4.1 and 0.28 to 8.9, respectively. Lung revealed the highest THC concentrations while brain and liver exhibited the most stable THC concentrations in tissues over time. Minor cannabinoids, cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN) and Δ^9-tetrahydrocannabivarin (THCV), were also detected in rabbit lung samples. This is the first study to consider the potential for cannabinoids to undergo PMR after inhaled cannabis administration. The data add substantially to understanding postmortem cannabinoids and can aid toxicologists in the interpretation of cannabinoid concentrations in medicolegal death investigations.

Introduction

Postmortem redistribution (PMR) is a well-known phenomenon documented in forensic toxicology case reports for three decades (99). When PMR occurs, postmortem drug concentrations can differ significantly from antemortem concentrations, depending on blood collection site and postmortem interval (PMI), rendering interpretation of results difficult (99). Drug concentrations in heart (central) blood can increase over the PMI due to diffusion of drug from lungs, myocardium, or liver along a concentration gradient into the surrounding area (106). The degree of PMR is influenced by drug physiochemical properties, including lipophilicity, volume of distribution (V_d), ionization and pKa (98). Cell death, putrefaction, body position and body movement after death also affect the degree of PMR (97).

Many reports on PMR are available for tricyclic antidepressants, cocaine, opiates and amphetamines (106). These research studies indicate that drugs likely to undergo PMR are weak bases ($pK_a > 7$), highly lipophilic (Log P > 0.5) with a large apparent V_d ($V_d > 3$) (97-99, 106). Heart blood samples can have drug concentrations more than five times greater than blood samples collected from a peripheral site like the femoral vein (101). The cannabinoid, Δ^9-tetrahydrocannabinol (THC), shares several of these characteristics that favor PMR.

Cannabis sativa is the most widely used drug in the world and is commonly detected in postmortem forensic toxicology specimens from individuals involved in motor vehicle or plane crashes (185, 186). The primary psychoactive component of cannabis, THC, is highly lipophilic (Log P – 5.648) with a large V_d (4-14 L/kg) and a pK_a of 10.6 (163, 187). The expectation is that THC is a good candidate for PMR due to these chemical properties; however, few experimental reports are available describing redistribution of THC in animals or humans. Historically, the scarcity of information was attributed to the idea that cannabis use rarely plays a role in cause and manner of death determination combined with interpretative difficulties associated with cannabis (90).

More recently, however, increases in THC potency revealed a greater negative impact on human performance, psychological disorders and physiological effects (188-190). Worryingly, deaths were attributed to cannabis ingestion (77-79). These changes in cannabis use and effects spotlight the need for more research into postmortem cannabinoid interpretation, including the outcomes of PMR.

A central:peripheral (C:P) ratio is utilized as a tool to determine if a drug exhibits PMR, with a ratio greater than one associated with PMR (103). In the last decade, researchers proposed a liver:peripheral (L:P) ratio as another indicator of a drug's tendency for PMR, with L:P ratios greater than 20-30 suggesting such a propensity (105). Several studies identified C:P ratios for THC and two major metabolites, 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (THCCOOH) (90, 92, 109). No L:P ratios for cannabinoids are currently reported in the literature.

Lemos and Ingle published cannabinoid concentrations in central and peripheral blood for 30 human postmortem cases (90). Central:peripheral ratios ranged from 0 – 2.6, 0.23 – 3.86 and 0 – 1.64 for THC, 11-OH-THC and THCCOOH, respectively. Results from 19 medical examiner cases revealed C:P ratios between 1-2 for all three of these analytes, suggesting modest PMR, albeit much less than expected based on the chemical nature of cannabinoids (109). Within the 19 cases analyzed by Holland et al., a trend of increased redistribution with increased PMI was observed. Another study showed minimal PMR for THC and THCCOOH in 25 human postmortem cases and showed no correlation between PMI and C:P ratios (92). Median C:P ratios determined by Hoffman et al. in this study was 1.1 and 1.3 for THC and THCCOOH, respectively.

One of the only animal studies of postmortem redistribution of THC was performed in the Large White pig (108). An intravenous dose of THC was administered via the intrajugular vein to 15 pigs and samples collected at 0, 6, 15, 24 and 48 h after death. Time related changes in postmortem blood concentrations occurred by the earliest tested PMI of 6 h. Tissue analysis revealed that spleen, muscle and heart concentrations

decreased with increasing PMI. Brain was the tissue in which THC concentrations exhibited the most stability over the tested PMI.

Postmortem redistribution is not only dependent on blood site collection, time since death, and physicochemical drug properties, but also on physical changes that occur after death. Ambient conditions and the condition of the corpse can affect postmortem bacterial degradation and ultimately postmortem drug concentration changes (97). During the putrefaction process, some substances may be metabolized by microorganisms; however, refrigerated temperatures (4°C) may slow this process (191). One recent study evaluated postmortem concentration changes of THC in pigs following administration by inhaled nebulization and storage of the carcass under two conditions, room temperature or 4°C for 24, 48 and 72 h (125). Bile was the only specimen in which significantly different mean concentrations were observed between the two storage temperatures.

We previously reported on the disposition of cannabinoids in various postmortem fluids and tissues after cannabis was administered to rabbits by inhalation via a smoking machine (192). Building upon that work, the current study aims to determine whether there are time- and temperature-dependent changes in the concentrations of cannabinoids after rabbit carcasses were stored under room temperature or refrigerated conditions for various times after the inhalation of cannabis smoke.

Materials and Methods

Animals

All experiments were performed at Oklahoma State University (OSU) under the approval of the University's Institutional Animal Care and Use Committee. Adult New

Zealand white rabbits (Charles River Laboratories, Canada) weighing approximately 2.5 kg were used. Rabbits were housed individually in cages with food (LabDiet 5321, St. Louis, MO) and water available *ad libitum* prior to experimental testing.

Drugs

Cannabis cigarettes were obtained through the National Institute on Drug Abuse Drug Supply Program. Cigarettes contained 6.8% THC, 0.20% CBD and 0.41% CBN and were stored at -20°C until testing. Cigarettes were removed from storage about 24 h before dosing and placed under 75% humidity at room temperature. Xylazine hydrochloride (20 mg/mL) and ketamine hydrochloride (100 mg/mL).

Experimental design

Rabbits were sedated with xylazine (2 mg/kg) by intramuscular injection in order to counter breath holding and aversive behavior of the rabbits when exposed to smoke. Two rabbits were housed together in each exposure chamber, separated by a wire rack, and two exposure chambers were used during each cannabis smoke administration session. Cannabis smoke was generated using a microprocessor-controlled cigarette smoking machine (model TE-10, Teague Enterprises, Davis, CA). Mainstream and sidestream smoke were transported to a mixing and diluting chamber before being introduced into two exposure chambers. During each exposure session, four rabbits were exposed to smoke from 6 cannabis cigarettes over an approximately 40 min period. Two cigarettes were burned simultaneously with the constant puff machine setting for approximately 13 min, then removed and 2 more cigarettes loaded in the machine, until all 6 cigarettes were burned. Once the cannabis exposure was complete, the rabbits remained in the chamber for 5 minutes for venting of smoke. Rabbits were anesthetized

with an intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg) and then euthanized by cervical dislocation, with the time of death occurring at approximately 65 min after beginning the cannabis administration or 10 minutes after leaving the smoking chamber.

Necropsy procedures

Sample collection was performed immediately after euthanasia in five rabbits. All other rabbits were placed in dorsal recumbency within closed plastic bags at ambient temperature (18-22°C) or refrigerated temperature (0-8°C). Sample collections were performed from three room temperature stored and three refrigerated rabbit carcasses at 2, 6, 16, 24 and 36 h after death. No samples were collected from rabbits stored for 36 h at the ambient temperature due to the extent of cell autolysis and putrefaction that occurred in these rabbits. At least 0.5 mL of heart blood was collected from the left ventricle, when available. No blood was present in the ventricle for rabbits stored for 24 h; therefore, clotted blood from the right auricle was collected. The heart was removed and rinsed with water. All lung lobes were excised and rinsed with water. Cavity blood was obtained from the pooled blood in the chest cavity after removing the heart and lungs. Urine was collected and its total volume in the urinary bladder was measured. A peripheral blood sample was obtained by exposing the caudal vena cava at its bifurcation into the external iliac veins. Blood from the femoral, iliac and caudal veins was milked to the iliac bifurcation where 0.5 mL was collected. Bile was collected before removal of the entire left medial lobe of the liver. Spleen, right kidney and approximately 5 g of skeletal muscle from the psoas major were excised. Vitreous humor was obtained from one eye followed by removal of the entire brain. Blood samples were stored in 10 mL

grey top vacutainers (MedEx Supply, Passaic, NJ) containing potassium oxalate and sodium fluoride, whereas bile, urine and vitreous humor were stored in 15 mL polypropylene conical tubes (Beckton Dickson, Franklin Lakes, NJ) and tissues were stored in 30 mL polypropylene tubes (OMNI International, Kennesaw, GA). All collected samples were stored at -20°C until analysis.

Determination of cannabinoids concentration

Cannabinoids were quantified via a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method (144). Briefly, protein precipitation was performed by adding ice-cold acetonitrile to 0.5 mL of blood or 0.5 g of tissue homogenate (1:4 *w:w)*. Urine, vitreous humor and bile (0.5 mL) were not subjected to protein precipitation. All samples were pre-treated with 0.2% ammonium hydroxide and glacial acetic acid prior to solid-phase extraction (Agilent Plexa PCX, 30mg/3mL). Eluents were evaporated to dryness with nitrogen, then reconstituted in mobile phase and injected onto an LCMS 8040 (Shimadzu Corp). Limit of quantification (LOQ) was 0.25 ng/mL for THC-glucuronide; 0.5 ng/mL for THC, 11-OH-THC, THCCOOH, CBD, CBN and CBG; 1 ng/mL for 8β-diOH-THC, 8β-OH-THC, THCV, THCVCOOH and THCCOOH-glucuronide. Bias determinations were between -15.1% and 13.6% of target concentrations; imprecision calculations were ≤18.1% CV.

Statistical analyses

Statistical analyses were performed using SAS Version 9.4 (SAS Institute, Cary NC). Analysis of variance (ANOVA) procedures were performed to assess the differences in THC concentrations in various samples. A two factor ANOVA with postmortem interval (PMI) and storage temperature as separate factors was conducted

and the simple effects of each factor were assessed (effects of PMI given storage temperature and storage temperature given PMI). Means and standard errors were reported and protected pairwise comparisons were made. For each test, p values less than 0.05 were considered to be statistically significant.

For the C:P ratios, L:P ratios and minor cannabinoid statistical analyses, a t-test was performed using Minitab® (version 19, Minitab, LLC, State College, PA). A p value less than 0.05 was considered to be statistically significant.

Results

Cannabinoid concentrations were determined in fluids and tissues from 27 rabbits stored under refrigerated and room temperature conditions and collection times at 2, 6, 16, 24 and 36 h after death. Data obtained from rabbit samples collected immediately upon death (PMI 0 h) were described previously in a related study (192). The major psychoactive cannabinoid, THC, was present in multiple blood, bile and tissue samples (Table 16). Statistical analyses revealed no significant differences ($p>0.05$) in THC concentrations for blood, bile and tissues between refrigerated and room temperature storage conditions. In contrast, PMI significantly ($p<0.05$) affected THC concentrations in heart blood, peripheral blood, lung, heart, kidney and muscle samples.

Fluids

All heart and cavity blood samples and all but one peripheral blood sample were positive for THC above the assay's LOQ. One peripheral blood sample collected at PMI 24 h and stored at room temperature had no detectable THC. A separate rabbit stored under refrigeration and with samples collected at PMI 24 h exhibited a surprisingly high heart blood THC concentration at 94.1 ng/mL. Concentrations in the other corresponding

specimens from this rabbit were not elevated as compared to the other rabbit samples, and review of cannabis administration, sample collection procedures, and the laboratory analysis data did not reveal a cause for this apparent outlier. As the heart blood concentration for this rabbit was greater than three standard deviations away from the overall mean THC heart blood concentrations in rabbits, and THC concentrations in corresponding specimens with this rabbit were not elevated, this heart blood value was excluded from table results and statistical analyses.

Highly variable THC concentrations were observed in cavity blood samples with a range of 2.1 to 69.2 ng/mL. Heart blood THC concentrations ranged from 0.5 to 18.9 ng/mL, excluding the outlier. Rabbits stored under refrigerated conditions with samples collected at PMI 2 h showed a significant increase ($p<0.05$) in heart blood THC concentrations compared to heart blood THC concentrations at PMI 0 h (Table 16). Peripheral blood THC concentrations ranged from 0 to 11.6 ng/mL with a significant increase ($p<0.05$) observed in THC concentrations from peripheral samples collected in refrigerated rabbits at PMI 16 h compared to those samples collected at PMI 0 h (Table 16).

Urine was present at the time of sample collection in sufficient volume to permit quantification in 25 of the 27 rabbits. Only three rabbit urine samples were positive for THC with concentrations ranging from 0.6 to 1.2 ng/mL. Vitreous humor was collected in all rabbits; THC was quantified in only nine vitreous humor samples with concentrations ranging from 0.6 to 4.1 ng/mL in positive samples. At the time of sample collection, bile was present in sufficient volume to allow analyte quantification in 23 of

Table 16. Mean ± SEM THC concentrations in blood (ng/mL), bile (ng/mL), and tissues (ng/g).

		Room Temperature (21°C)				Refrigerated (4°C)				
	PMI 0 h (n=5)	PMI 2 h (n=3)	PMI 6 h (n=3)	PMI 16 h (n=3)	PMI 24 h (n=3)	PMI 2 h (n=3)	PMI 6 h (n=3)	PMI 16 h (n=3)	PMI 24 h (n=3)	PMI 36 h (n=3)
HB	4.3±0.8	6.3±3.3	3.9±0.16	3.5±1.2	0.6±0.1	10.7±4.1*	6.2±0.3	3.7±1.6	1.1±0.2[a]	1.6±0.16
PB	2.1±0.4	1.8±0.7	2.8±0.5	6.3±3.1	2.1±0.5	3.4±0.9	6.4±2.4	8.3±3.1*	2.8±0.5	1.7±0.4
CB	6.3±2.3	8.2±2.7	26.8±7.0	11.5±5.7	5.6±0.7	20.9±5.4	48.6±9.8	10.7±5.4	20.3±7.1	7.3±1.2
Bile	1.9±0.8	1.0±0.5	1.5±0.0	1.6±0.2	1.2±0.2	1.9±0.5	0.94±0.5	2.3±1.7	1.3±0.2	0±0.4
Lung	391±82.3	368±126	253±16.6	260±56.0	220±37.6	372±155	365±117	290±61.1	165±79.2	143±22.6*
Heart	19.9±6.3	13.0±5.3	15.0±5.4	9.4±4.7	2.0±2.0*	9.3±1.9	13.1±7.0	5.9±2.9	16.1±2.6	13.7±3.2
Brain	19.0±4.0	9.6±2.3	17.1±5.2	13.8±1.9	9.3±2.8	17.5±3.2	17.6±6.4	10.0±4.6	10.7±0.7	12.3±4.5
Liver	6.8±1.4	4.0±1.4	8.9±1.5	9.2±1.4	6.6±0.9	9.9±1.8	8.3±2.8	8.3±2.6	6.2±1.2	4.4±0.2
Kidney	17.3±6.1	2.5±1.3*	10.6±0.6	9.5±5.3	6.1±6.1	16.0±5.3	11.1±8.1	7.7±3.4	8.1±1.4	6.0±1.1
Muscle	12.1±6.3	2.0±2.0	8.8±6.9	6.3±3.3	0±0*	4.8±2.4	2.8±1.5	4.4±2.5	5.6±0.5	2.0±2.0
Spleen	13.9±5.1	8.0±5.7[a]	10.0±2.4	9.5±2.6	3.3±3.3[a]	9.0±5.9	16.6±4.6[a]	8.9±4.7	0±0[a]	6.9±1.7[a]

SEM: Standard error of the mean; THC: Δ^9-tetrahydrocannabinol; PMI: postmortem interval; HB: Heart Blood; PB: Peripheral blood; CB: Cavity blood

*Significant difference ($p<0.05$) compared to PMI 0 h concentration

[a]n=2 for statistical analyses; Heart blood data point excluded due to outlier; Spleen samples excluded due to unsuitable specimen

the 27 rabbits. Bile contained quantifiable THC in 20 rabbits with concentrations ranging from 0.6 to 4.0 ng/mL.

Tissues

Tissue analysis revealed the highest THC concentrations in lungs and the lowest THC concentrations in liver (Table 16). Brain, kidney, spleen, muscle and heart exhibited moderate THC concentrations at PMI 0 h. A significant decrease ($p<0.05$) in THC concentrations was observed in lung at PMI 36 h in refrigerated rabbits compared to THC lung concentrations at PMI 0 h (Table 16). Heart, kidney and muscle THC concentrations significantly ($p<0.05$) decreased in room temperature rabbits at PMI 24 h, PMI 2 h and PMI 24 h, respectively (Table 16). In five of the 27 spleen samples, the extracted samples showed no recovery for drugs or internal standards. After three unsuccessful attempts to quantify cannabinoids, the samples were deemed unsuitable for analysis.

Ratios

Figure 12 displays the C:P ratios for all postmortem intervals and storage conditions tested. Significant differences from PMI 0 h were observed for C:P ratios at PMI 16 h, PMI 24 h and PMI 36 h in refrigerated conditions and at PMI 24 h for room temperature conditions. Mean±SEM for C:P ratios in the study was 1.6±0.3. Figure 13 shows the L:P ratios for the entire study. No significant differences in the L:P ratios were observed at any postmortem intervals as compared with the control samples. Mean±SEM for L:P ratios in the study was 2.9±0.5. No significant differences were noted between the two storage conditions for either C:P and L:P ratios.

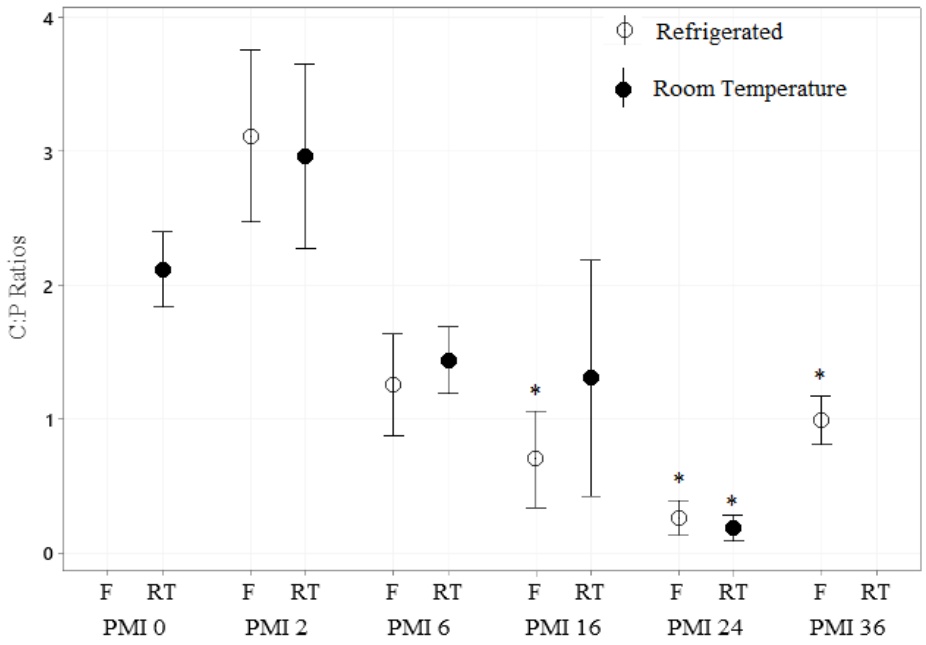

Figure 12. The mean and standard error for central:peripheral blood ratios for rabbits necropsied at various postmortem intervals (PMI) and stored under two different temperature conditions, refrigerated (F, 4°C) and room temperature (RT, 21°C). Significant difference ($p<0.05$, *) compared to PMI 0 h.

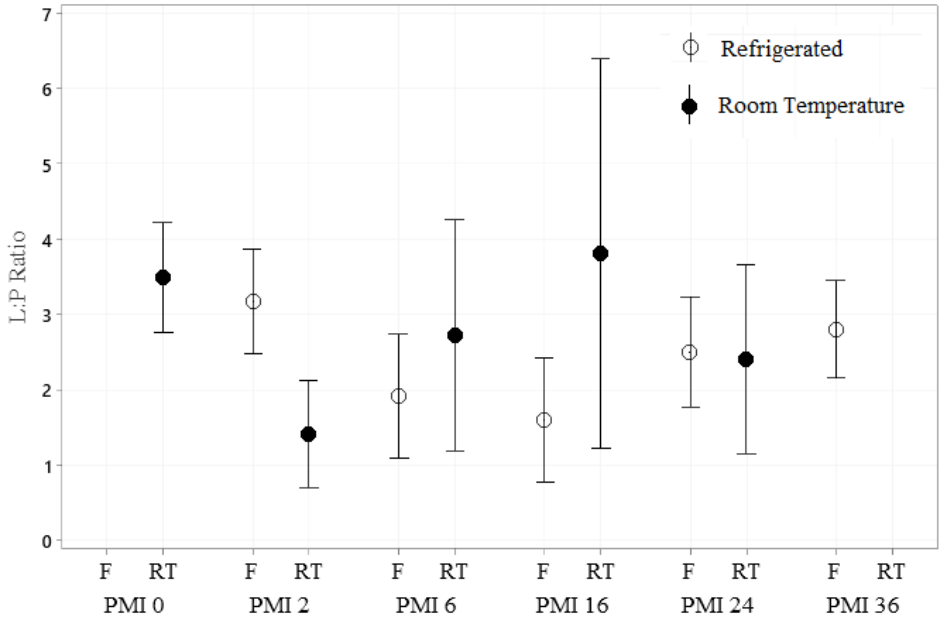

Figure 13. The mean and standard error for liver:peripheral blood ratios for rabbits necropsied at various postmortem intervals and stored under two different temperature conditions, refrigerated (F, 4°C) and room temperature (RT, 21°C).

THC Metabolites

The Phase I and II metabolites, THCCOOH, THC-g and THCCOOH-g, were quantified in various biological specimens throughout the study; however, there were not enough positive results for any metabolite in a fluid or tissue sample to perform statistical analyses. No 11-OH-THC, 8β-diOH-THC, or 8β-OH-THC analytes were detected in any rabbit samples collected from the study. Despite the inability to perform statistical analyses, some general observations for metabolites were noted. Liver exhibited high THCCOOH concentrations, whereas kidney exhibited high concentrations for THCCOOH-g. THC-g was prevalent in bile and urine with considerably higher concentrations than THCCOOH and THCCOOH-g in urine specimens.

Minor Cannabinoids

Cannabis cigarettes contain small amounts of minor cannabinoids. All four minor cannabinoids within the assay, CBD, CBN, CBG and THCV were quantified in rabbit lung samples. High concentrations were noted for CBN with the lowest concentrations among these four minor cannabinoids observed for THCV. Although the mean lung concentrations of minor cannabinoids appeared to linearly decrease in refrigerated and room temperature samples as the PMI increased from 2 to 24 h (Figure 14), PMI did not significantly affect these values ($P>0.05$). However, lung concentrations of CBG, CBN and THCV under refrigerated conditions at PMI 36 h were significantly lower than those at PMI 0 h.

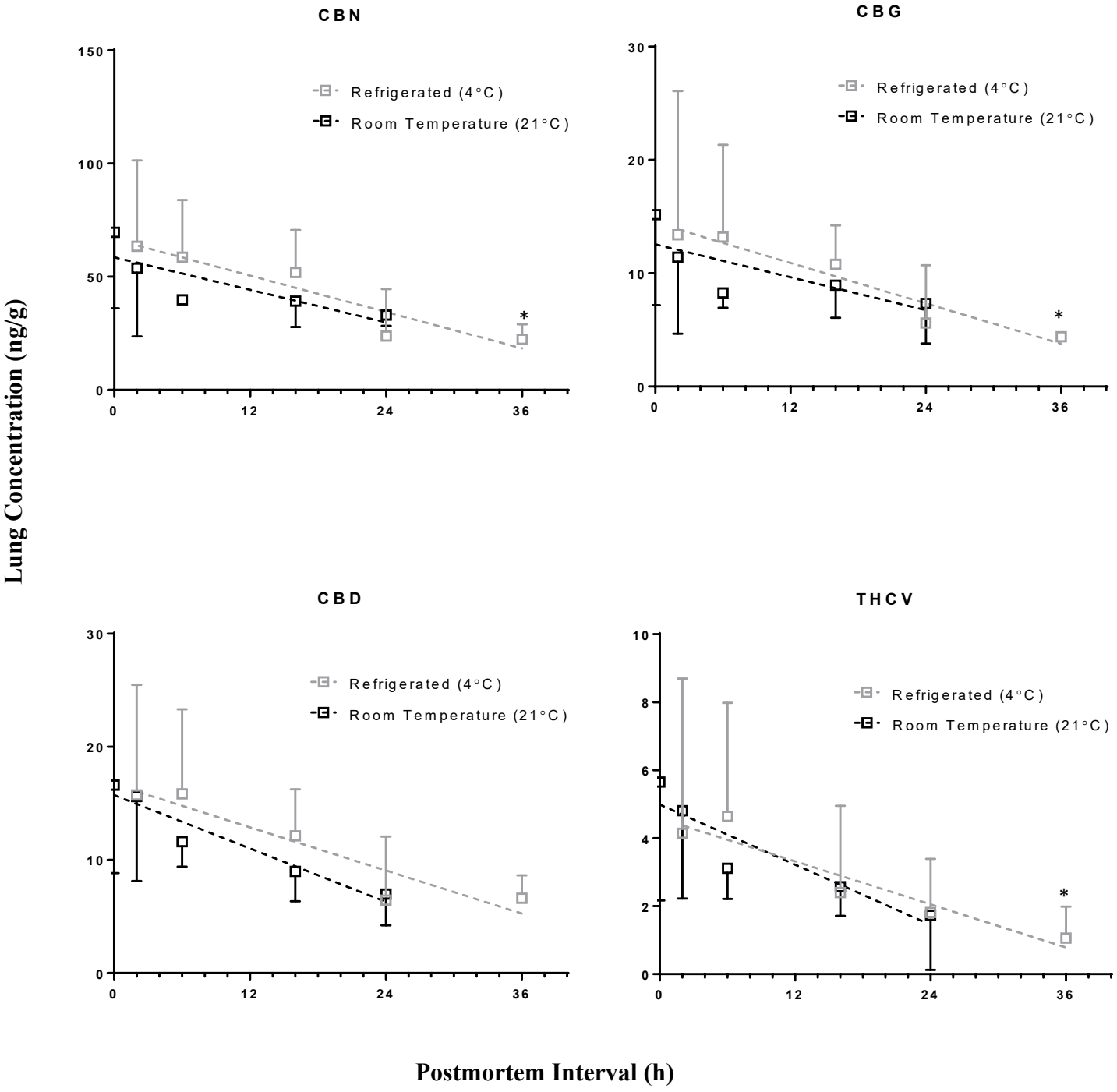

Figure 14. Minor cannabinoid concentrations (mean±SD) in lung (ng/g) at different time points and at different storage conditions after rabbits' death. No differences were observed with each cannabinoid between refrigerated and room temperature conditions. Statistical significance ($p<0.05$, *) is noted with three cannabinoids at PMI 36 h compared to PMI 0 h. CBD: cannabidiol; CBG: cannabigerol; CBN: cannabinol; THCV: Δ^9-tetrahydrocannabivarin.

Discussion

To the authors' knowledge, this is the first controlled inhaled cannabis administration to rabbits to evaluate both time- and temperature-dependent changes in post-mortem sample concentrations of cannabinoids. A strength of this study was the use of the inhalation route of administration for smoked cannabis, as this is the most common route of administration in man and allows for determination of minor cannabinoids from the cannabis material. Most other animal studies utilized oral, intravenous injection, or nebulized cannabis administration, which incompletely model human exposure by smoking (107, 108, 125). Additionally, the current study evaluated drug concentration changes while the carcasses were stored at two different temperatures, which is an important component of medicolegal death investigations. Limitations of the study included the small sample size for each time and storage point and the low cannabinoid concentrations observed. Blood THC concentrations did not reach values seen in human clinical studies. This is most likely due to the experimental conditions that used the smoking machine and exposure chamber to expose the rabbits to continuous smoked air, which was not necessarily comparable to an individual smoking a cigarette. Since THC concentrations were low and the time between THC administration and death was short, metabolites were also low or not detected, hindering the evaluation of the effect of PMR on these metabolite sample concentrations.

Immediately upon death, several physical and chemical changes occur within the deceased body. Decomposition of soft tissues is the result of cell membrane degradation and the release of cellular contents into the extracellular space (97). After autolysis, bacterial proliferation and consumption furthers the decomposition process. The rate of

decomposition is notably variable; however, the progress may be slowed by artificial means of preservation (193). Refrigeration at 4°C reduced the bacterial metabolism rate for nitrobenzodiazepines, suggesting that corpses stored at cooler temperatures may exhibit fewer postmortem drug concentration changes (191). The present study evaluated this idea by storing rabbit carcasses at a refrigerated temperature (4°C) or room temperature (21°C) prior to sample collection. THC concentrations were not affected by temperature as concentrations showed no significant difference between the two storage conditions, which is not surprising since stability of free and glucuronidated cannabinoids after controlled smoked cannabis administration in man showed that THC concentrations were stable in pooled blood samples for 1 week at room temperature and for 12 weeks at 4°C (194).

Changes in THC heart blood concentrations appeared to be an early phenomenon with a significant increase occurring at 2 h in refrigerated rabbits. Similarly, Brunet et al. observed that THC concentration changes in postmortem cardiac blood occurred by 6 h after death in pigs (108). Results from a recent study evaluating changes in THC concentrations over time in deceased people also showed a large increase in THC blood concentration within the early postmortem period, followed by a decline (195). Early PMR studies with rats and amitriptyline revealed a rise in heart blood concentrations within the first two hours after death due to redistribution from the lungs (196). Lungs are a major source of PMR as they serve as drug reservoirs storing highly lipophilic drugs prior to death. Upon death, diffusion of drugs occur along its concentration gradient from the lungs into surrounding areas, especially the heart blood (97). In our study design, with the route of administration and lipophilic properties of THC, the rabbit lung tissues

provided an ideal situation for THC to redistribute from the lungs into heart blood in the early postmortem phase.

In peripheral blood, a significant increase was observed for THC at 16 h after death in the refrigerated rabbit. Peripheral blood, particularly femoral blood, is thought to show fewer concentration changes than heart blood in the postmortem interval as it is farther away from tissues with high drug concentrations (106). However, PMR can occur in femoral blood samples due to proximity with the bladder, skeletal muscle, and body fat. Fluoxetine, mirtazapine, sertraline and methadone all showed significant drug increases in femoral blood after death (197). One possible explanation for increased THC peripheral blood concentrations is that THC is highly lipophilic and rapidly penetrates into fat and muscle tissue after administration (12). Under normal conditions in the body, THC seems to diffuse from fat back into the bloodstream over time (50). Passive diffusion of THC from rabbit fat and muscle into the peripheral blood could explain the increased blood concentrations at the 16 h PMI in the peripheral sample.

Thirty years ago, Prouty and Anderson were the first toxicologists to identify over 50 drugs with the common characteristic of significantly higher drug concentrations in heart blood than blood collected from peripheral sites (101). Since that time, others reported lists of drug concentrations from cardiac, or central, blood compared to peripheral blood samples, establishing an observed C:P ratio for numerous drugs (103, 198). This ratio is used as a measure of the potential for postmortem drug redistribution, with a higher ratio suggesting a greater potential for PMR (103). In the current study, the average C:P ratio for THC at the time of death was 2.1. Although the ratio is greater than 1, we do not believe this initial C:P ratio is indicative of PMR. Since rabbits inhaled the

cannabis dose, and lung THC concentrations were quite high, we instead hypothesize that the experimental design did not allow enough time for complete drug absorption from the site of administration, causing a higher heart blood THC concentration than its corresponding peripheral sample.

As reports regarding postmortem drug concentrations and PMR accumulated in the literature, some studies showed that the C:P ratio can be misleading (105). Some drugs with a reported C:P ratio greater than 1.0 are not susceptible to PMR. For example, inaccurate ratios may be obtained in cases where a drug has not fully completed the absorption or distribution phases; therefore, toxicologists recently suggested that liver drug concentrations compared to peripheral blood drug concentrations may be helpful in understanding postmortem drug redistribution (199). McIntyre reported previously published C:P ratios, along with L:P ratios for 13 drugs, in order to evaluate the usefulness of a L:P ratio in determining a drug's propensity for PMR (105). Tricyclic antidepressants, drugs that are well-documented for their potential to redistribute, exhibited an L:P ratio greater than 20. Drugs suspected to have a low to moderate propensity for PMR exhibited intermediate L:P ratios ranging from 5 to 19, whereas drugs that exhibit little to no PMR reportedly have L:P ratios less than 5. In the current study, the mean (±SD) L:P ratio across all time and temperature conditions was 2.9 (±1.7), which puts THC in the category of exhibiting little to no PMR. To our knowledge, this study was the first to evaluate L:P ratios for THC after controlled administration in an animal model. More studies are needed to substantiate this finding and to determine if an L:P ratio is a suitable marker for identifying postmortem redistribution of THC.

Analysis of drug concentrations in tissues is particularly important in traumatic injury deaths or severely decomposed bodies in which a blood sample may not be available for toxicology testing. Identifying the tissue that exhibits drug stability over time would provide valuable information to forensic toxicologists. With this study, we were able to determine THC concentrations in multiple tissues for 36 h after death. Results showed that THC concentrations in lung, kidney, brain and heart remained the most stable over time, whereas muscle and spleen THC concentrations diminished substantially as the PMI increased. Postmortem redistribution of THC in the pig revealed that brain THC concentrations were the most stable compared to other tissues 48 h after death (108). In cases where cannabis is expected and no blood sample is available, toxicologists can rely on testing lung, kidney, brain or heart as these tissues reveal high and moderate THC concentrations that remain stable hours after death.

Cannabis is a complex plant containing more than 500 chemical compounds, of which more than 100 are identified as cannabinoids (158). Researchers studied several phytocannabinoids including CBG, CBN and THCV to identify potential markers of cannabis use (30, 129). Minor phytocannabinoids were detected in rabbit lung samples with high concentrations for CBN, moderate concentrations for CBD and CBG, and low concentrations for THCV. A limitation of this study was that minor cannabinoids were not detected in biological specimens other than lungs. While concentrations for all minor cannabinoids decreased in lung as PMI increased, the lack of cannabinoids in blood or other tissues prevented a complete evaluation of concentration changes after death. More research is needed to determine if minor cannabinoids undergo PMR similar to THC.

Conclusion

In this study, we determined THC, its phase I and glucuronidated phase II metabolites, and minor cannabinoids in biological specimens collected from rabbits following inhaled controlled cannabis administration to evaluate time- and temperature-dependent changes after death. No significant difference for THC concentrations was observed between rabbits stored at room temperature or refrigerated conditions. Comparing THC concentrations from various postmortem intervals to those observed at PMI 0 h, significant THC concentration changes in heart blood and peripheral blood were noted only in refrigerated samples collected 2 and 16 h after death, respectively. Average C:P and L:P ratios for THC were 1.6 and 2.9, respectively. The data presented suggest that THC is not prone to substantial postmortem redistribution in the rabbit.

CHAPTER VI

CONCLUSION

Understanding the complexity of cannabinoid pharmacokinetics in humans requires studying their distribution in a broad array of biological specimens. Studies of this type are crucial for the analysis and interpretation of cannabinoid concentrations in forensic casework. Although cannabis is commonly abused worldwide, few studies exist to characterize cannabinoid distribution or redistribution in postmortem fluids and tissues. We hypothesize that an analytical method can be validated for the simultaneous quantification of 12 cannabinoids in postmortem biological specimens. Using the validated method, tissue analysis of pilots fatally injured in aviation crashes will show the greatest cannabinoid concentrations in highly perfused tissues. The method was employed to determine cannabinoid concentrations in biological specimens from rabbits after controlled cannabis administration. We hypothesize that concentrations from the rabbits will correlate to predicted concentrations from a physiologically-based pharmacokinetic model developed for rabbits and will reveal time- and temperature-dependent changes after death.

The major aims of this research were: 1) Develop and validate an LC-MS/MS analytical method for the simultaneous detection and quantification of THC, 11-OH-THC, THCCOOH, 8β-OH-THC, 8β-diOH-THC, THC-g, THCCOOH-g, CBD, CBG, CBN, THCV, and THCVCOOH in postmortem biological specimens, allowing investigation of cannabinoid distribution. 2) Apply the validated method to authentic forensic casework by determining cannabinoid concentrations in blood and tissue samples from 10 pilots fatally injured in aviation crashes. 3) Develop the first PBPK model for THC in rabbits using the inhalation route of administration. Determine cannabinoid concentrations in postmortem fluids and tissues from rabbits following controlled cannabis administration and compare cannabinoid concentrations to the developed PBPK model. 4) Evaluate postmortem redistribution of THC in rabbits stored at two different temperature conditions.

Analytical Method

We developed an LC-MS/MS method utilizing solid phase extraction to quantify 12 cannabinoids, as well as their free and glucuronidated metabolites in postmortem blood, vitreous humor, bile, urine, liver, lung, kidney, spleen, muscle, brain, and heart. Analytes were separated by liquid chromatography with quantification performed by multiple reaction monitoring in positive ion mode using electrospray ionization. The linear range was from 0.25-50 ng/mL (THC-g), 0.5-100 ng/mL (CBN), 0.5-250 ng/mL (THC, 11-OH-THC, THCCOOH, CBD, and CBG), 1-100 ng/mL (8β-diOH-THC, THCVCOOH, 8β-OH-THC, and THCV), and 1-250 ng/mL (THCCOOH-g). To our knowledge, this is the first method reported for the determination of CBG, THCV and THCVCOOH in postmortem biological specimens. The method achieved adequate

sensitivity, specificity, accuracy, and precision, indicating that the method is applicable to postmortem forensic cases.

Method Application to Authentic Forensic Casework

We applied the method for quantitation of cannabinoids in biological specimens to cases received by the Federal Aviation Administration's Forensic Sciences Section. We determined cannabinoid concentrations in a broad array of biological specimens from 10 pilots who were fatally-injured in plane crashes. Their mean ± SEM THC blood concentration was 4.3±1.5 ng/mL. Phase I metabolites, 11-OH-THC and THCCOOH and the Phase II glucuronidated metabolite, THCCOOH-g had mean ± SEM blood concentrations of 1.3 ± 0.2, 17.9 ± 6.5, and 47.3 ± 13.3 ng/mL, respectively. Urine analyses revealed positive results for THCCOOH, THC-g, THCCOOH-g, and THCVCOOH. The major cannabinoid, THC, was readily distributed to lung, brain, kidney, spleen, and heart. The psychoactive THC metabolite, 11-OH-THC, was identified in liver and brain with mean ± SEM concentrations of 7.1 ± 1.6 and 3.1 ± 0.7 ng/g, respectively. Substantial THCCOOH and THCCOOH-g concentrations were observed in liver, kidney, spleen, and heart. These data improve our understanding of postmortem distribution and analysis by identifying which fluids and tissues are useful for determining cannabinoid concentrations after cannabis exposure.

Cannabinoid Distribution in Rabbits/Physiologically-Based Pharmacokinetic Model

We describe a controlled inhaled cannabis protocol that addresses questions regarding cannabinoid distribution after inhaled administration. The experimental design consisted of cannabis administration to rabbits via a smoking machine and exposure

chamber. Cannabinoid concentrations were determined in a broad array of biological specimens collected immediately upon death. Mean THC concentration in heart blood was 4.3 ng/mL while mean concentration in peripheral blood was 2.1 ng/mL. THCCOOH was detected in heart, cavity, and peripheral blood samples in four of the five rabbits with mean concentrations of 1.1, 1.2 and 0.9 ng/mL, respectively. Both phase II glucuronide metabolites, THC-g and THCCOOH-g, were detected in blood, urine, and bile, with considerable THC-g concentrations noted in urine (4.2-33.6 ng/mL) and bile (14.3-48.6 ng/mL). Tissue analysis revealed high THC concentrations in lungs (103-601 ng/g), moderate THC concentrations in the brain (5.1-32.4 ng/g), heart (6.3-42.4 ng/g), and kidneys (4.5-40.1 ng/g), and low THC concentrations in the liver (2.3-10.0 ng/g). Minor phytocannabinoids, CBN, CBD, CBG and THCV, were detected in lung samples with only CBN detected in blood and bile samples. This is the first animal study focusing on distribution following controlled inhaled cannabis administration. Tissue characterization showed that lungs, brain, heart, and kidneys are the most suitable specimens for THC analysis after cannabis exposure. Furthermore, this study shows that urine and bile are ideal specimens for detection of THC-g after an acute cannabis dose. The distribution data from this study adds substantially to the limited information available for cannabinoid distribution.

Using rabbit physiological parameters, THC chemical-specific parameters, and the cannabis administration protocol, we developed a PBPK model for THC in rabbits via the inhalation route. To our knowledge, this is the first PBPK model for THC in any animal with inhaled cannabis administration. Chapter 4 explains the equations and coding utilized in the Berkeley Madonna software to simulate THC concentrations in various

tissues. We compared the predicted concentrations from the nine compartment, flow-limited model to THC concentrations observed experimentally in rabbit samples collected for the distribution study. Observed THC concentrations in blood and tissues from rabbits sampled immediately upon death following cannabis administration were similar to the simulated THC concentrations from the PBPK model.

Postmortem Redistribution of THC in Rabbits

We describe the experimental design to evaluate time- and temperature-dependent THC concentration changes after death in rabbits. After cannabis administration and euthanasia, we stored the study rabbits in either refrigerated or room temperature conditions and biological samples collected at 2, 6, 16, 24 or 36 h after death. No statistically significant difference was observed for THC concentrations between rabbits stored at room temperature or refrigerated conditions. When THC concentrations from various postmortem intervals were compared to THC concentrations from control animals, significant THC concentration changes in heart and peripheral blood concentrations were noted only in refrigerated samples collected 2 and 16 h after death, respectively. Mean C:P and L:P ratios for THC were 1.6 and 2.9, respectively. The data presented suggest that THC is not prone to substantial postmortem redistribution in the rabbit.

Future Directions

Extrapolation from the rabbit PBPK model to a human PBPK model would provide valuable information regarding tissue concentrations expected after cannabis administration. The data can reveal which tissues are expected to have high THC

concentrations following inhaled cannabis exposure. This information will guide forensic toxicologists testing postmortem samples for the presence of cannabinoids in the selection of tissues that are most suitable for determination of THC concentrations and may aid in interpretation of THC concentrations in tissue samples.

Results from Chapter 5 are the first to report L:P ratios for THC in animals. Mean (range) L:P ratios for THC were 2.9 (0.3 – 8.9) for refrigerated and room temperature rabbits across all postmortem intervals tested. As the mean is below 5, the findings suggest that little to no postmortem redistribution exists for THC; however, more research projects reporting L:P ratios, particularly in human subjects, will help substantiate this finding.

REFERENCES

1. United Nations Office on Drugs and Crime. 2020. World Drug Report 2020. In *United Nations publication, Sales No. E.20.XI.6*.
2. Adams IB, Martin BR 1996. Cannabis: pharmacology and toxicology in animals and humans. *Addiction* 91:1585-614.
3. Substance Abuse and Mental Health Services Administration. 2020. Key substance use and mental health indicators in the United States: Results from the 2019 National Survey on Drug Use and Health Rockville, MD.
4. Meich RA, Schulenberg JE, Johnston LD, Bachman JG, O'Malley PM, Patrick, ME. 2019. National Adolescent Drug Trends in 2019: Findings Released. Ann Arbor, MI.
5. National Conference State Legislatures. 2020. *State Medical Marijuana Laws*. https://www.ncsl.org/research/health/state-medical-marijuana-laws.aspx.
6. Chandra SR, Radwan MM, Majumdar, CG, Church, JC, Freeman TP, ElSohly MA. 2019. New trends in cannabis potency in USA and Europe during the last decade (2008-2017). *Eur Arch Psychiatry Clin Neurosci* 269:5-15.
7. Kahn CA. 2015. National Highway Traffic Safety Administration (NHTSA) Notes. Results of the 2013-2014 National Roadside Survey of Alcohol and Drug Use by Drivers. *Ann Emerg Med* 66:669.
8. Asbridge MH, Hayden JA, Cartwright JL. 2012. Acute cannabis consumption and motor vehicle collision risk: systematic review of observational studies and meta-analysis. *Bmj* 344:e536.

9. McKay MP, Groff L. 2016. 23 years of toxicology testing fatally injured pilots: Implications for aviation and other modes of transportation. *Accid Anal Prev* 90:108-17.
10. Farnsworth NR. 1969. Pharmacognosy and chemistry of "cannabis sativa". *J Am Pharm Assoc* 9:410-4 passim.
11. Bonn-Miller MO, ElSohly MA, Loflin, MJ, Chandra S, Vandrey R. 2018. Cannabis and cannabinoid drug development: evaluating botanical versus single molecule approaches. *Int Rev Psychiatry* 30:277-84.
12. Sharma P, Murthy P, Bharath MM. 2012. Chemistry, metabolism, and toxicology of cannabis: clinical implications. *Iran J Psychiatry* 7:149-56.
13. Taura FS, Sirikantaramas S, Shoyama Y, Shoyama Y, Morimoto S. 2007. Phytocannabinoids in Cannabis sativa: recent studies on biosynthetic enzymes. *Chem Biodivers* 4:1649-63.
14. Taura FM, Morimoto S, Shoyama Y. 1996. Purification and characterization of cannabidiolic-acid synthase from Cannabis sativa L.. Biochemical analysis of a novel enzyme that catalyzes the oxidocyclization of cannabigerolic acid to cannabidiolic acid. *J Biol Chem* 271:17411-6.
15. Taura FM, Morimoto S, Shoyama Y, Mechoulam R. 1995. First direct evidence for the mechanism of .DELTA.1-tetrahydrocannabinolic acid biosynthesis. *Journal of the American Chemical Society* 117:9766-7.
16. ElSohly MA, Mehmedic Z, Foster S, Gon C, Chandra S, Church JC. 2016. Changes in Cannabis Potency Over the Last 2 Decades (1995-2014): Analysis of Current Data in the United States. *Biol Psychiatry* 79:613-9.
17. Cinnamon Bidwell L, YorkWilliams SL, Mueller RL, Bryan AD, Hutchison KE. 2018. Exploring cannabis concentrates on the legal market: User profiles, product strength, and health-related outcomes. *Addictive Behaviors Reports* 8:102-6.
18. Freeman TP, Winstock AR. 2015. Examining the profile of high-potency cannabis and its association with severity of cannabis dependence. *Psychol Med* 45:3181-9.

19. Roberts BA. 2019. Legalized Cannabis in Colorado Emergency Departments: A Cautionary Review of Negative Health and Safety Effects. *West J Emerg Med* 20:557-72.
20. Turner CE, Hadley KW, Fetterman PS, Doorenbos NJ, Quimby MW, Waller C. 1973. Constituents of Cannabis sativa L. IV. Stability of cannabinoids in stored plant material. *J Pharm Sci* 62:1601-5.
21. Yamamoto I, Watanabe K, Kuzuoka K, Narimatsu S, Yoshimura H. 1987. The pharmacological activity of cannabinol and its major metabolite, 11-hydroxycannabinol. *Chem Pharm Bull (Tokyo)* 35:2144-7.
22. Englund A, Morrison PD, Nottage J, Hague D, Kane F, Bonaccorso S, Stone JM, Reichenberg A, Brenneisen R, Holt D, Feilding A, Walker L, Murray RM, Kapur S. 2013. Cannabidiol inhibits THC-elicited paranoid symptoms and hippocampal-dependent memory impairment. *J Psychopharmacol* 27:19-27.
23. Freeman AM, Petrilli K, Lees R, Hindocha C, Mokrysz C, Curran HV, Saunders R, Freeman TP. 2019. How does cannabidiol (CBD) influence the acute effects of delta-9-tetrahydrocannabinol (THC) in humans? A systematic review. *Neurosci Biobehav Rev* 107:696-712.
24. Scuderi C, Fillipis DD, Iuvone T, Blasio A, Steardo A, Esposito G. 2009. Cannabidiol in medicine: a review of its therapeutic potential in CNS disorders. *Phytother Res* 23:597-602.
25. Karschner EL, Darwin WD, Goodwin RS, Wright S, Huestis MA. 2011. Plasma cannabinoid pharmacokinetics following controlled oral delta9-tetrahydrocannabinol and oromucosal cannabis extract administration. *Clin Chem* 57:66-75.
26. Karschner EL, Darwin WD, McMahon RP, Liu F, Wright S, et al. 2011. Subjective and physiological effects after controlled Sativex and oral THC administration. *Clin Pharmacol Ther* 89:400-7.
27. Colizzi M, Bhattacharyya S. 2017. Does Cannabis Composition Matter? Differential Effects of Delta-9-tetrahydrocannabinol and Cannabidiol on Human Cognition. *Curr Addict Rep* 4:62-74.

28. ElSohly MA, Slade D. 2005. Chemical constituents of marijuana: The complex mixture of natural cannabinoids. *Life Sciences* 78:539-48.
29. Nachnani R, Raup-Konsavage WM, Vrana KE. 2021. The Pharmacological Case for Cannabigerol. *Journal of Pharmacology and Experimental Therapeutics* 376:204.
30. Newmeyer MN, Swortwood MJ, Barnes AJ, Abulseoud OA, Scheidweiler KB, Huestis MA. 2016. Free and Glucuronide Whole Blood Cannabinoids' Pharmacokinetics after Controlled Smoked, Vaporized, and Oral Cannabis Administration in Frequent and Occasional Cannabis Users: Identification of Recent Cannabis Intake. *Clin Chem* 62:1579-92.
31. AbbVie Inc. *Marinol (dronnabinol) [package insert]*. https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/018651s029lbl.pdf
32. ElSohly MA, deWit H, Wachtel SR, Feng S, Murphy TP. 2001. Delta9-tetrahydrocannabivarin as a marker for the ingestion of marijuana versus Marinol: results of a clinical study. *J Anal Toxicol* 25:565-71.
33. ElSohly MA, Feng S, Murphy TP, Warrington AW, Ross S, Nimrod A, Mehmedic Z, Fortner N. 2001. Identification and quantitation of 11-nor-delta9-tetrahydrocannabivarin-9-carboxylic acid, a major metabolite of delta9-tetrahydrocannabivarin. *J Anal Toxicol* 25:476-80.
34. Devane WA, Dysarz FA, Johnson MR, Melvin LS, Howlett AC. 1988. Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* 34:605-13.
35. Lowin T, Straub RH. 2015. Cannabinoid-based drugs targeting CB1 and TRPV1, the sympathetic nervous system, and arthritis. *Arthritis Res Ther* 17:226.
36. Zou S, Kumar U. 2018. Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *Int J Mol Sci* 19.
37. Raymon LP, Walls HC. 2005. Pharmacology of cannabinoids. In *Marijuana and the Cannabinoids*, ed. MA ElSohly:97-123. New York: Humana Press. Number of 97-123 pp.
38. Cabral GA, Ferreira GA, Jamerson MJ. 2015. Endocannabinoids and the Immune System in Health and Disease. *Handb Exp Pharmacol* 231:185-211.

39. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258:1946-9.
40. Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K. 1995. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 215:89-97.
41. Lu HC, Mackie K. 2016. An Introduction to the Endogenous Cannabinoid System. *Biol Psychiatry* 79:516-25.
42. Tsuboi K, Uyama T, Okamoto Y, Ueda N. 2018. Endocannabinoids and related N-acylethanolamines: biological activities and metabolism. *Inflammation and Regeneration* 38:28.
43. Blankman JL, Simon GM, Cravatt BF. 2007. A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem Biol* 14:1347-56.
44. Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. 1996. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384:83-7.
45. Ohlsson A, Lindgren JE, Wahlen A, Agurell S, Hollister LE, Gillespie HK. 1980. Plasma delta-9 tetrahydrocannabinol concentrations and clinical effects after oral and intravenous administration and smoking. *Clin Pharmacol Ther* 28:409-16.
46. Huestis MA, Henningfield JE, Cone EJ. 1992. Blood cannabinoids. I. Absorption of THC and formation of 11-OH-THC and THCCOOH during and after smoking marijuana. *J Anal Toxicol* 16:276-82.
47. Huestis MA. 2007. Human cannabinoid pharmacokinetics. *Chem Biodivers* 4:1770-804.
48. Grotenhermen F. 2003. Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin Pharmacokinet* 42:327-60.
49. Brunet B, Doucet C, Venisse N, Hauet T, Hébrard W, Papet Y, Mauco G, Mura P. 2006. Validation of Large White Pig as an animal model for the study of cannabinoids metabolism: application to the study of THC distribution in tissues. *Forensic Sci Int* 161:169-74.

50. Gunasekaran N, Long LE, Dawson BL, Hansen GH, Richardson DP, Li KM, Arnold JC, McGregor IS. 2009. Reintoxication: the release of fat-stored delta(9)-tetrahydrocannabinol (THC) into blood is enhanced by food deprivation or ACTH exposure. *Br J Pharmacol* 158:1330-7.
51. Mazur A, Lichti CF, Prather PL, Zielinska AK, Bratton SM, Gallus-Zawada A, Finel M, Miller GP, Radomińska-Pandya A, Moran JH. 2009. Characterization of human hepatic and extrahepatic UDP-glucuronosyltransferase enzymes involved in the metabolism of classic cannabinoids. *Drug Metab Dispos* 37:1496-504.
52. Huestis MA. 2005. Pharmacokinetics and metabolism of the plant cannabinoids, delta-9-tetrahydrocannabinol, cannabidiol, and cannabinol. In *Handbook of Experimental Pharmacology*, ed. RG Pertwee, 168:657-90. New York: Springer. Number of 657-90 pp.
53. Burstein S, Rosenfeld J, Wittstruck T. 1972. Isolation and characterization of two major urinary metabolites of 1 -tetrahydrocannabinol. *Science* 176:422-3.
54. Wall ME, Sadler BM, Brine D, Taylor H, Perez-Reyes M. 1983. Metabolism, disposition, and kinetics of delta-9-tetrahydrocannabinol in men and women. *Clin Pharmacol Ther* 34:352-63.
55. Williams PL, Moffat AC. 1980. Identification in human urine of delta 9-tetrahydrocannabinol-11-oic acid glucuronide: a tetrahydrocannabinol metabolite. *J Pharm Pharmacol* 32:445-8.
56. Scheidweiler KB, Desrosiers NA, Huestis MA. 2012. Simultaneous quantification of free and glucuronidated cannabinoids in human urine by liquid chromatography tandem mass spectrometry. *Clin Chim Acta* 413:1839-47.
57. Ashton CH. 1999. Adverse effects of cannabis and cannabinoids. *Br J Anaesth* 83:637-49.
58. McClure EA, Stitzer ML, Vandrey R. 2012. Characterizing smoking topography of cannabis in heavy users. *Psychopharmacology (Berl)* 220:309-18.
59. Schwope DM, Bosker WM, Ramaekers JG, Gorelick DA, Huestis MA. 2012. Psychomotor performance, subjective and physiological effects and whole blood Δ^9-tetrahydrocannabinol concentrations in heavy, chronic cannabis smokers following acute smoked cannabis. *J Anal Toxicol* 36:405-12.

60. Huestis MA. 2002. Cannabis (Marijuana) - Effects on Human Performance and Behavior. *Forensic Sci Rev* 14:15-60.
61. Ramaekers JG, Berghaus G, van Laar M, Drummer OH. 2004. Dose related risk of motor vehicle crashes after cannabis use. *Drug Alcohol Depend* 73:109-19.
62. Drummer OH, Gerostamoulous J, Batziris H, Chu M, Caplehorn J, Robertson MD, Swann P. 2004. The involvement of drugs in drivers of motor vehicles killed in Australian road traffic crashes. *Accid Anal Prev* 36:239-48.
63. Longo MC, Hunter CE, Lokan RJ, White JM, White MA. 2000. The prevalence of alcohol, cannabinoids, benzodiazepines and stimulants amongst injured drivers and their role in driver culpability: part ii: the relationship between drug prevalence and drug concentration, and driver culpability. *Accid Anal Prev* 32:623-32.
64. Drummer OH, Gerostamoulos D, Di Rago M, Woodford NW, Morris C, Frederiksen T, Jachno K, Wolfe R. 2020. Odds of culpability associated with use of impairing drugs in injured drivers in Victoria, Australia. *Accid Anal Prev* 135:105389.
65. Ramaekers JG. 2018. Driving Under the Influence of Cannabis: An Increasing Public Health Concern. *JAMA* 319:1433-4.
66. Hartman RL, Huestis MA. 2013. Cannabis effects on driving skills. *Clin Chem* 59:478-92.
67. Ramaekers JG, Robbe HW, O'Hanlon JF. 2000. Marijuana, alcohol and actual driving performance. *Hum Psychopharmacol* 15:551-8.
68. Janowsky DS, Meacham MP, Blaine JD, Schoor M, Bozzetti LP. 1976. Marijuana effects on simulated flying ability. *Am J Psychiatry* 133:384-8.
69. Yesavage JA, Leirer VO, Denari M, Hollister LE. 1985. Carry-over effects of marijuana intoxication on aircraft pilot performance: a preliminary report. *Am J Psychiatry* 142:1325-9.
70. Thompson GR, Rosenkrantz H, Schaeppi, Ulrich H, Braude MC. 1973. Comparison of acute oral toxicity of cannabinoids in rats, dogs and monkeys. *Toxicology and Applied Pharmacology* 25:363-72.

71. Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC. 990. Cannabinoid receptor localization in brain. *Proc Natl Acad Sci U S A* 87:1932-6.
72. Wong KU, Baum CR. 2019. Acute Cannabis Toxicity. *Pediatr Emerg Care* 35:799-804.
73. Noble MJ, Hedberg K, Hendrickson RG. 2019. Acute cannabis toxicity. *Clin Toxicol (Phila)* 57:735-42.
74. Jones RT. 2002. Cardiovascular system effects of marijuana. *J Clin Pharmacol* 42:58s-63s.
75. Latif Z, Garg N. 2020. The Impact of Marijuana on the Cardiovascular System: A Review of the Most Common Cardiovascular Events Associated with Marijuana Use. *J Clin Med* 9.
76. Rezkalla S, Kloner RA. 2019. Cardiovascular effects of marijuana. *Trends in Cardiovascular Medicine* 29:403-7.
77. Drummer OH, Gerostamoulos D, Woodford NW. 2019. Cannabis as a cause of death: A review. *Forensic Sci Int* 298:298-306.
78. Bachs L, Morland H. 2001. Acute cardiovascular fatalities following cannabis use. *Forensic Science International* 124:200-3.
79. Hartung B, Kauferstein S, Ritz-Timme S, Daldrup T. 2014. Sudden unexpected death under acute influence of cannabis. *Forensic Science International* 237:e11-e3.
80. Kudo K, Nagata T, Kimura K, Imamura T, Jitsufuchi N. 1995. Sensitive determination of delta 9-tetrahydrocannabinol in human tissues by GC-MS. *J Anal Toxicol* 19:87-90.
81. Karschner EL, Barnes AJ, Lowe RH, Scheidweiler KB, Huestis MA. 2010. Validation of a two-dimensional gas chromatography mass spectrometry method for the simultaneous quantification of cannabidiol, Delta(9)-tetrahydrocannabinol (THC), 11-hydroxy-THC, and 11-nor-9-carboxy-THC in plasma. *Anal Bioanal Chem* 397:603-11.

82. Castro AL, Tarelho S, Melo P, Franco JM. 2018. A fast and reliable method for quantitation of THC and its 2 main metabolites in whole blood by GC-MS/MS (TQD). *Forensic Sci Int* 289:344-51.
83. Scheidweiler KB, Newmeyer MN, Barnes AJ, Huestis MA. 2016. Quantification of cannabinoids and their free and glucuronide metabolites in whole blood by disposable pipette extraction and liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 1453:34-42.
84. Andrews R, Paterson S. 2012. A validated method for the analysis of cannabinoids in post-mortem blood using liquid-liquid extraction and two-dimensional gas chromatography-mass spectrometry. *Forensic Sci Int* 222:111-7.
85. Gronewold A, Skopp G. 2011. A preliminary investigation on the distribution of cannabinoids in man. *Forensic Sci Int* 210:e7-e11.
86. Saenz SR, Lewis RJ, Angier MK, Wagner JR. 2017. Postmortem Fluid and Tissue Concentrations of THC, 11-OH-THC and THC-COOH. *J Anal Toxicol* 41:508-16.
87. Schwope DM, Scheidweiler KB, Huestis MA. 2011. Direct quantification of cannabinoids and cannabinoid glucuronides in whole blood by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* 401:1273-83.
88. Rositano J, Harpas P, Kostakis C, Scott T. 2016. Supported liquid extraction (SLE) for the analysis of methylamphetamine, methylenedioxymethylamphetamine and delta-9-tetrahydrocannabinol in oral fluid and blood of drivers. *Forensic Sci Int* 265:125-30.
89. Żwir-Ferenc A, Biziuk M. 2006. Solid phase extraction technique – Trends, opportunities and applications. *Polish J. of Environ. Stud* 15:677-90.
90. Lemos NP, Ingle E. A. 2011. Cannabinoids in postmortem toxicology. *J Anal Toxicol* 35:394-401.
91. Andrews R, Murphy KG, Nahar L, Paterson S. 2015. Cannabinoid concentrations detected in fatal road traffic collision victims compared with a population of other postmortem cases. *Clin Chem* 61:1256-64.
92. Hoffman MA, Trochta A, Gary RD, Fitzgerald RL, McIntyre IM. 2020. An evaluation of postmortem concentrations of Δ9-tetrahydrocannabinol (THC) and

11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THCCOOH). *Forensic Science International* 315:110414.

93. Kemp PM, Cardona PS, Chaturvedi AK, Soper JW. 2015. Distribution of Δ(9)-Tetrahydrocannabinol and 11-Nor-9-Carboxy-Δ(9)-Tetrahydrocannabinol Acid in Postmortem Biological Fluids and Tissues From Pilots Fatally Injured in Aviation Accidents. *J Forensic Sci* 60:942-9.
94. Leuschner JT, Harvey DJ, Bullingham RE, Paton WD. 1986. Pharmacokinetics of delta 9-tetrahydrocannabinol in rabbits following single or multiple intravenous doses. *Drug Metab Dispos* 14:230-8.
95. Mura P, Kintz P, Dumestre V, Raul S, Hauet T. 2005. THC can be detected in brain while absent in blood. *J Anal Toxicol* 29:842-3.
96. Withey SL, Bergman J, Huestis MA, George SR, Madras BK. 2020. THC and CBD blood and brain concentrations following daily administration to adolescent primates. *Drug and Alcohol Dependence* 213:108129.
97. Pélissier-Alicot AL, Gaulier JM, Champsaur P, Marquet P. 2003. Mechanisms underlying postmortem redistribution of drugs: a review. *J Anal Toxicol* 27:533-44.
98. Yarema MC, Becker CE. 2005. Key concepts in postmortem drug redistribution. *Clin Toxicol (Phila)* 43:235-41.
99. Pounder DJ, Jones GR. 1990. Post-mortem drug redistribution--a toxicological nightmare. *Forensic Sci Int* 45:253-63.
100. Kennedy M. 2015. Interpreting postmortem drug analysis and redistribution in determining cause of death: a review. *Pathology and Laboratory Medicine International*:55-62.
101. Prouty RW, Anderson WH. 1990. The forensic science implications of site and temporal influences on postmortem blood-drug concentrations. *J Forensic Sci* 35:243-70.
102. Cook DS, Braithwaite RA, Hale KA. 2000. Estimating antemortem drug concentrations from postmortem blood samples: the influence of postmortem redistribution. *J Clin Pathol* 53:282-5.

103. Leikin JB, Watson WA. 2003. Post-mortem toxicology: what the dead can and cannot tell us. *J Toxicol Clin Toxicol* 41:47-56.
104. McIntyre IM, Sherrard J, Lucas J. 2012. Postmortem carisoprodol and meprobamate concentrations in blood and liver: lack of significant redistribution. *J Anal Toxicol* 36:177-81.
105. McIntyre IM. 2014. Liver and peripheral blood concentration ratio (L/P) as a marker of postmortem drug redistribution: a literature review. *Forensic Sci Med Pathol* 10:91-6.
106. Drummer OH. 2004. Postmortem toxicology of drugs of abuse. *Forensic Sci Int* 142:101-13.
107. Hilberg T, Rodge S, Mørland, J. 1999. Postmortem drug redistribution--human cases related to results in experimental animals. *J Forensic Sci* 44:3-9.
108. Brunet B, Hauet T, Hébrard W, Papet Y, Mauco G, Mura P. 2010. Postmortem redistribution of THC in the pig. *Int J Legal Med* 124:543-9.
109. Holland MG, Schwope DM, Stoppacher R, Gillen SB, Huestis MA. 2011. Postmortem redistribution of Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (THCCOOH). *Forensic Sci Int* 212:247-51.
110. Sager JE, Yu J, Ragueneau-Majlessi I, Isoherranen N. 2015. Physiologically Based Pharmacokinetic (PBPK) Modeling and Simulation Approaches: A Systematic Review of Published Models, Applications, and Model Verification. *Drug Metab Dispos* 43:1823-37.
111. Jones H, Rowland-Yeo K. 2013. Basic concepts in physiologically based pharmacokinetic modeling in drug discovery and development. *CPT Pharmacometrics Syst Pharmacol* 2:e63.
112. Kuepfer L, Niederalt C, Wendl T, Schlender JF, Willmann S, Lippert J, Block M, Eissing T, Teutonico D. 2016. Applied Concepts in PBPK Modeling: How to Build a PBPK/PD Model. *CPT Pharmacometrics Syst Pharmacol* 5:516-31.
113. Peters S. 2012. Modeling in the Pharmaceutical Industry. In *Physiologically-Based Pharmacokinetic (PBPK) Modeling and Simulations*:1-12. Number of 1-12 pp.

114. Andersen ME, Yang R, Clewell SH, Harvey J, Reddy M B. 2005. Introduction: A Historical Perspective of the Development and Applications of PBPK Models. In *Physiologically Based Pharmacokinetic Modeling*:1-18. Number of 1-18 pp.
115. Methaneethorn J, Naosang K, Kaewworasut P, Poomsaidorn C, Lohitnavy M. 2020. Development of a Physiologically-Based Pharmacokinetic Model of Δ(9)-Tetrahydrocannabinol in Mice, Rats, and Pigs. *Eur J Drug Metab Pharmacokinet* 45:487-94.
116. Bornheim LM, Kim KY, Li J, Perotti BY, Benet LZ. 1995. Effect of cannabidiol pretreatment on the kinetics of tetrahydrocannabinol metabolites in mouse brain. *Drug Metab Dispos* 23:825-31.
117. Klausner HA, Dingell JV. 1971. The metabolism and excretion of delta 9-tetrahydrocannabinol in the rat. *Life Sci I* 10:49-59.
118. Methaneethorn J, Poomsaidorn C, Naosang K, Kaewworasut P, Lohitnavy M. 2020. A Δ(9)-Tetrahydrocannabinol Physiologically-Based Pharmacokinetic Model Development in Humans. *Eur J Drug Metab Pharmacokinet* 45:495-511.
119. Hunault CC, Mensinger TT, de Vries I, Kelholt-Dijkman HH, Hoek J, Kruidenier M, Leenders ME, Meulenbelt J. 2008. Delta-9-tetrahydrocannabinol (THC) serum concentrations and pharmacological effects in males after smoking a combination of tobacco and cannabis containing up to 69 mg THC. *Psychopharmacology (Berl)* 201:171-81.
120. Naef M, Russmann S, Petersen-Felix S, Brenneisen R. 2004. Development and pharmacokinetic characterization of pulmonal and intravenous delta-9-tetrahydrocannabinol (THC) in humans. *J Pharm Sci* 93:1176-84.
121. Wall ME, Perez-Reyes M. 1981. The metabolism of delta 9-tetrahydrocannabinol and related cannabinoids in man. *J Clin Pharmacol* 21:178s-89s.
122. Meneses V, Hernandez M. 2021. Cannabinoid distribution in postmortem samples. *Forensic Chemistry* 23:100303.
123. Al-Asmari AI. 2019. Method for Postmortem Quantification of Δ9-Tetrahydrocannabinol and Metabolites Using LC-MS-MS. *J Anal Toxicol* 43:703-19.

124. Al-Asmari AI. 2020. Method for Postmortem Tissue Quantification of Δ9-Tetrahydrocannabinol and Metabolites Using LC-MS-MS. *J Anal Toxicol* 44:718-33.
125. Schaefer N, Kroll AK, Körbel C, Laschke MW, Menger MD, Maurer HH, Meyer MR, Schmidt PH. 2020. Time- and temperature-dependent postmortem concentration changes of the (synthetic) cannabinoids JWH-210, RCS-4, as well as Δ9-tetrahydrocannabinol following pulmonary administration to pigs. *Arch Toxicol* 94:1585-99.
126. Bornheim LM, Lasker JM, Raucy JL. 1992. Human hepatic microsomal metabolism of delta 1-tetrahydrocannabinol. *Drug Metab Dispos* 20:241-6.
127. Aizpurua-Olaizola O, Soydaner U, Öztürk E, Schibano D, Simsir Y, Navarro P, Etxebarria N, Usobiaga A. 2016. Evolution of the Cannabinoid and Terpene Content during the Growth of Cannabis sativa Plants from Different Chemotypes. *J Nat Prod* 79:324-31.
128. Desrosiers NA, Hines SK, Scheidweiler KB, Concheiro-Guisan M, Gorelick DA, Huestis MA. 2014. Phase I and II cannabinoid disposition in blood and plasma of occasional and frequent smokers following controlled smoked cannabis. *Clin Chem* 60:631-43.
129. Schwope DM, Karschner EL, Gorelick DA, Huestis MA. 2011. Identification of recent cannabis use: whole-blood and plasma free and glucuronidated cannabinoid pharmacokinetics following controlled smoked cannabis administration. *Clin Chem* 57:1406-14.
130. Abraham TT, Lowe RH, Pirnay SO, Darwin WD, Huestis MA. 2007. Simultaneous GC-EI-MS determination of Delta9-tetrahydrocannabinol, 11-hydroxy-Delta9-tetrahydrocannabinol, and 11-nor-9-carboxy-Delta9-tetrahydrocannabinol in human urine following tandem enzyme-alkaline hydrolysis. *J Anal Toxicol* 31:477-85.
131. Kemp PM, Abukhalaf IK, Manno JE, Manno BR, Alford DD, Abusada, GA. 1995. Cannabinoids in humans. I. Analysis of delta 9-tetrahydrocannabinol and six metabolites in plasma and urine using GC-MS. *J Anal Toxicol* 19:285-91.

132. Stout PR, Horn CK, Klette KL. 2001. Solid-phase extraction and GC-MS analysis of THC-COOH method optimized for a high-throughput forensic drug-testing laboratory. *J Anal Toxicol* 25:550-4.
133. Arzimanoglou A, Brandi U, Cross JH, Gil-Nagel A, Lagae L, Landmark CJ, Specchio N, Nabbout R, Thiele EA, Gubbay O. The Cannabinoids International Experts, Panel. 2020. Epilepsy and cannabidiol: a guide to treatment. *Epileptic Disord* 22:1-14.
134. 2013. Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. *J Anal Toxicol* 37:452-74.
135. Hoffman MA, Trochta A, Gary RD, Fitzgerald RL, McIntyre IM. 2020. An evaluation of postmortem concentrations of Δ(9)-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-Δ(9)-tetrahydrocannabinol (THCCOOH). *Forensic Sci Int* 315:110414.
136. Kerrigan S. 2013. Sampling, storage and stability. In *Clarke's Analytical Forensic Toxicology*, ed. AC Negrusz, G,335-56: Pharmaceutical Press. Number of 335-56 pp.
137. Ho S. 2013. Challenges of atypical matrix effects in tissue. *Bioanalysis* 5:2333-5.
138. Hubbard JA, Smith BE, Sobolesky PM, Kim S, Hoffman MA, Stone J, Huestis MA, Grelotti DJ, Grant I, Marcotte TD, Fitzgerald RL. 2020. Validation of a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to detect cannabinoids in whole blood and breath. *Clin Chem Lab Med* 58:673-81.
139. Hanisch S, Paulke A, Toennes SW. 2017. 11-nor-9-carboxy-Δ(9)-tetrahydrocannabinol glucuronide exhibits acyl-migration isomers. *J Pharm Biomed Anal* 146:261-5.
140. Janowsky DS, Meachamn MP, Blaine JD, Schoor M, Bozzetti LP. 1976. Simulated flying performance after marihuana intoxication. *Aviat Space Environ Med* 47:124-8.
141. Meacham MP, Janowsky DS, Blaine JD, Bozetti LP, Schorr M. 1974. Letter: Effects of marihuana on flying ability. *Jama* 230:1258.
142. Lin DL, Lin RL. 2005. Distribution of 11-nor-9-carboxy-Delta9-tetrahydrocannabinol in traffic fatality cases. *J Anal Toxicol* 29:58-61.

143. Fabritius M, Staub C, Mangin P, Giroud C. 2012. Distribution of free and conjugated cannabinoids in human bile samples. *Forensic Sci Int* 223:114-8.
144. Cliburn KD, Huestis MA, Wagner JR, Kemp PM, . 2021. Identification and Quantification of 12 Cannabinoids in Postmortem Fluids and Tissues by LC-MS/MS.
145. Huestis MA, Henningfield JE, and Cone E.J. 1992. Blood cannabinoids I. Absorption of THC and formation of 11-OH-THC and THCCOOH during and after smoking marijuana. *Journal of Analytical Toxioclogy* 16:276-82.
146. Desrosiers NA, Lee D, Concheiro-Guisan M, Scheidweiler K B, Gorelick DA, Huestis MA. 2014. Urinary cannabinoid disposition in occasional and frequent smokers: is THC-glucuronide in sequential urine samples a marker of recent use in frequent smokers? *Clin Chem* 60:361-72.
147. Kemp PM, Abukhalaf IK, Manno JE, Manno BR, Alford DD, McWilliams ME, Nixon FE, Fitzgerald MJ, Reeves RR, Wood MJ. 1995. Cannabinoids in humans. II. The influence of three methods of hydrolysis on the concentration of THC and two metabolites in urine. *J Anal Toxicol* 19:292-8.
148. Huestis MA, Sempio C, Newmeyer MN, Andersson M, Barnes AJ, Abulseoud OA, Blount BC, Schroeder J, Smith ML. 2020. Free and Glucuronide Urine Cannabinoids after Controlled Smoked, Vaporized and Oral Cannabis Administration in Frequent and Occasional Cannabis Users. *J Anal Toxicol* 44:651-60.
149. Jones G. 2013. Postmortem Specimens. In *Encyclopedia of Forensic Sciences*, ed. JS Siegel, PJ: Houck, MM, pp. 270-4: Elsevier Ltd.
150. Peres MD, Pelicao FS, Caleffi B, De Martinis BS. 2014. Simultaneous quantification of cocaine, amphetamines, opiates and cannabinoids in vitreous humor. *J Anal Toxicol* 38:39-45.
151. Jenkins AJ, Oblock J. 2008. Phencyclidine and cannabinoids in vitreous humor. *Leg Med (Tokyo)* 10:201-3.
152. Garza AZ, Park SB, Kocz R. 2020. Drug Elimination. In *StatPearls*. Treasure Island (FL): StatPearls Publishing: Copyright © 2020, StatPearls Publishing LLC. Number of.

153. Hložek T, Uttl L, Kadeřábek L, Balíková M, Lhotková E, et al. 2017. Pharmacokinetic and behavioural profile of THC, CBD, and THC+CBD combination after pulmonary, oral, and subcutaneous administration in rats and confirmation of conversion in vivo of CBD to THC. *European Neuropsychopharmacology* 27:1223-37.
154. Cataldi M, Vigliotti C, Mosca T, Cammarota M, Capone D. 2017. Emerging Role of the Spleen in the Pharmacokinetics of Monoclonal Antibodies, Nanoparticles and Exosomes. *Int J Mol Sci* 18.
155. Levine WG. 1978. Biliary excretion of drugs and other xenobiotics. *Annu Rev Pharmacol Toxicol* 18:81-96.
156. Bergamaschi MM, Karschner EL, Goodwin RS, Scheidweiler KB, Hirvonen J, Queiroz RH, Huestis MA. 2013. Impact of prolonged cannabinoid excretion in chronic daily cannabis smokers' blood on per se drugged driving laws. *Clin Chem* 59:519-26.
157. Kogan NM, Mechoulam R. 2007. Cannabinoids in health and disease. *Dialogues Clin Neurosci* 9:413-30.
158. ElSohly MA, Radwan MM, Gul W, Chandra S, Galal A. 2017. Phytochemistry of Cannabis sativa L. *Prog Chem Org Nat Prod* 103:1-36.
159. Beal JE, Olson R, Lefkowitz L, Laubenstein L, Bellman,P, Yangco B, Morales JO, Murphy R, Powderly W, Plasse TF, Mosdell KW, Shepard KV. 1997. Long-term efficacy and safety of dronabinol for acquired immunodeficiency syndrome-associated anorexia. *J Pain Symptom Manage* 14:7-14.
160. Russo EB. 2019. Cannabis and Pain. *Pain Med* 20:2083-5.
161. Maccarrone M, Battista N, Centonze D. 2007. The endocannabinoid pathway in Huntington's disease: a comparison with other neurodegenerative diseases. *Prog Neurobiol* 81:349-79.
162. Huestis MA, Smith ML. 2005. Human cannabinoid pharmacokinetics and interpretation of cannabinoid concentrations in biological fluids and tissues. In *Marijuana and the Cannabinoids*, ed. MA ElSohly:205-35. New York: Humana Press. Number of 205-35 pp.

163. Baselt RC. 2020. *Disposition of Toxic Drugs and Chemicals in Man, 12th Edition*. Seal Beach, CA: Biomedical Publications.
164. Lemberger L, Axelrod J, Kopin IJ. 1971. Metabolism and disposition of delta-9-tetrahydrocannabinol in man. *Pharmacol Rev* 23:371-80.
165. Garrett ER, Hunt CA. 1977. Pharmacokinetics of delta9-tetrahydrocannabinol in dogs. *J Pharm Sci* 66:395-407.
166. Perlin E, Smith CG, Nichols AI, Almirez R, Flora KP, Cradock JC, Peck CC. 1985. Disposition and bioavailability of various formulations of tetrahydrocannabinol in the rhesus monkey. *J Pharm Sci* 74:171-4.
167. Davies B, Morris T. 1993. Physiological parameters in laboratory animals and humans. *Pharm Res* 10:1093-5.
168. Patilea-Vrana GI, Unadkat JD. 2019. Quantifying Hepatic Enzyme Kinetics of (-)-Δ(9)-Tetrahydrocannabinol (THC) and Its Psychoactive Metabolite, 11-OH-THC, through In Vitro Modeling. *Drug Metab Dispos* 47:743-52.
169. Hunt CA, Jones RT. 1980. Tolerance and disposition of tetrahydrocannabinol in man. *J Pharmacol Exp Ther* 215:35-44.
170. Mumtaz MM, Ray M, Crowell SR, Keys D, Fisher J, Ruiz P. 2012. Translational research to develop a human PBPK models tool kit-volatile organic compounds (VOCs). *J Toxicol Environ Health A* 75:6-24.
171. DeLorme MP, Himmelstein MW, Kemper RA, Kegelman TA, Gargas ML, Kinzell JH. 2009. Evaluation of respiratory parameters in rats and rabbits exposed to methyl iodide. *Inhal Toxicol* 21:505-11.
172. Johnson RL, Gilbert M, Meschia G, Battaglia FC. 1985. Cardiac output distribution and uteroplacental blood flow in the pregnant rabbit: a comparative study. *Am J Obstet Gynecol* 151:682-6.
173. Sweeney LM, Kirman CR, Gannon SA, Thrall KD, Gargas ML, Kinzell JH. 2009. Development of a physiologically based pharmacokinetic (PBPK) model for methyl iodide in rats, rabbits, and humans. *Inhal Toxicol* 21:552-82.
174. Grotenhermen F. 2003. Clinical Pharmacokinetics of Cannabinoids. *Journal of Cannabis Therapeutics* 3:3-51.

175. Rodgers T, Leahy D Rowland M. 2005. Physiologically based pharmacokinetic modeling 1: predicting the tissue distribution of moderate-to-strong bases. *J Pharm Sci* 94:1259-76.
176. Badyal DK, Desai C. 2014. Animal use in pharmacology education and research: the changing scenario. *Indian J Pharmacol* 46:257-65.
177. Higashiyama H, Uemura M, Igarashi H, Kurohmaru M, Kanai-Azuma M, Kanai Y. 2018. Anatomy and development of the extrahepatic biliary system in mouse and rat: a perspective on the evolutionary loss of the gallbladder. *J Anat* 232:134-45.
178. Harvey DJ, Brown NK. 1991. Comparative in vitro metabolism of the cannabinoids. *Pharmacology Biochemistry and Behavior* 40:533-40.
179. Lemberger LS, Axelrod J, Kopin IJ. 1970. Marihuana: studies on the disposition and metabolism of delta-9-tetrahydrocannabinol in man. *Science* 170:1320-2.
180. Ben-Zvi Z, Burstein S. 1974. 7-Oxo-delta1-tetrahydrocannabinol: a novel metabolite of delta1-tetrahydrocannabinol. *Res Commun Chem Pathol Pharmacol* 8:223-9.
181. Moriya F, Hashimoto Y. 1999. Redistribution of basic drugs into cardiac blood from surrounding tissues during early-stages postmortem. *J Forensic Sci* 44:10-6.
182. Jones H, Rowland-Yeo K. 2013. Basic concepts in physiologically based pharmacokinetic modeling in drug discovery and development. *CPT Pharmacometrics Syst Pharmacol* 2:e63.
183. Poulin P, Theil FP. 2000. A priori prediction of tissue:plasma partition coefficients of drugs to facilitate the use of physiologically-based pharmacokinetic models in drug discovery. *J Pharm Sci* 89:16-35.
184. Willmann S, Lippert J, Schmitt W. 2005. From physicochemistry to absorption and distribution: predictive mechanistic modelling and computational tools. *Expert Opinion on Drug Metabolism & Toxicology* 1:159-68.
185. McKay MP, Groff L. 2016. 23 years of toxicology testing fatally injured pilots: Implications for aviation and other modes of transportation. *Accident Analysis & Prevention* 90:108-17.

186. Li G, Brady JE, Chen Q. 2013. Drug use and fatal motor vehicle crashes: A case-control study. *Accident Analysis & Prevention* 60:205-10.
187. Information NCfB. 2021. *PubChem Compound Summary for CID 16078, Dronabinol*. https://pubchem.ncbi.nlm.nih.gov/compound/Dronabinol.
188. Ramaekers JG, Kauert G, van Ruitenbeek P, Theunissen EL, Schneider E, Moeller MR. 2006. High-potency marijuana impairs executive function and inhibitory motor control. *Neuropsychopharmacology* 31:2296-303.
189. Hines LA, Freeman TP, Gage SH, Zammit S, Hickman M, et al. 2020. Association of High-Potency Cannabis Use With Mental Health and Substance Use in Adolescence. *JAMA Psychiatry* 77:1044-51.
190. Goyal H, Awad HH, Ghali JK. 2017. Role of cannabis in cardiovascular disorders. *J Thorac Dis* 9:2079-92.
191. Robertson MDD, O. H. 1998. Postmortem distribution and redistribution of nitrobenzodiazepines in man. *J Forensic Sci* 43:9-13.
192. Cliburn KD. 2021. Application of Physiologically Based Pharmacokinetic Modeling for Tetrahydrocannabinol in Rabbits following Controlled Cannabis Administration.
193. Brooks JW. 2016. Postmortem Changes in Animal Carcasses and Estimation of the Postmortem Interval. *Vet Pathol* 53:929-40.
194. Scheidweiler KB, Schwope DM, Karschner EL, Desrosiers NA, Gorelick DA, Huestis MA. 2013. In vitro stability of free and glucuronidated cannabinoids in blood and plasma following controlled smoked cannabis. *Clin Chem* 59:1108-17.
195. Chu MR, M. D, Mantinieks, D, Glowacki, L, Woodford, N. W, Gerostamoulos, D, Drummer, O. H. 2021. Time-Dependent Changes in THC Concentrations in Deceased Persons. *J Anal Toxicol* 45:1-7.
196. Hilberg TM, J, Bjørneboe, A. 1994. Postmortem release of amitriptyline from the lungs; a mechanism of postmortem drug redistribution. *Forensic Sci Int* 64:47-55.
197. Gerostamoulos DB, J, Staikos, V, Tayler, P, Woodford, N, Drummer, O. H. 2012. The effect of the postmortem interval on the redistribution of drugs: a comparison of mortuary admission and autopsy blood specimens. *Forensic Sci Med Pathol* 8:373-9.

198. Dalpe-Scott MD, M, Garbutt, D, Drost, M. 1995. A Comparison of Drug Concentrations in Postmortem Cardiac and Peripheral Blood in 320 Cases. *Canadian Society of Forensic Science Journal* 28:113-21.

199. McIntyre IMS, J, Lucas, J. 2012. Postmortem carisoprodol and meprobamate concentrations in blood and liver: lack of significant redistribution. *J Anal Toxicol* 36:177-81.

VITA

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