Trends in Molecular Medicine

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Special Issue: Biomarkers of Substance Abuse

Opinion

Cannabinoid Markers in Biological Fluids and Tissues: Revealing Intake

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Understanding cannabis and synthetic cannabinoid intake history is vital for treating drug dependence, investigating cannabinoid effects, and providing information to healthcare personnel, medical examiners, and public health officials; this is particularly relevant today with cannabis medicalization and legalization. Required information includes identifying exposure, time of use, frequency of use, relapse, withdrawal, and predicting cannabinoid effects. Recent controlled cannabinoid administration studies enable the development of models and markers to better identify patterns of intake and exposure. Future challenges include developing behavioral markers of cannabis impairment, bringing to market breathalyzers for cannabinoid detection, and identifying markers of recent cannabis intake in diverse biological matrices. We posit that biological monitoring of cannabinoids and metabolites will improve the characterization of cannabis and synthetic cannabinoid intake history.

Highlights

Medicinal cannabinoids are approved in 29 US states as of late 2016. Therapeutic drug monitoring of cannabinoids will become important in determining if these new drugs are safe and efficacious. If successful pharmacotherapies are approved, it will be necessary to define optimal cannabinoid concentrations of new markers for achieving desired effects.

Additional research is necessary to define the pharmacokinetics of cannabinoid markers in a variety of biological matrices after controlled cannabinoid administration by new routes including e-cigarettes and 'dabbing'.

Analysis of minor blood cannabinoids will become essential in identifying recent cannabis intake in frequent and occasional cannabis users.

Improved behavioral markers of cannabis intake are necessary to identify recent intake in driving under the influence of cannabis and other accident investigations.

Our understanding of the development of tolerance to cannabinoid effects will be improved following more extensive research.

A sensitive and specific breathalyzer for Δ9-tetrahydrocannabinol (THC) is being developed to determine recent cannabis intake.

Potent synthetic cannabinoids will continue to be introduced into the drug abuse market, creating continued challenges for clinicians, toxicology laboratories, and public health programs.

From Δ9-Tetrahydrocannabinol (THC) to Synthetic Cannabinoid Markers

Cannabis is a complex plant containing more than 100 **cannabinoids** (see Glossary) and >500 other chemicals, including hydrocarbons, terpenes, flavonoids, and non-cannabinoid phenols [1]. THC is the primary psychoactive cannabis component, binds to endogenous cannabinoid receptors, and hijacks the normal functioning of the endocannabinoid system [2]. THC is absorbed rapidly from the lungs in humans, and peak THC concentrations occur before the last puff of a cannabis joint or blunt reflecting the ability of the individual to titrate their dose [3]. The subjective and physiological effects of inhaled THC begin immediately and influence the **smoking topography** of the user by the way cannabis is smoked. Smoking topography changes with user experience and desired effects. After the peak, THC blood concentrations decrease rapidly as the lipophilic compound is distributed into tissues [3]. THC absorption and elimination profiles can be characterized and correlation of concentrations with observed effects attempted. Unlike alcohol, the pharmacokinetics of THC are nonlinear, and a long terminal elimination phase makes it difficult to directly correlate blood concentrations and effects [4]. With chronic frequent exposure, THC accumulates in tissues, creating a large THC body burden that is slowly excreted over time, leading to major differences in THC pharmacokinetics in occasional (less than daily) and chronic frequent (daily) cannabis users, complicating the interpretation of THC and THC metabolite concentrations [5].

Controlled cannabinoid administration studies expand our knowledge of the pharmacodynamics and pharmacokinetics of the drug, identifies potential markers of exposure, time and frequency of exposure, as well as differences between occasional and frequent intake. Controlled administration data can also enable the simultaneous collection of data on drug effects and concentrations in blood and oral fluid (OF), providing a scientific database for interpreting individual drug tests. These advances facilitate model development that can predict

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Box 1. Cannabinoid Detection Methods: Immunoassays, Chromatography, Mass Spectrometry

Gas chromatography with MS (GC-MS) has been the most common analytical approach for cannabinoid detection, but currently liquid chromatography tandem MS (LC-MS/MS) and high-resolution MS (HR-MS) offer many advantages in our search for more informative cannabinoid markers. Indeed, LC-MS/MS enables simultaneous quantification of free and conjugated (glucuronides or sulfates) analytes in a single assay. HR-MS can preliminarily identify a compound based on its accurate mass.

Δ9-Tetrahydrocannabinol (THC) pharmacokinetics have been characterized by controlled cannabinoid administration studies. THC is primarily metabolized to the equipotent 11-hydroxy-THC (11-OH-THC) and the inactive THCCOOH, followed by metabolite glucuronidation to increase drug hydrophilicity and excretion [3].

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the drug use history of an individual and suggest ongoing drug effects. Examples include models for determining time of last cannabis intake, distinguishing between occasional and frequent cannabis users, and identifying recent cannabis intake and cannabis relapse [6–8]. Moreover, blood and OF cannabinoids can identify recent ingestion of causative agents in impaired driving cases.

Modern instrumentation, including **liquid chromatography with tandem mass spectrometry** (LC-MS/MS) and **high-resolution mass spectrometry** (HR-MS), can offer high sensitivity and specificity to identify informative cannabinoid markers (Box 1) [9]. The **Phase II THC metabolite**, THC-glucuronide, and other cannabis constituents including cannabigerol (CBG), cannabinol (CBN), and tetrahydrocannabivarin (THCV), are markers of recent cannabis intake in frequent and occasional cannabis users (Figures 1,2) [10–12]. These improved methodologies have provided information on the movement of cannabinoids (cannabinoid disposition) and their metabolites into **alternative matrices** such as oral fluid, sweat, and hair at low μg/l to ng/l concentrations. Marker concentrations in these matrices may provide unique information about cannabinoid intake and can address questions of longer timeframes of exposure, frequency of use, and relapse.

With the focus on improved **phytocannabinoid** marker interpretation, a major new drug abuse challenge has occurred, namely, the emergence of potent and toxic **synthetic cannabinoids** such as AB-FUBINACA, 5F-PB-22, AB-PINACA, BB-22, and EG-018. These compounds may vary widely from the structure of THC, but bind with high affinity to cannabinoid receptors [13–15]. The challenge to find markers of exposure for hundreds of potent synthetic cannabinoids has overwhelmed the field. From 2008 to December 2016 the European Monitoring Centre for Drugs and Drug Abuse (EMCDDA) reported 169 new synthetic cannabinoids [16]. Confronting this epidemic and educating the public on the dangers of these new drugs is dependent on identifying the drug and tying the toxicity to a specific agent. In most cases we cannot initially identify the drug because we do not know how the drug is metabolized and what markers to investigate in humans. Thus, determining metabolism of potent synthetic cannabinoids might reveal candidate urinary markers necessary to characterize the dangers of these **novel psychoactive substances** (NPS). A further challenge in the field is the lack of reference standards for the drugs in question, and their metabolites, because these are needed for positive identification and quantification [17].

Cannabinoid Markers

Blood and Plasma

One of the simplest cannabinoid markers for defining drug intake history is detection time in different biological fluids and tissues. Initially, experiments on occasional cannabis users indicated that blood THC was detected for ~6 h after smoking cannabis [3]; thus, finding THC in blood indicated recent cannabis intake. Investigators found that cannabis effects were

more easily related to time after ingestion than to specific THC concentrations [18]. Recent cannabis use explained the observed behavior, including impaired operation of an automobile, train, or aircraft, workplace or home accidents, or poor academic performance [19,20]. Mathematical models were developed to predict time of last cannabis use from blood and plasma THC (model I) and THC and 11-nor-9-carboxy-THC (THCCOOH) concentrations (model II) [6]. These models accurately predicted time of last cannabis use within a 95% confidence interval for all published blood and plasma controlled cannabis administration data, and greatly improved blood and plasma cannabinoid results interpretation [6]. Later model refinement documented time after last use following multiple cannabis intakes, and following oral ingestion [7,21]. Simultaneous cannabinoid monitoring in blood and plasma in humans showed that cannabinoids did not distribute well into erythrocytes, yielding cannabinoid plasma concentrations approximately twice those of blood cannabinoids [22].

In a placebo-controlled double-blind crossover study of 18 participants in the most advanced driving simulator worldwide, the effects of cannabis with and without low dose (0.05%) alcohol intake attempted to determine if a correlation existed between impaired driving and blood THC concentrations [19]. Each participant inhaled two THC doses (2.9 and 6.7% THC cigarettes) *ad libitum*, but owing to titration the delivered doses were not significantly different, and hence both active THC doses were considered together. Maximum THC blood concentrations (10 minutes after the start of inhalation) were significantly higher when administered with alcohol than alone (median 38.2 vs 47.9 μg/l), most likely because alcohol increases THC absorption by dilating blood vessels [23,24]. Another key finding was that the **alcohol T_{max}** occurred significantly later when THC and alcohol were coadministered, as opposed to alone, consistent with THC slowing gastric emptying, although this was not directly tested. Many toxicologists back-extrapolate the alcohol concentration to the time of a crash or police stop, but this may not be accurate when cannabis is coingested with alcohol. Because alcohol is primarily absorbed from the small intestine, T_{max} was found to be delayed when cannabis was present [24]. Moreover, a blood THC of 8.2 and 13.1 μg/l during driving produced the same impairment as 0.05% and 0.08% alcohol, respectively [19]. However, these values reflected THC concentrations at the time of driving impairment, not at 1.4–4 h after a crash or traffic stop was recorded, a time when blood samples are typically collected [25–27]. THC concentrations decreased rapidly, with a 74% decrease in 30 minutes and 90% decrease in 1.4 h, clearly highlighting the importance of rapid blood collection to document THC intake [23]. These marker data convinced the International Association of Chiefs of Police to recommend that the collection of blood is moved to the first step in the evaluation of drugged driving, where previously it constituted the last step. Back-extrapolation of THC concentrations are not considered to be accurate owing to the pharmacokinetics of THC [23]. Indeed, THC is a lipophilic compound that is rapidly distributed from blood into lipophilic adipose tissues, brain, and organs with high blood flow. Furthermore, THC is not like hydrophilic alcohol which can be eliminated at a constant zero-order rate. Accordingly, chronic frequent cannabis use results in the storage of a large body burden of THC which is slowly released over time, even during cannabis abstinence.

After the November 2016 elections, medicinal cannabis was approved in 29 US states, with recreational use being approved in eight states and in Washington DC. There are now many more daily cannabis users, challenging the predictive models for this population. Indeed, these models were accurate for frequent cannabis smokers during cannabis use, but inaccurate during sustained cannabis abstinence [5]. In 28 frequent cannabis smokers abstaining from cannabis for 7 consecutive days, five maintained blood THC concentrations above 1 μg/l for an entire week [5]. In this study participants resided in a closed research unit with no access to cannabis. This was the first indication that THC in the blood of frequent users exhibited a much

Glossary

Alcohol T_{max}: the time after alcohol ingestion when blood ethanol concentration is highest.
Alternative matrices: biological samples other than blood, serum, plasma, or urine. Examples include oral fluid, sweat, hair, placenta, meconium, and umbilical cord.
Anti-doping: drug testing in sports to deter athletes from ingesting prohibited drugs to achieve an unfair advantage in competition.
Cannabinoids: a class of closely related compounds of the cannabis plant including Δ9-tetrahydrocannabinol (THC, the primary psychoactive chemical in cannabis), more than 100 other structurally related chemicals in the plant, and the endocannabinoid neurotransmitters produced by the human body and many other living organisms, as well as synthetic cannabinoids produced by clandestine chemists, all of which interact with cannabinoid receptors.
Cannabinoid disposition: the movement of cannabinoids from the blood into tissues, urine, feces, and bile, as well as into alternative matrices such as oral fluid, sweat and hair.
CB1 and CB2 receptors: G protein-coupled receptors located in the brain and body that bind to endogenous cannabinoid neurotransmitters such as anandamide and 2-arachidonoylglycerol, THC, and other plant cannabinoids, as well as synthetic cannabinoids.
Controlled cannabinoid administration: the dosing of a known potency and amount of a cannabinoid by a specific route of administration with appropriate controlled conditions and subject to required regulatory controls. Refers here specifically to randomized, placebo-controlled dosing to known cannabis users with approvals from an ethical committee, the FDA, and the US Drug Enforcement Agency (DEA).
High-resolution mass spectrometry (HR-MS): mass spectrometry in which *m/z* for each ion is measured to several decimal places (i.e., exact rather than nominal masses are measured).
Human liver microsomes: the subcellular fraction of human liver

longer detection time than in occasional users [5]. Subsequently, THC was quantified in the blood and plasma of 27 frequent cannabis users over 30 days of sustained abstinence [4]. All blood samples presented THC concentrations of ≤5 μg/l within 24 h of abstinence, but in two participants blood THC exceeded 0.3 μg/l for 30 days. Moreover, these daily smokers slowly excreted their large THC body stores for as long as 30 days, resulting in failure of the models to predict use within 95% CI during sustained abstinence [18]. Consequently, the plasma and blood predictive models were not considered to be reliable in predicting time of last use in chronic frequent cannabis users [18].

From another perspective, there is also value in identifying frequent cannabis intake. For example, THCCOOH blood concentrations of ≤3 μg/l are considered to be a marker of occasional cannabis intake, and ≥40 μg/l a marker of near-daily cannabis use [28]. For individuals arrested for driving under the influence of cannabis in Switzerland, different rehabilitation programs and penalties have been recommended based on the blood THCCOOH concentrations of the individual [28]. These limits have been useful in categorizing the cannabis intake history of some individuals, but many blood THCCOOH concentrations in this study fell within the 3–40 μg/l range, and therefore the intake frequency was indeterminate [28]. Thus, it is frequently impossible to differentiate occasional from chronic frequent cannabis use when considering a single blood specimen.

A newly developed LC-MS/MS method has enabled the simultaneous quantification of blood THC, THC-glucuronide, 11-hydroxy-THC (11-OH-THC), THCCOOH, THCCOOH-glucuronide, cannabidiol (CBD), and cannabinol (CBN) following cannabis smoking, showing that THC-glucuronide, CBD, and CBN can exhibit short detection times of less than 4 h, even in frequent users [10]. Furthermore, our group demonstrated that THC could be found in the blood of some chronic cannabis users for as long at 30 days after last use [4]. Indeed, identifying a marker of recent cannabis intake in daily users is of great importance when time of ingestion is needed. Knowing when the drug was taken can enable the prediction on whether the drug may have impaired the individual while driving a car, for instance in 'driving under the influence of drugs' (DUID) cases, or alternatively on whether the drug may have contributed to carrying out a crime in a legal investigation. If these markers are not present, recent use cannot be ruled out.

Recently, using another new method, blood cannabinoids from occasional and frequent cannabis users were quantified following controlled smoked, vaporized, and oral cannabis use that added cannabigerol (CBG) and THCV as markers [12]. The study revealed that the markers of recent use that exhibited the highest likelihood of detection were CBG and CBN, while lower detectability was noted for THC-glucuronide and THCV [12]. THCV-carboxylic acid exhibited a much longer detection time (several days) in blood than CBG and CBN [12]. Of note, CBD cannot be currently included as a recent use marker because high-potency CBD strains continue to be evaluated [29]. Another combination of markers and cutoffs (THC ≥5 μg/l and THCCOOH/11-hydroxy-THC ratio <20) indicated detection windows <8 h for all consumption routes in frequent smokers; occasional smokers were positive 1.5 h or 12 h following inhaled or oral cannabis, respectively, with this combination of cutoffs [12]. The cannabinoid marker Δ9-tetrahydrocannabinolic acid A, a biosynthetic precursor of THC, was recently identified in the blood and plasma of cannabis smokers; however, it did not correlate with the degree of intoxication cited by police reports, and is thus not useful for predicting time of last use or for measuring cannabis-induced impairment [30]. Cannabinoid markers can also differentiate cannabis intake from oral dronabinol (Marinol) intake. Dronabinol is a legal synthetic THC pharmacotherapy approved for the treatment of autoimmune deficiency syndrome (AIDS) wasting disease, and to combat nausea and vomiting during chemotherapy [31]. Moreover, finding concentrations of CBD, CBN, THCV, or other minor

cells that contains membrane-bound metabolizing enzymes.
Hysteresis curves: the relationship between the effects of a drug and its concentration in a biological fluid, demonstrating different relationships between drug concentrations and effects during drug absorption, distribution, and elimination.
Liquid chromatography with tandem mass spectrometry (LC-MS/MS): an analytical technique combining separation of analytes in a liquid mobile phase passing through a stationary phase column with detection of the analyte(s) in mass spectrometers placed in series.
Median visual analog scores (VAS): the median score on a visual analog scale for different subjective effects from 0 to 100.
Novel psychoactive substances (NPS): according to the UN Office on Drugs and Crime, NPS are 'substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat.'
Partial tolerance: when the response of an organism to a drug is reduced following repeated exposure; the development of tolerance may be different for each drug effect and tolerance is never complete.
Phase II THC metabolite: the product of metabolic enzymes that make a drug more hydrophilic and more easily excreted, such as the glucuronide or sulfate product of a drug.
Phytocannabinoids: cannabinoids that occur naturally in the cannabis plant.
Synthetic cannabinoids: a class of chemicals that bind to cannabinoid receptors in the body, but that are different from the natural cannabinoids in cannabis plants.
Smoking topography: the manner in which a drug is smoked; affects the amount and speed of drug delivery, and includes the numbers of puffs, length of inhalation, hold time in the lungs, exhalation time, and time between puffs.
THC C_{max}: the maximum concentration of THC in a biological fluid after cannabis administration.

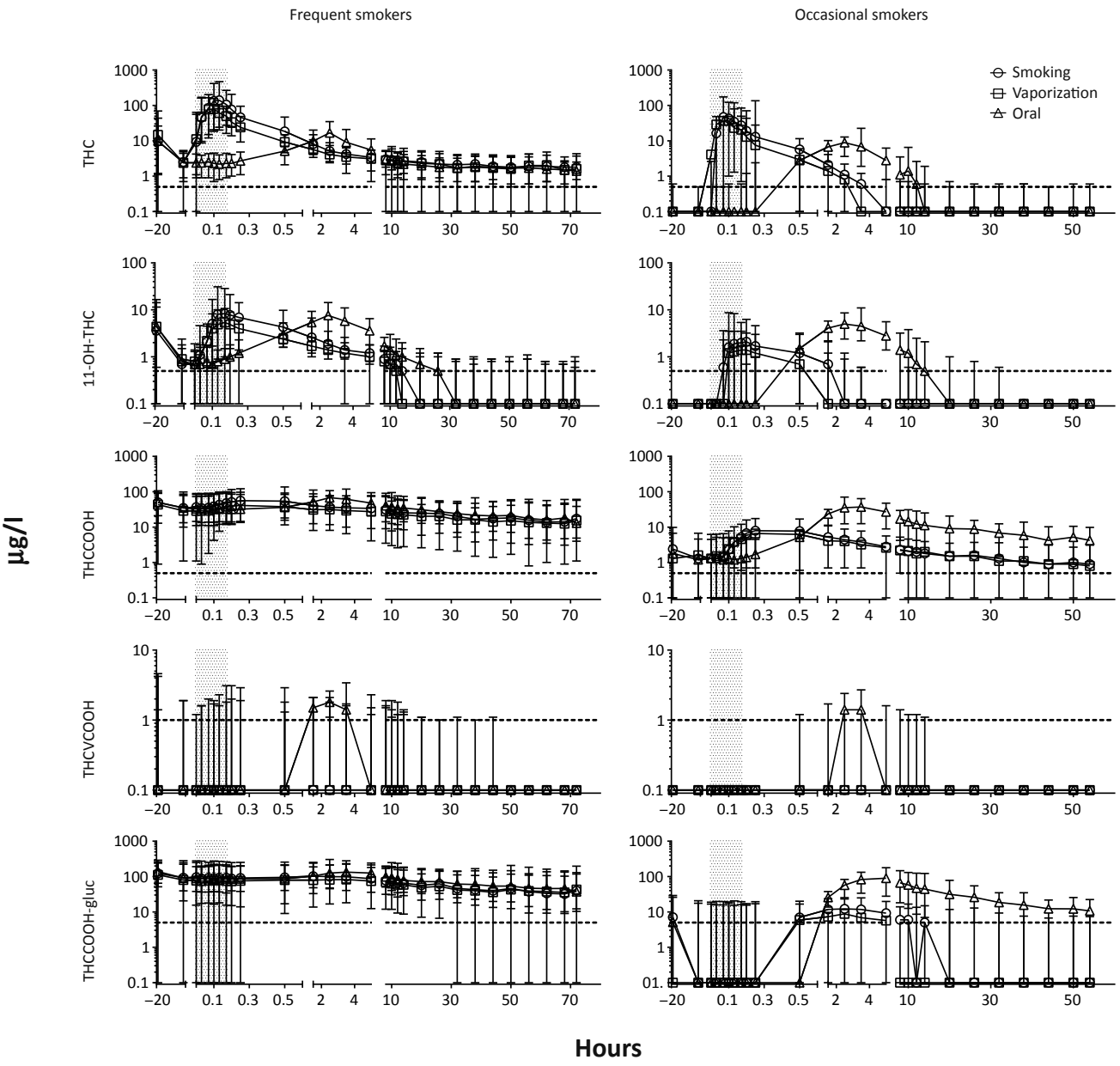

Figure 1. Example of Blood Cannabinoid Concentrations and Pharmacokinetics Relative to Routes of Administration. Representative graphs of mean + SD blood cannabinoid concentrations from 11 frequent and nine occasional cannabis smokers following administration of cannabis containing 6.9% Δ^9-tetrahydrocannabinol (THC) via smoked, vaporized, and oral routes. The different cannabinoid pharmacokinetics following 50.6 mg smoked, vaporized, and oral THC are shown [12]. The shaded area designates 10 minutes of smoking time. The broken line is the limit of quantification. Data are presented on a log scale. Abbreviations: 11-OH-THC, 11-hydroxy-THC; THCCOOH, 11-nor-9-carboxy-THC; THCVCOOH, 11-nor-9-carboxy-Δ^9-tetrahydrocannabivarin; THCCOOH-gluc, THCCOOH-glucuronide.

cannabinoids in human samples refutes the claim that these result only from taking the legal medication (Marinol) [32]. Thus, the quantification of additional cannabinoid markers and metabolites can improve the interpretation of blood cannabinoid results, and inform clinicians on the history and recency of cannabis intake.

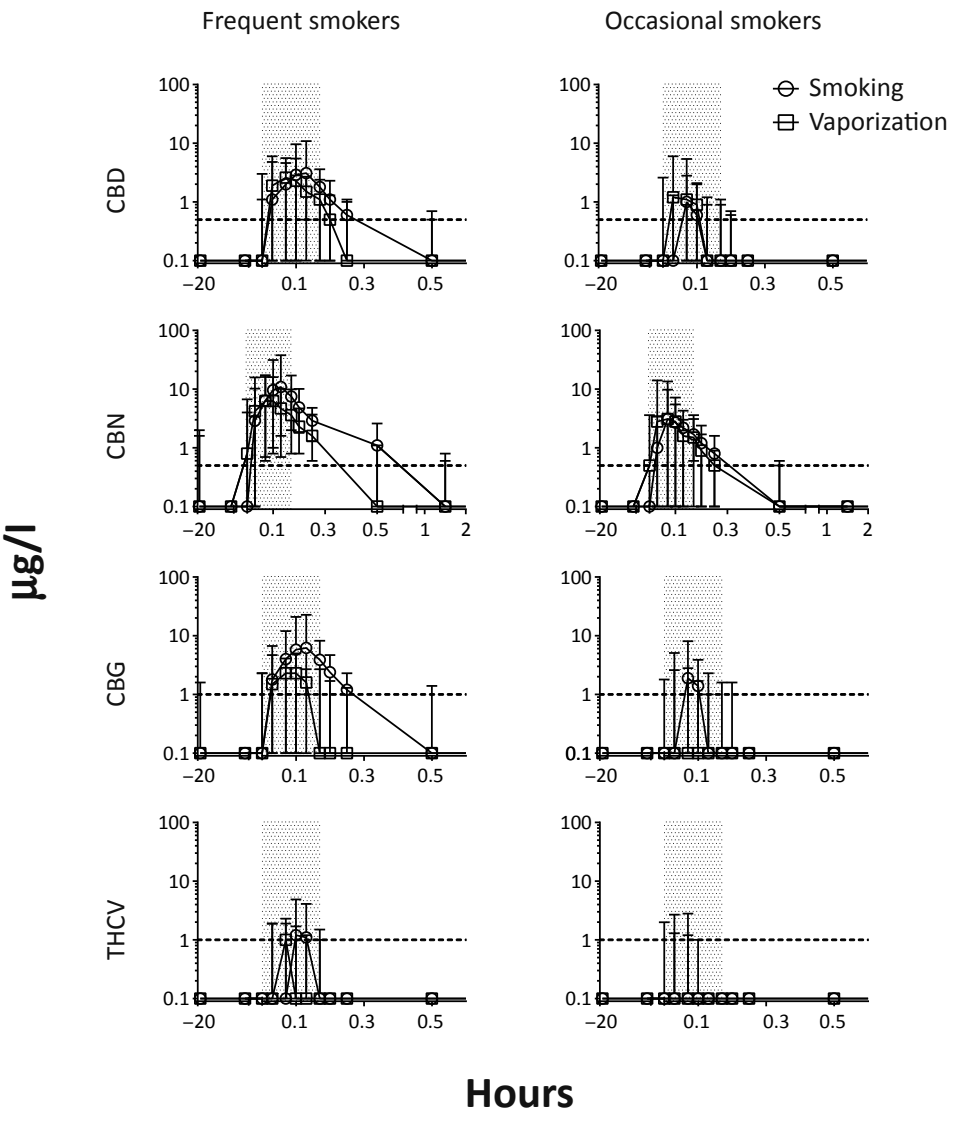

Figure 2. Example of Minor Cannabinoid Concentrations and Pharmacokinetics Relative to Routes of Administration. Representative graphs of mean + SD blood concentrations of minor cannabinoids from 11 frequent and nine occasional cannabis smokers following administration of cannabis containing 6.9% Δ^9-tetrahydrocannabinol (THC) via smoked and vaporized routes. The shaded area designates 10 minutes of smoking time. The broken line is the limit of quantification (LOQ). Data are presented on a log scale. Abbreviations: CBD cannabidiol, CBN cannabinol, CBG cannabigerol, THCV Δ^9-tetrahydrocannabivarin. The concentrations of these analytes did not exceed the LOQ after oral THC administration [12].

Urine Markers

The primary urinary cannabinoid is THCCOOH-glucuronide; analytical methods generally include an alkaline or enzymatic hydrolysis step to liberate free THCCOOH [33]. In occasional cannabis users, mathematical models have been developed to determine if cannabis has been ingested by comparing two urine sample collections [33]. This model has successfully identified cannabis reuse in **anti-doping**, civilian, and military cases. The creatinine-normalized THCCOOH concentration in a later urine sample is divided by the creatinine-normalized

THCCOOH concentration in an earlier urine sample [33]. This model was later improved to compare this ratio to established minimum, median, and maximum creatinine-normalized ratios for each 24 h time-period after last use; this enables a much tighter control on the ratios that are possible each day, and a more useful model for predicting new use in occasional cannabis users [34].

When studying THCCOOH urinary excretion in frequent cannabis users, our laboratory group observed positive urine tests for weeks after last use, making it difficult to determine if individuals were abstaining or relapsing in drug treatment [35]. Studying cannabinoid distribution in frequent cannabis users is difficult because ethical and safety concerns prohibit administering the expected amount and frequency of cannabis taken by this population. Nevertheless, this has led to several studies where every urine sample can be analyzed for THCCOOH and creatinine during sustained abstinence to determine THCCOOH pharmacokinetics in frequent users [36]. From thousands of datapoints, mathematical models have been developed to determine from two urine samples if a chronic frequent cannabis user has relapsed [8]. This model is more complex than the model used to identify new use in occasional users because it aims to account for the contribution of residual drug excretion. In addition, creatinine-normalized urine THCCOOH concentration is important for selecting the appropriate model formula to utilize; there are also rules that need to be applied to the data to ensure accurate predictions [8]. This model appears to be especially useful in cannabis treatment, employee assistance, and criminal justice programs to identify when drug relapse occurs and for providing a deterrent for new drug use [8].

Investigators proposed that $\geq$1.5 μg/l urine THC-glucuronide indicated cannabis intake within 8 h [37]. Later, investigators reported that $\geq$2.3 μg/l THC in urine indicated recent cannabis use, after adding the uncertainty of measurement to the previously proposed concentration [38]. Others later suggested that finding THC and 11-OH-THC in hydrolyzed urine predicted recent cannabis use; this was applied to anti-doping urine samples to determine if cannabis use occurred during competition events (e.g., sports) [39]. However, we have observed that THC can be detected up to 24 days after last cannabis use in hydrolyzed urine at up to 14.8 μg/l, and 11-OH-THC for more than 24 days at up to 132.8 μg/l, in urine samples of frequent cannabis users [35]. Free THC and 11-OH-THC were not found in non-hydrolyzed urine [40]. Therefore, neither urine THC, 11-OH-THC, nor their glucuronides can be considered as markers of recent cannabis intake.

THC, 11-OH-THC, THCCOOH, CBD, CBN, THC-glucuronide, and THCCOOH-glucuronide disposition in the urine of frequent and occasional cannabis smokers after smoking a 6.8% THC cigarette have also been determined [40]. No urine samples were found to contain measurable THC, 11-OH-THC, CBD, or CBN; but THCCOOH, THC-glucuronide, and THCCOOH-glucuronide were measurable in the urine of all frequent smokers, as well as in 60%, 100%, and 100% of occasional smokers, respectively [40]. From these data our laboratory group determined that cannabis use within 6 h can be predicted if there is an absolute difference of 50% between two consecutive THC-glucuronide-positive urine samples, and if the creatinine-normalized concentration in the first sample is at least 2 μg/g. This criterion has identified cannabis use within 6 h in urine samples from 93.1% of frequent and 76.9% of occasional marijuana smokers [40].

Oral Fluid

OF is a good alternative matrix for workplace, drug treatment, pain management, and DUID testing. OF offers many advantages over blood or urine sampling because of its easy, non-

invasive, and gender-neutral collection. Finding 2 μg/l THC in OF (the Substance Abuse Mental Health Services Administration proposed confirmation THC cutoff) is generally considered to be a marker of cannabis intake within the past 24 h, even in chronic frequent smokers [41]. Smoked, vaporized, or edible cannabis contaminates the oral mucosa, producing high THC concentrations initially that drop rapidly, precluding dose–concentration and concentration–effect relationships [42,43]. Cannabinoid OF markers THC, THCCOOH, THCV, CBD, and CBG have been quantified after occasional and frequent users ingested 50.6 mg THC by these three routes of administration (Figure 3) [43]. In this study the **THC C_{max}** occurred during or immediately after cannabis consumption as a result of oral mucosa contamination. Last detection times for THCCOOH were >72 h for frequent and >54 h for occasional users at a 15 ng/l cutoff. THCV and CBG marker concentrations ≥limit of quantification (LOQ) of 0.3 μg/l yielded detection windows indicative of recent cannabis intake. The last positive THCV was recorded up to 8 h for occasional, and 12 h for frequent, cannabis users, and for CBG at up to 26 h for occasional, and 20 h for frequent, cannabis users. Moreover, CBD concentrations were positive in OF at 0.3 μg/l for up to 20 h in both groups [43]. However, CBD has not been recommended as a marker of recent cannabis use because high-potency CBD cannabis has not been evaluated.

Passive THC oral mucosa contamination has been demonstrated following extended exposure of 10 non-smokers to cannabis smoke for 3 h in a Dutch coffee shop [44]. Seven participants exhibited OF THC concentrations >2 μg/l after 2 h in the smoke-filled environment. Because THCCOOH is not present in cannabis smoke, it was not identified in any OF samples after extended exposure to cannabis smoke [44]. However, other studies documented that a low 15 ng/l cutoff concentration is necessary to identify THCCOOH in OF from occasional users [45,46]. Following oral synthetic THC (Marinol, dronabinol) ingestion, OF THC reflects previously self-administered inhaled cannabis, with little contribution from ingested THC [45]. Thus, OF THCCOOH is considered a good marker of cannabis use because it can differentiate actual cannabis use from possible passive environmental exposure to THC, it identifies oral cannabis ingestion, and has a long window of detection in chronic frequent cannabis users [45,46].

Sativex is an oral mucosal spray containing 1:1 THC:CBD approved in some countries for treating neurogenic pain, sequelae from multiple sclerosis, and nausea. Our laboratory group attempted to document compliance with the therapeutic regimens of the patients by monitoring THC, THCCOOH, and CBD in OF [47]. High OF CBD/THC ratios could distinguish Sativex from cannabis ingestion, but only for a few hours after cannabis administration. Low CBD/THC and CBN/THC ratios were indicative of cannabis administration; however, high-potency CBD cannabis preparations have not been tested to determine if the values of these markers can change [47]. Thus, it is currently not possible to distinguish if patients prescribed Sativex also self-administer cannabis.

Our knowledge of the disposition of cannabinoids in OF has matured to the point that OF is currently in the final stages of approval for inclusion in federally regulated workplace programs. Government agencies will be able to select OF rather than urine testing of employees in security- and safety-sensitive positions for drug use detection and deterrence. OF has represented a biological fluid of choice for drug testing for many years in other countries [48]. However, one of the final issues to resolve is whether OF THCCOOH should be tested to rule out passive exposure.

Hair Markers

Hair is a biological matrix that is non-invasive in terms of collection, and has the advantage of long detection times for drug intake. Basic drugs preferentially transfer into hair and remain

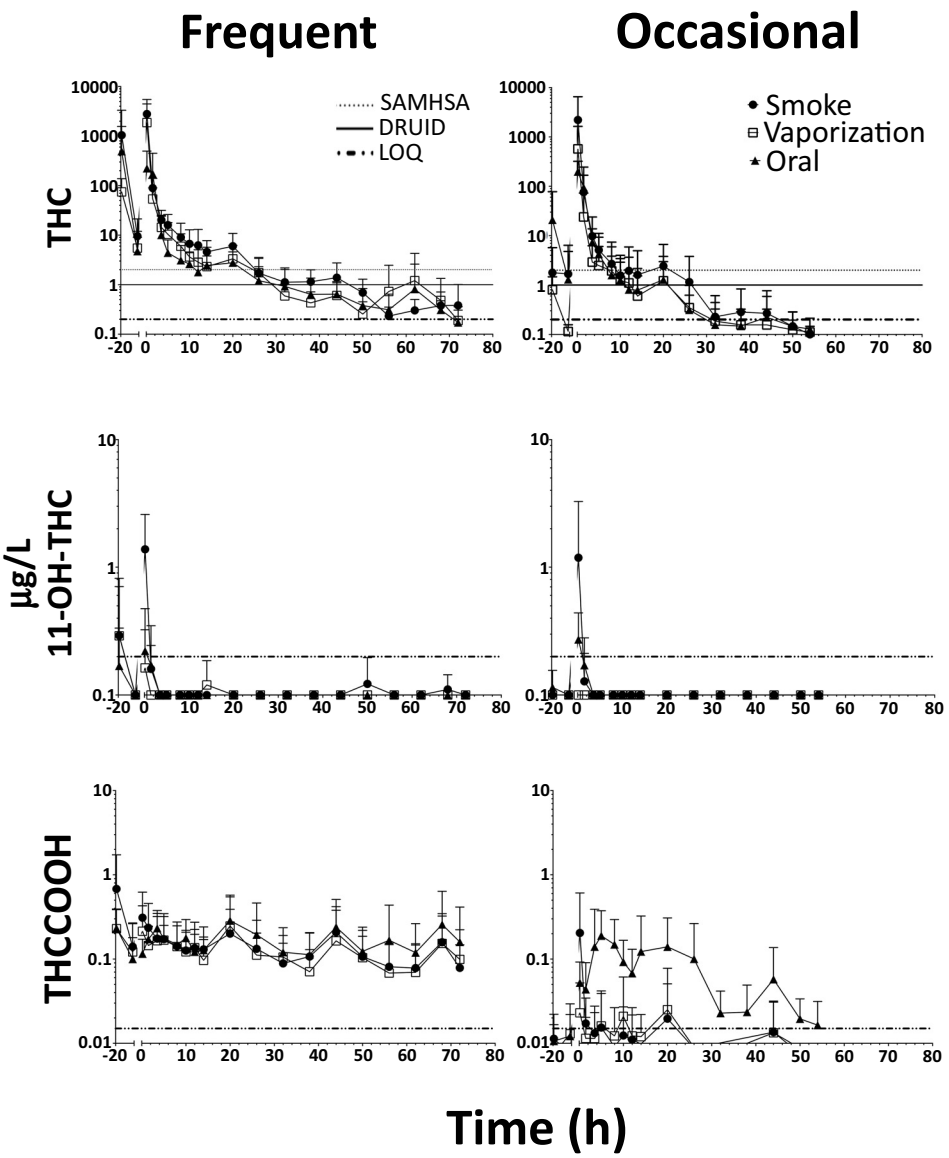

Figure 3. Example of Minor Cannabinoid Concentrations and Pharmacokinetics Relative to Routes of Administration. Representative graphs of mean + SD oral fluid (OF) concentrations on a log-scale for Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (THCCOOH) in n = 11 frequent (left) and n = 9 occasional (right) smokers up to 72 h and 54 h, respectively, after smoked, vaporized, and oral cannabis (6.9% THC; ~50.6 mg THC) administration (0 h) [43]. Horizontal lines represent the limits of quantification (LOQ; 0.2 µg/l for all cannabinoids, except 15 ng/l for THCCOOH) and OF THC cut-offs for the EU research project on Driving Under the Influence of Drugs, Alcohol, and Medicines (DRUID; 1 µg/l) and the US Substance Abuse and Mental Health Services Administration (SAMHSA; 2 µg/l).

within the hair for years; a most extreme example being a cocaine metabolite detected in the hair of pre-Columbian mummies from 2000 BC [49]. Cannabinoids are acidic drugs and do not incorporate well into hair, but are quantifiable in pg/mg concentrations [50,51]. In one study, hair THC, 11OH-THC, THCCOOH, CBN, and CBD concentrations were compared to

self-reported cannabis use [50]. Hair analysis was a good qualitative indicator of heavy (daily or near daily) cannabis consumption for the previous 3 months, but was not sensitive in the identification of occasional cannabis use [50]. Our laboratory group collected hair from 18 daily and 20 occasional cannabis users before and after each smoked two 2.7% THC cigarettes [50]. Using cutoffs for THC and THCCOOH of 1 and 0.1 pg/mg, respectively, 85% of daily and 52% of occasional cannabis users were identified [50]. In addition, contamination of hair with THC but not THCCOOH by side-stream smoke was reported [52]. Recently, one study reported that THC, THCCOOH, and the THC precursor, Δ9-tetrahydrocannabinolic acid A, could all be present in hair samples from non-consuming individuals owing to transfer of cannabinoids from cannabis consumers via their hands, sebum/sweat, or cannabis smoke (e.g., exhaled) [53]. Given the poor incorporation of THC in hair and the possibility of contamination from environmental smoke, THCCOOH is considered to be the best hair marker for identifying cannabis use.

Sweat Markers

Sweat testing offers an intermediary matrix for detecting cannabis use. Sweat patches are generally worn for one week and continuously monitor drug intake from approximately 3 days before patch application until removal. This is especially useful for individuals in drug treatment or criminal justice programs because they only need to report once per week to exchange sweat patches [54]. In one study, THC was quantified in sweat patches from frequent users during sustained abstinence. In many frequent cannabis users only the patch applied during the week abstinence had initiated was positive for THC, and in other patches that were applied the second, third, and fourth weeks of abstinence patch cannabinoids documented extended excretion of THC. However, no THC was found in test patches following oral ingestion of up to 14.8 mg of THC [54]. Nevertheless, sweat testing is a good method for evaluating drug use within a 1 week wear period, and has been best utilized for monitoring drug use of parolees between weekly parole officer appointments. However, only a single commercial laboratory routinely offers sweat patch testing, limiting its widespread use.

Breath Markers

Breath testing is most frequently utilized by law enforcement officials, but this technology may offer much wider application. THC was first reported in breath specimens when individuals blew into a bag collecting breath that was subsequently passed through a trapping filter [55]. THC was removed selectively from the filter and quantified by LC-MS/MS; breath samples from 18 chronic and 11 occasional cannabis users were examined following smoking of a 6.5% THC cigarette [56]. THC $\geq$50 pg/filter was detected up to 4 h after cannabis smoking in frequent cannabis users and for a shorter time in occasional users. Thus, while breath is a good matrix for identifying recent cannabis use, no THCCOOH has been identified in breath [56]. Many companies are pursuing the development of cannabis breathalyzers for roadside DUID testing. Of note, it is important not to contaminate breath specimens with oral fluid because cannabis concentrations may be in the thousands of μg/l immediately after intake. Taken together, breath THC represents a promising future matrix for monitoring recent THC intake, provided that manufacturers can develop on-site instruments that are specific and sensitive in identifying low pg/filter THC concentrations.

Synthetic Cannabinoid Markers

As scientists refined markers of phytocannabinoid intake history, a major new challenge appeared in 2008. The first reports of drugs with cannabis-like effects that tested negative in urine cannabinoid tests occurred in Germany, where great analytical efforts were expended to identify the first synthetic cannabinoid, JWH-018 [57]. J.W. Huffman synthesized a series of

synthetic cannabinoids based on a naphthoylindole structure in the 1980s, attempting to develop useful pharmacotherapies. His published synthetic pathways were first utilized by clandestine chemists to market the high-potency drugs on the Internet [58]. As governments attempted to schedule drugs that produced overdoses, kidney failures, heart attacks, and death, manufacturers continuously modified drugs to evade scheduling regulations, often creating more life-threatening compounds [59].

Identifying new high-potency parent synthetic cannabinoids is difficult because doses are low and detection times in blood and OF can be as short as a few hours to maximum of 1–2 days [17]. It is more common to try to determine the cause of intoxications or overdoses by identifying novel psychoactive substances in urine [13]. This is highly challenging because the metabolism of these compounds in humans is unknown. It is crucially important to identify the source of drug toxicity and cause of death to inform public health officials and the public of the danger of these highly potent analogs. Metabolite identification can be performed in several different ways, including using **human liver microsomes** or *in vivo* rodent drug administration studies, but both approaches have important limitations. Rodents may have different metabolic pathways, and liver microsome studies may suggest an array of metabolites without indicating which are the best targets in human urine, and membrane transporters and Phase II metabolism are not accounted for in this system [17]. NPS incubation with human hepatocytes offers the best approach because not only is the breadth of metabolic possibilities identified but also the most abundant metabolites produced in humans can be selected by using this technology [17]. HR-MS and sophisticated software are necessary to identify mass fragments of these compounds by accurate mass measurements. It is important to follow human hepatocyte studies with analysis of authentic urine samples to document the accuracy of predicted metabolites. In a cooperative effort with the US Drug Enforcement Agency (DEA), we utilized human hepatocyte incubations of new synthetic cannabinoids frequently seized in DEA operations and HR-MS to identify the best urinary targets for NPS (Figure 4) [9,13,17,60–62]. This enabled us to rapidly publish the HR-MS spectrum of NPS metabolites but also to identify those with the highest prevalence and those that specifically identified a particular parent drug. This is important because there may be common metabolites from several closely related NPS analogs. These data not only enabled laboratories around the world to enter these spectra into their libraries and search biological samples for their presence but also provided data for standard reference manufacturers to select the best NPS metabolites to prepare.

LC-MS/MS screening for new synthetic cannabinoids in a targeted method is a good approach and an achievable one based on the available instrumentation and personnel resources to identify NPS markers, but this approach is also limited by the time that is necessary to keep analytical methods current with newly marketed synthetic cannabinoid compounds, and by the constant need for new reference standards that may not yet be available [63,64]. Unfortunately, this is almost an impossible task. A different approach utilizes HR-MS and a consistent acquisition program to facilitate the addition of newly introduced NPS [9]. An innovative recent approach employs a G protein-coupled receptor activation assay with chemiluminescent detection to detect **CB1 and CB2 receptor** activity rather than identifying the chemical structure of each new analyte [65]. This screening approach can quickly rule out the presence of synthetic cannabinoids in a biological specimen, but confirmation of positive results will require a chromatography and MS approach [17]. Thus, identifying the presence of synthetic cannabinoid metabolites in biological samples is a challenging task that requires highly sensitive and specific MS approaches as well as well-trained innovative scientists. New G protein-coupled receptor activation assays offer the promise of a more rapid means to screen biological samples for the presence of any natural or synthetic cannabinoid drug that activates

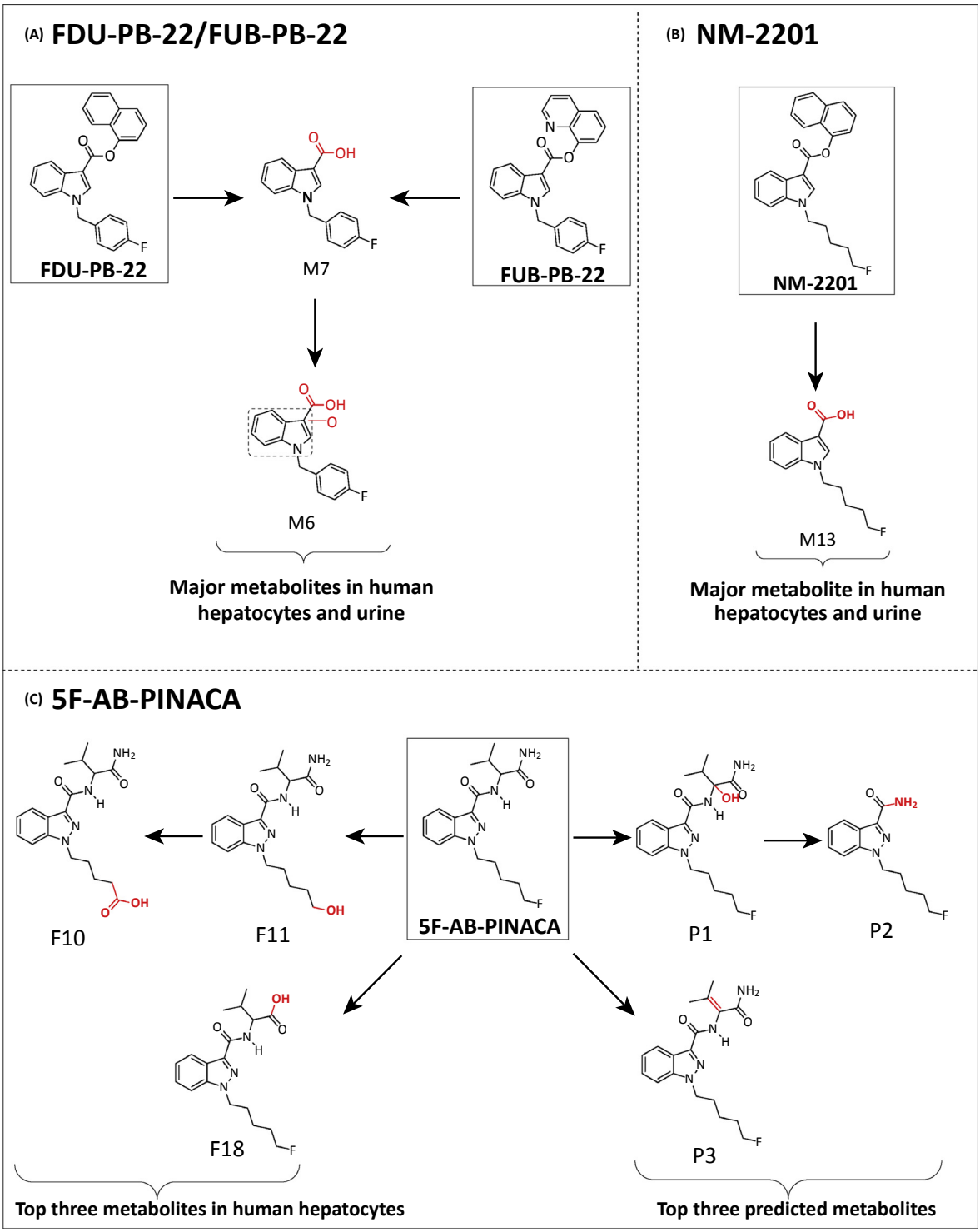

Figure 4. Major Metabolites of Synthetic Cannabinoids. FDU-PB-22/FUB-PB-22 (A), NM-2201 (B), and 5F-AB-PINACA (C) are incubated with human hepatocytes, identified by mass spectroscopy, and validated in suspect human urine samples [17].

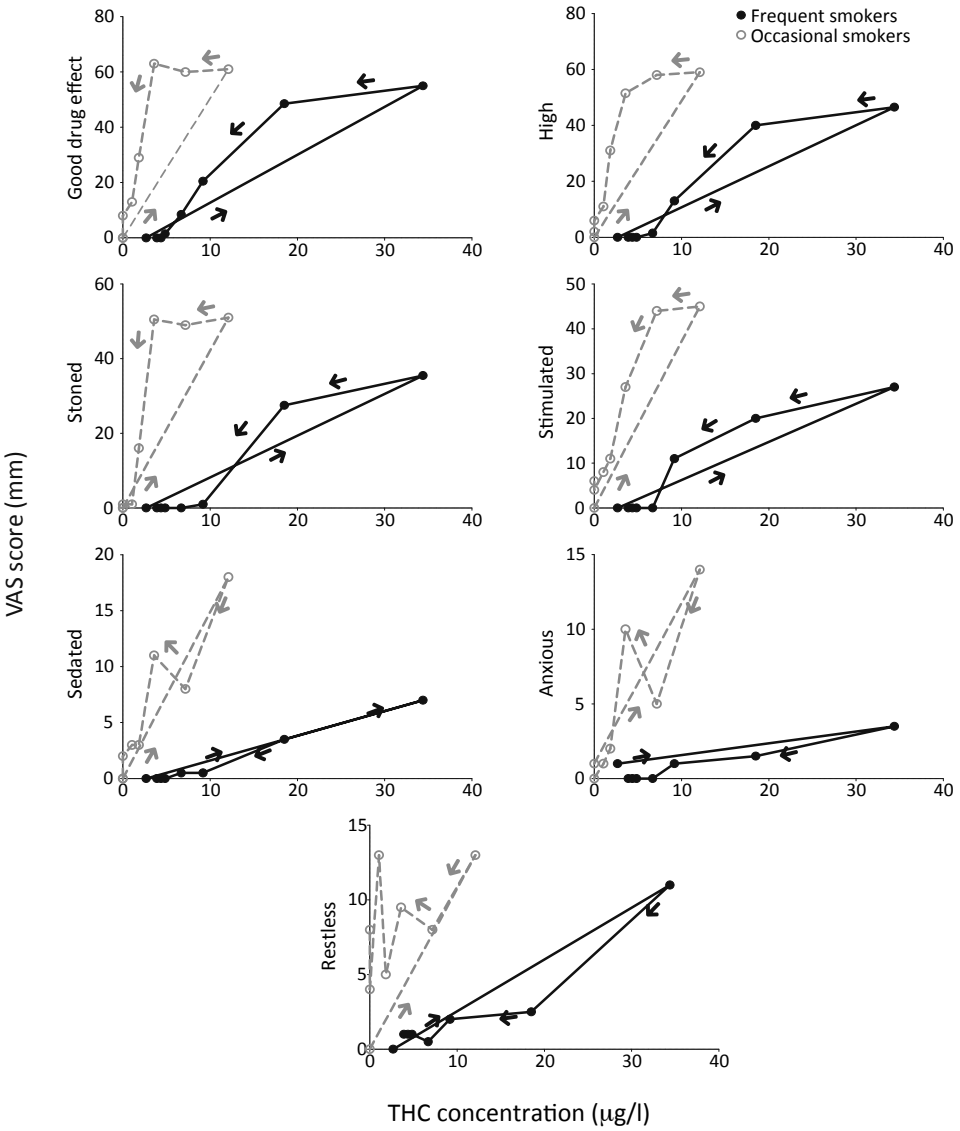

Figure 5. Median Visual Analog Scores (Computer Screen Tests) Relative to THC Concentrations in Cannabis Smokers. **Median visual analog scores** (VAS) from computer screen tests relative to blood THC concentrations were assessed for 'good drug effects', 'stoned', 'sedated', 'high', 'stimulated', 'anxious', and 'restless' in 14 frequent and 11 occasional cannabis smokers following controlled smoking of a 6.8% Δ9-tetrahydrocannabinol (THC, 54 mg) cannabis cigarette. Reproduced, with permission, from [69].

cannabinoid receptors; however, MS will be necessary to identify the specific compound leading to this activation [64].

Challenges in Interpreting Cannabinoid Use Findings

The interpretation of cannabinoid effects is even more difficult than identifying the presence or concentration of natural or synthetic cannabinoid markers in a diverse array of biological samples. Interpretation is complex because the onset, peak, and duration of effects are different based on whether the route of cannabis administration is inhalation, oral, or rectal,

and on whether the individual is an occasional or chronic frequent cannabis user [12]. Chronic frequent cannabis use can lead to the development of **partial tolerance** to some cannabis effects, but it is important to remember that tolerance is never complete and does not occur for all effects [66,67]. Partial tolerance to cannabinoid effects (both natural and synthetic cannabinoids) can develop with repetitive frequent cannabinoid exposure; however, more data on tolerance will be necessary to improve our interpretation of cannabinoid markers. Other challenges for interpreting cannabinoid results comprise the windows of cannabinoid detection that vary by the biological matrix tested and the analyte(s) selected for monitoring. In this manuscript we have discussed the importance of recent use markers to identify the timeframe of cannabinoid intake, especially in chronic frequent cannabis users. Blood is considered to be the biological matrix that best reflects ongoing pharmacological effects, but blood cannabinoid concentrations decrease rapidly. Distribution of the highly lipophilic cannabinoids into tissues and out of the blood can result in negative or low blood cannabinoid concentrations that are more difficult to interpret, especially owing to the residual excretion of cannabinoids after chronic frequent intake [4]. In addition, the continuous introduction of highly potent and toxic synthetic cannabinoids makes interpretation of these data highly difficult because their pharmacology has not yet been established. Another difficult interpretation issue is that cannabis users titrate their dose to their desired effects by changing their smoking topography [3]. Concentration–effect curves are helpful in interpreting the effects of a drug that may be present at specific concentrations; however, for cannabinoids, concentration–effect curves are not linear but are counterclockwise **hysteresis curves** [68]. Indeed, there is a concentration effect of drug 'high' versus THC blood concentration in occasional and frequent cannabis smokers (Figure 5) [69] which denotes a lack of a linear response. In fact, individual experiences reflect two different levels of drug 'high' at the same blood concentration of THC, namely a low 'high' effect in the absorption phase during cannabis inhalation, and a much higher effect later during the distribution phase owing to the lag time for full distribution of the active THC to the site of action – in this case, the brain. To reach a similar level of 'high', the chronic frequent cannabinoid user must achieve higher blood THC concentrations because of the development of partial tolerance to the effects of THC [69].

Concluding Remarks

Advances in biological monitoring and controlled cannabinoid administration studies have greatly improved markers of cannabinoid intake, and our interpretation of cannabinoid and metabolite concentrations has enabled the development of predictive models. However, the prediction of cannabinoid cognitive, psychomotor, and subjective effects remains a future need.

Of note, there are currently many new methods for cannabis self-administration, including e-cigarettes and 'dabs' – which are highly concentrated (up to 80%) THC following plant extraction with solvents that are later evaporated. Data on drug delivery through these new methods does not yet exist, making it impossible to know what new markers might be available and how to interpret their concentrations and toxicity. The need for behavioral and biological cannabinoid markers is expanding with cannabis medicalization and legalization. Therapeutic drug monitoring of effective cannabinoid pharmacotherapies will be required in the future when new cannabinoids are proven safe and efficacious. What markers will be best for establishing therapeutic ranges? (see Outstanding Questions and Box 2).

Currently, science does not support the development of cannabinoid limits *per se* because of in motor vehicles drivers because of the many factors influencing concentration–effect relationships. Thus, the development of sensitive and specific behavioral and motor impairment markers collected onsite is needed, with cannabinoid biological markers defining the agent

Outstanding Questions

What are the major factors differentiating cannabinoid pharmacokinetics in frequent and occasional cannabis users?

What are the best cannabinoid markers for the identification of new cannabis use, time of last use, and frequency of cannabis use in occasional and frequent cannabis users?

What are the mechanisms of action of tolerance to cannabinoid effects? Can complete tolerance to the performance-impairing effects of cannabis occur? For which effects can tolerance be demonstrated, and with which cannabinoid markers?

Can behavioral tests for cannabis impairment be developed for use at the roadside? Simple and rapid assessment instruments are needed.

Are there better markers of recent cannabis intake to assist in detecting impaired driving now that cannabis is legal for use in many US states? New markers include cannabigerol and cannabinol; however, these cannabinoid markers may not be present in all individuals after recent use.

Are there receptor assays available that will detect any CB1 cannabinoid receptor agonist despite the wide variety of synthetic cannabinoid structures? This may be possible, but if routine cannabis also produces positive tests, expensive confirmatory procedures will be necessary to differentiate routine cannabis use from novel synthetic cannabinoid use.

Box 2. Clinician's Corner

Understanding the cannabis and synthetic cannabinoid intake history of an individual is vital for clinicians treating drug dependence, emergency department and primary care personnel, researchers investigating cannabinoid effects, medical examiners, and public health officials.

Required information about cannabis and synthetic cannabinoid intake history includes identifying cannabinoid exposure, time of last use, frequency of use, and relapse to drug use.

Markers of cannabinoid intake history include Δ9-tetrahydrocannabinol (THC) and minor cannabinoids that are present in the cannabis plant, as well as their metabolites, in various biological matrices.

A variety of biological matrices are available, including blood, urine, oral fluid, sweat, hair and breath; each matrix and analyte may have different pharmacokinetic profiles that can provide unique data to answer a variety of basic and clinically relevant questions.

Minor cannabinoids cannabigerol and cannabinol are markers of recent cannabis intake, and can provide crucial information to improve the interpretation of 'driving under the influence' tests for cannabinoids, as well as for other accident- and toxicity-related investigations.

Synthetic cannabinoids are novel psychoactive substances (NPS) that are often more potent and toxic than cannabis, and are readily available via the Internet. They are often structurally unrelated to THC, have higher affinity for CB1 receptors, produce more deleterious effects, and are difficult to detect owing to their high potency and short detection time in blood and oral fluid. Initially, urinary metabolites of new synthetic cannabinoids are unknown and rarely produce positive results on standard drug tests.

(s) responsible for observed performance impairments. It is clear that continued development of biological and behavioral cannabinoid markers is needed now and for the foreseeable future.

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