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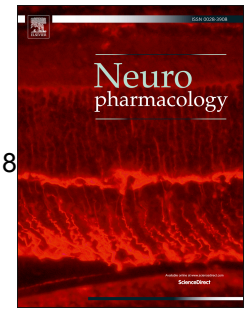

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METHODOLOGY FOR CONTROLLED ADMINISTRATION OF SMOKED SYNTHETIC CANNABINOIDS JWH-018 AND JWH-073

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ABSTRACT:

Synthetic cannabinoids (SCs) are a significant public health concern given their widespread use and severe effects associated with intoxication. However, there is a paucity of controlled human studies investigating the behavioral and physiological effects and pharmacokinetics of these compounds. Designing a reliable method to administer consistent, concentration-dependent synthetic cannabinoids is an integral component of controlled study of these compounds. Further, optimizing methods to assess the parent compounds and metabolites in plasma is critical in order to be able to establish their pharmacokinetics after administration. To develop a reliable method to administer smokable, concentration-dependent SCs, cigarettes were prepared with plant matter adulterated with increasing concentrations of the first generation cannabinoids found in SC products, JWH-018 and JWH-073. Cigarettes were assessed 1 - 6 months after preparation using ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to determine compound stability over time and concentration consistency throughout the cigarettes. Optimal conditions to detect metabolites in human plasma as a function of storage temperature (-4°C - -80°C) and time (24 hrs - 1 month) were also determined. Analyses verified that the method utilized to develop SC cigarettes yielded consistent, concentration-dependent products within 25% of the expected concentrations. JWH-018, JWH-073 and metabolites in spiked plasma were stable under the time and temperature conditions; concentrations were within ± 20% of target values. These studies provide techniques and methods to conduct controlled investigations of the dose-dependent effects of first generation SCs to begin understanding risks associated with use.

1. INTRODUCTION:

Synthetic cannabinoids (SCs), commonly referred to as 'Spice' or 'K2,' have been an emerging class of abused drugs in Europe and the United States for close to a decade (EMCDDA, 2016; DEA, 2016). The widespread use and severe adverse effects of SCs in the US was recently documented by a 330% increase in calls associated with SC use to poison control centers in 2015 (Law et al., 2015). Although the most serious cases of intoxication are those that receive attention by the media and scientific literature, others use SC without experiencing adverse effects. For example, among current cannabis smokers recruited for human laboratory studies of cannabis dependence in New York City, 32.3% reported SC use; close to half of respondents reported to have used the products repeatedly, and nearly 10% endorsed frequent to heavy use (Cooper, 2016). People use SCs for a variety of reasons including its availability, low cost, and as a substitute for cannabis as they are not detected by conventional urine toxicology screens (Zawilska et al., 2014; Ralphs, et al, 2017). When these products first emerged on the black market, they were not scheduled by the Drug Enforcement Administration. However, between July 2012 and April 2017 over 10 compounds (and their isomers) that were associated with adverse health consequences, including seizures, psychosis, and loss of consciousness (Cooper et al., 2016) and death, have been classified as Schedule I substances in accordance with the Synthetic Drug Abuse Prevention Act (US DEA, 2012; 2015; 2017). Despite the scheduling of these compounds and the attention regarding their extreme risks, calls to Poison Control Centers have decreased since 2015 with 7,779 calls, but have not been eliminated as evidenced by 2,695 calls in 2016 (American Association of Poison Control Center, 2017). Furthermore, recent population-based surveys reveal that the drugs continue to be used. For example, 5.2% of 12th graders reported SC use according the 2015 Monitoring the Future survey (Monitoring the Future, 2015). Over the last decade, the compounds identified in these products have changed to reflect the scheduling of an increasing number of compounds. As such, compounds detected in products span multiple chemical classes including indazole carboxamides (AB-PINACA), cyclohexylphenols (CP-55,940), benzoylindoles (AM694), tetramethylcyclopropylindole (UR-144) , naphthoylindoles (JWH-018 and JWH-073) (Castaneto et al., 2014; Wiley et al., 2015), and newer indole- (MDMB-CHMICA) and indazoles (AMB-FUBINACA, 5F-AMB, and MDMB-FUBINACA) (Banister et al., 2016) that have been associated with toxicity (Adams et al., 2017; Shanks and Behonick, 2016; Westin et al., 2015). JWH-018 and JWH-073 were the commonly detected psychoactive constituents of the first generation SC

products (Auwärter et al., 2009); as such their in-vitro and cross-species pharmacology has been studied to a greater extent than the more recently identified compounds, although very little has been reported relating to their pharmacology in human subjects.

The compounds comprising SC products are cannabinoid-receptor 1 (CB1) agonists similar to the primary psychoactive component of cannabis, Δ^9-tetrahydrocannabinol (THC) (Atwood et al., 2011, 2010; Banister et al., 2016; Wiley et al., 2015). However, *in vitro* and *in vivo* assessments of the pharmacology of the first-generation SCs, JWH-018 and JWH-073, demonstrate that these compounds differ from THC in their CB1 receptor affinity and efficacy. *In vitro* (Wiley et al., 1998) and laboratory animal studies (Ginsburg et al., 2012; Järbe et al., 2011; Wiley et al., 2017) demonstrate that JWH-018 and JWH-073 have pharmacodynamic and pharmacokinetic differences from THC that suggest that synthetic cannabinoids pose a greater risk for abuse and dependence than cannabis. Furthermore, many metabolites of JWH-018 and JWH-073 have been shown to retain *in vitro* and *in vivo* activity, which have been suggested to contribute to the toxic effects of these drugs warranting future study of both parent compounds and their metabolites (Brents et al., 2012, 2011; Fantegrossi et al., 2014).

Despite the widespread use of SC products, there is a paucity of literature assessing the acute effects of these compounds in human subjects. There are two published reports investigating the pharmacokinetics of JWH-018 in a total of 8 participants (Teske et al., 2010; Toennes et al., 2017), yet no studies to date have reported on the dose-dependent behavioral or physiological effects of smoked JWH-018 or JWH-073 relative to placebo. Furthermore, there have been no studies comparing SC products to cannabis, an important comparator given that SCs are reported to be used as substitutes for cannabis or for the more intense cannabis-like high (Zawilska and Andrzejczak, 2015). Assessing the effects of SCs under controlled conditions and relating these effects to the pharmacokinetics of the parent compounds and metabolites is critical to 1) understanding their behavioral and physiological risks and 2) fundamental to future investigations of potential pharmacotherapies for acute intoxication and longer-term adverse effects including synthetic cannabinoid use disorder. In effort to establish the methodological parameters to establish the dose-dependent physiological and behavioral effects of SCs, a procedure was developed and validated to produce consistent and reproducible synthetic cannabinoid cigarettes for future studies of controlled smoked administration in humans.

For future studies relating SC effects to their pharmacokinetics, optimal conditions to detect parent compounds and metabolites in spiked plasma were investigated.

2. METHODS

2.1 Synthesis of synthetic cannabinoids: JWH-073 and JWH-018 were synthesized according to previous reports (Blaazer et al., 2011) in high yield (**Figure 1)**. The intermediate (1H-indol-3-yl)(naphthalene-1-yl)methanone (**2**) was prepared by Friedel-Crafts acylation of indole (**1**) with 1-naphthoyl chloride in the presence of diethylaluminium chloride in toluene. In order to obtain (**2**) in high yield, the optimal reaction conditions were performed with different molar ratios of 1-naphthoyl chloride, indole and amount of diethylaluminum chloride and purification was achieved via column chromatography or recrystallization. Each of the final compounds was purified using hexane and ethyl acetate (95:5, v/v) by column chromatography to give JWH-073 and JWH-018 as an oily yellow gum. For the intermediate, a molar ratio of 1.2 - 1.4 times of indole to 1-naphthoyl chloride and diethylaluminium chloride achieved a yield of 85 - 93%. Subsequent N-alkylation with 1-bromobutane and 1-bromopentane using potassium hydroxide and dimethylformamide in acetone provided JWH-073 and JWH-018. This sequence of classical reaction procedures generated pure products of JWH-073 and JWH-018 and gave satisfactory to good yield (JWH-073, 65 - 75%; JWH-018, 75 - 80%). Identification and structural characterization of JWH-073 and JWH-018 was established by proton NMR spectroscopy (1H NMR) and high-resolution mass spectrometry (HRMS), as well as high performance liquid chromatography (HPLC). The purities of both compounds were estimated to be more than 99% based on 1H NMR and HPLC.

1H NMR spectra were recorded on a Bruker NMR 400 MHz spectrometer. The details interpreting every peak in the spectrum were as follows: 1H NMR for JWH-073 (400 MHz, $CDCl_3$), δ 8.48 (m, 1H), 8.18 (d, J = 8.4 Hz, 1H), 7.97 (d, J = 8.0 Hz, 1H), 7.90 (d, J = 7.6 Hz, 1H), 7.65 (dd, J = 1.2 Hz, 1H), 7.547.44 (m, 3H), 7.40-7.33 (m, 4H), 4.06 (t, J = 7.2 Hz, 2H), 1.82-1.75 (q, 2H), 1.33-1.25 (q, 2H), 0.91-0.88 (t, J = 7.2 Hz, J = 7.6 Hz, 3H); 1H NMR for JWH-018 (400 MHz, $CDCl_3$), δ 8.49 (m, 1H), 8.20-8.18 (d, J = 8.8 Hz, 1H), 7.96 (d, J = 8.4 Hz, 1H), 7.90 (d, J = 7.2 Hz, 1H), 7.66 (dd, J = 1.2 Hz, 1H), 7.54-7.44 (m, 3H), 7.41-7.33 (m, 4H), 4.06 (t, J = 7.2 Hz, 2H), 1.84-1.76 (q, 2H), 1.33-1.22 (m, 4H), 0.86-0.83 (t, J = 6.8 Hz, J = 7.2 Hz, 3H). The HRMS mass spectra were obtained on JKS-HX 11UHF/HX110 HF Tandem Mass Spectrometer in the FAB+ mode. The

averaged molecular weights of both compounds were determined to be 328.1694 for $C_{23}H_{21}NO$ (JWH-073) and 342.1873 for $C_{24}H_{23}NO$ (JWH-018), respectively, and the theoretically calculated (FAB+) was 328.1701 for JWH-073 and 342.1858 for JWH-018. HPLC analyses were performed on a system consisting of a Waters model 515 pump, a Waters model 2487 UV detector, and a Phenomenex Prodigy C18 column (5 µm, 4.6mm × 250 mm). The mobile phase consisted of 75% acetonitrile (ACN) and 25% 0.1M ammonium formate (AMF) (v/v). The flow rate of the mobile phase was 2 mL/min. UV detection was performed at wavelength of 220 nm. Data acquisition was accomplished using PeakSimple Chromatography integration software. Under these conditions, both JWH-073 and JWH-018 gave a single peak on analytical HPLC and had a retention time of 6.33 min and 8.27 min, respectively.

2.2 Cigarette Preparation: Concentration-dependent synthetic cannabinoid-laced cigarettes (800 mg) were prepared using placebo cannabis plant matter (0.0%THC, 0.0% cannabidiol, 0.0% cannabinol) obtained from NIDA. Compounds were dissolved in 95% ethanol for concentrations of 0.32, 1.0, 3.2, and 10.0 mg/ml for JWH-018, and 1.0, 3.2, and 10.0 mg/ml JWH-073. 1 ml of each concentration was added to 800 mg of placebo cannabis plant matter in a glass vial for each cigarette. For the higher JWH-073 concentrations (32.0 mg), 3.2 mls of the 10 mg/ml stock solution was added to 800 mg plant matter. Adulterated plant matter was mixed using a vortex for 10-15 seconds and dried for 2 days; cigarettes were prepared using non-filtered cigarette tubes (Daughters & Ryan, Inc, Smithfield, NC) and an electric cigarette injector (Powermatic, Brea CA). Cigarettes were shipped to the reference laboratory overnight and tested at 1, 3, and 6 months after production to establish compound concentration and its uniform distribution over time. During this time, cigarettes were stored in the dark at -20°C. Prior to analysis, cigarettes were brought to room temperature for 24 hours and split in half. Drug concentrations from both segments of the cigarettes were analyzed as described in **Section 2.3**.

One-way analyses of variance (ANOVA) were conducted to determine whether JWH-018 and JWH-073 concentrations in the cigarettes changed as a function of storage duration (1, 3, and 6 months) to assess for potential degradation (GraphPad Prism, 6.0, La Jolla, CA). Data from cigarette segments for each respective drug (JWH-018 and JWH-073) were pooled for each time point (n = 8 segments per time point). One-way ANOVAs were also conducted to determine uniformity in drug distribution between cigarette segments as a function of drug concentration (n = 3 cigarettes per drug concentration) for each drug. The JWH cigarette

strengths were based upon potency ratios reported by *in vitro* (Wiley et al., 1998) and *in vivo* studies (Jarbe et al., 2011; Ginsburg et al., 2012) suggesting that JWH-018 is about 10-times more potent than THC, whereas JWH-073 and THC are approximately equipotent. The dose-range of JWH-018 cigarettes were prepared to provide 0.04 - 1.25% (0.32 – 10.0 mg per 800 mg plant material) and 0.125 – 4.0% for JWH-073 cigarettes (1.0 - 32 mg per 800 mg plant material). Cigarettes were prepared according to these strengths for future comparisons of smoked JWH-018, JWH-073, and equipotent cannabis cigarettes. That is the low strength of JWH-018 and JWH-073 (0.125 and 1.25%, respectively) would be compared to low strength cannabis (1.25% THC) whereas the higher strength of JWH-018 and JWH-073 (0.40 and 4.0%, respectively) would be compared to a higher strength cannabis (4.0% THC).

2.3 Determination of concentration and uniform distribution over time of Naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018) and naphthalen-1-yl-(1-butylindol-3-yl)methanone (JWH-073) in SC-spiked cigarettes using a UPLC/MS/MS analysis:

2.3.1 Reagents: The primary reference materials for JWH-018, JWH-073, and the internal standards (ISTD); THC-d_3 (Δ^9-Tetrahydrocannabinol-d_3) were purchased from Cerilliant (Round Rock, Texas). Ammonium formate, acetonitrile, ammonium hydroxide, methanol, formic acid and deionized (DI) water were purchased from Fisher Scientific (Hanover Park, Illinois). All reagents were ACS grade. Medical grade nitrogen was purchased from National Welders Supply Company (Richmond, Virginia). *Sample Preparation:* For each cigarette strength, cigarettes were split in half and ~80 mg of each half of the cigarette were homogenized and extracted into methanol over a 24-hour period. An aliquot of this extraction brought up to 100 µL with methanol and 10 µL of a 10 µg/mL THC-d_3 ISTD was added. These samples were then analyzed with an unextracted 5 point calibration curve with a linear range of 200 to 5000 ng/mL in methanol.

2.3.2 Instrumental Analysis: The UPLC/MS/MS analysis of JWH-018, JWH-073 and their metabolites (for the following study) was performed on the Waters Xevo TQD LC/MS mass spectrometer attached to a ACQUITY UPLC® System controlled by MassLynx software (Milford, Massachusetts). Chromatographic separation was performed on an Agilent Zorbax 4.6 x 75 mm, 3.5 µm XDB-C18 column (Agilent, Santa Clara, CA). The column was kept at 40ºC and 5 µL of sample was injected. The mobile phase consisted of A: deionized water

with 10 mM ammonium formate and 0.1% formic acid and B: 90:10 acetonitrile:deionized water with 10 mM ammonium formate and 0.1% formic acid. The following gradient was used: 0.0 to 1.5 mins at 25% A and 75% B, 1.6 to 4.5 mins at 0% A and 100% B held for 4.5 mins and then returned to 25% A and 75% B at 10.0 minutes and held for 0.5 mins. The flow rate was 0.5 mL/minute. The source temperature was set at 150ºC with a capillary voltage of 3.00 kV. The desolvation temperature was set at 600ºC with a gas flow rate of 650 L/hour. The cone flow rate was set at 100 L/hour. The acquisition mode used was Multiple Reaction Monitoring (MRM). The retention times (min), cone voltage (V), transition ions (*m/z*) and corresponding collection energies (eV) for all the compounds can be found in **Supplementary Table 1**. The total run time for the analytical method was 10.5 minutes. Calibrators were the following concentrations: 200, 500, 1000, 2500 and 5000 ng/mL for JWH-018 and JWH-073. A linear regression of the ratio of the peak area counts of the drug and drug metabolites to that of the ISTDs versus corresponding concentration ratios was used to construct the calibration curves. The lower limit of quantitation (LOQ) was administratively set at 200 ng/mL. The method yielded a linear regression correlation coefficients (r^2) for the all the calibration curves in the range of > 0.9995 for JWH-018 and JWH-073.

2.3.3 Carryover: Sample carryover of the JWH-018 and JWH-073 was evaluated in each of batches by injecting of the highest calibrator (5000 ng/mL for JWH-018 and JWH-073) followed by the analysis of a negative control (drug-free methanol). Lack of carryover was confirmed as JWH-018 and JWH-073 were not detected in the negative control.

2.4 Determination of optimal conditions to assess for plasma concentrations of naphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-018), naphthalen-1-yl-(1-butylindol-3-yl)methanone (JWH-073) and their metabolites using UPLC/MS/MS:

2.4.1 Reagents: The primary reference materials for JWH-018 4-hydroxypentyl metabolite (JWH-018 4OH), JWH-018 5-hydroxypentyl metabolite (JWH-018 5OH), JWH-073 3-hydroxybutyl metabolite (JWH-018 3OH), JWH-073 4-hydroxybutyl metabolite (JWH-018 4OH) and their internal standards (ISTD); JWH-018 *N*-(4-hydroxypentyl) metabolite-d_5 (JWH-018 4OH-d_5) and JWH-073 *N*-(3-Hydroxybutyl) metabolite-d_5 (JWH-073 3OH-d_5) were purchased from Cerilliant (Round Rock, TX). Additional information regarding reference material

and reagents can be found in section 2.3.1. Drug-free expired whole plasma was obtained from Transfusion Medicine Laboratory at Virginia Commonwealth University Health System. The plasma was certified in-house as drug free by scanning of ammonium hydroxide and methylene chloride liquid-liquid basic extract for drugs by GC/MS operated in scan mode (40-500 m/z) and UPLC/MS/MS for JWH-018, JWH-073 and metabolites prior to use.

2.4.2 Sample Extraction: The extractions were performed using a modification of the procedure of Foltz et al. (1983) as previously described for analysis of JWH-018 and JWH-073 (Poklis et al., 2012). 10 µL of ISTD was added to 1 mL aliquots of calibrators, controls and samples. These samples were mixed and allowed to equilibrate overnight. The following day 2 mL of ice cold acetonitrile was added drop by drop to each sample while vortexing. The samples were then centrifuged at 3500 rpm for 10 min. After centrifuging, the samples were placed in a −40 °C freezer for at least 2 h. The top layer of each sample containing the acetonitrile was then removed via a disposable glass pipette and placed in clean test tube. Extracts were then dried using a Savant AES1000. The resultant residues were reconstituted with 100 µL of mobile phase and placed in auto-sampler vials for UPLC/MS/MS analysis.

2.4.3 Instrumental Analysis: The UPLC/MS/MS analysis of JWH-018, JWH-073 and their metabolites was performed as described above. The retention times (min), cone voltage (V), transition ions (*m/z*) and corresponding collection energies (eV) for the compounds can be found in the **Supplementary Table 1**.

2.4.4 Method Validation Procedure: The evaluation of the plasma assay was conducted over three days. Sample batches were analyzed for linearity, lower limit of quantitation (LOQ), accuracy/bias (i.e., systematic error), precision, carryover, selectivity and matrix, absolute recovery. Validation sample batches contained calibrators, drug free controls (negative control) with internal standard (ISTD) added and a double negative control containing neither drugs, drug metabolites, nor ISTDs. Aliquots of plasma quality control (QC) specimens were analyzed in triplicate. Plasma QC specimens were prepared with the following target values: limit of quantitation quality control (LOQC), 1 ng/mL of JWH-O18 and JWH-073 and 0.1 ng/mL of the metabolites; low control (LQC), 3 ng/mL of JWH-O18 and JWH-073 and 0.3 ng/mL of the metabolites; medium control (MQC), 30 ng/mL of JWH-O18 and JWH-073 and 3 ng/mL of the metabolites; and high control (HQC),

75 ng/mL of JWH-O18 and JWH-073 and 7.5 ng/mL of the metabolites. All QC specimens were stored at -20°C until testing.

2.4.5 Linearity, limit of quantitation: Linearity of the assay was verified from seven point calibration curves prepared in certified in-house drug-free plasma. Calibrators were the following concentrations: 1, 2, 5, 10, 20, 50 and 100 ng/mL for JWH-018 and JWH-073, 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mL for the hydroxyl metabolites. A linear regression of the ratio of the peak area counts of the drug and drug metabolites to that of the ISTDs versus corresponding concentration ratios was used to construct the calibration curves. The lower limit of quantitation (LOQ) was administratively set at 1 ng/nL for JWH-018 and JWH-073 and 0.1 ng/mL for the metabolites. The method yielded linear regression correlation coefficients (r^2) for the all the calibration curves in the range of > 0.9920 for JWH-018, JWH-073 and their metabolites. The LOQC samples was within ± 20% of the target value and had a response at least ten times greater than the signal to noise ratio of drug-free plasma.

2.4.6 Accuracy/bias and precision: Accuracy/bias and precision were determined from the prepared QC plasma samples. The Accuracy/bias and precision was calculated for each concentration over the three batches by combining all the data from all days (**Table 1**).

Table 1. Accuracy / Precision and Bias.

Bias/Precision	JWH-018		JWH-073	
QC (n = 9)	Bias	%CV	Bias	%CV
LOD (1 ng/mL)	1	9	12	5
LQC (3 ng/mL)	-3	15	1	12
MQC (30 ng/mL)	-9	13	5	11
HQC (75 ng/mL)	-8	12	-2	13

Bias/Precision	JWH-018 4OH		JWH-018 5OH	
QC (n = 9)	Bias	%CV	Bias	%CV
LOD (0.1 ng/mL)	2	4	-2	6
LQC (0.3 ng/mL)	-3	10	-3	9
MQC (3.0 ng/mL)	<1	7	2	6
HQC (7.5 ng/mL)	<1	7	-1	7

Bias/Precision	JWH-073 3OH	JWH-073 4OH

QC (n = 9)	Bias	%CV	Bias	%CV
LOD (0.1 ng/mL)	3	5	6	5
LQC (0.3 ng/mL)	-2	9	<1	9
MQC (3.0 ng/mL)	<1	8	2	8
HQC (7.5 ng/mL)	-2	4	-2	4

QC = Quality Control; LOD = limit of quantitation quality control; LQC = low control; MQC = medium control; HQC = high control; % CV = Percent Coefficient of Variation.

2.4.7 Absolute recovery: The absolute percent recovery of the assay was determined at 10 ng/mL JWH-018, JWH-073 and their ISTDs (n = 3), and 1 ng/mL for their metabolites and corresponding ISTDs (n = 3). The absolute percent recovery was determined by preparing matrix and extracted samples. The matrix samples were prepared by first extracting drug free plasma by the presented method and then adding JWH-018, JWH-073, their metabolites and ISTDs at the appropriate concentrations. The addition of the drugs, metabolites and ISTDs to the extracted plasma was used to mitigate any matrix effects in the recovery studies. Plasma specimens with the drugs, drug metabolites and ISTDs added at the appropriate concentrations were then extracted using the presented method. The absolute recovery of the assay was determined by comparing the absolute area of the extracted samples to the absolute peak area of matrix samples (**Table 2**).

Table 2. Absolute Recovery.

Drug	Recovery %
JWH-018	83 ± 11
JWH-018 4OH	91 ± 10
JWH-018 5OH	90 ± 11
JWH-018 4OH-d_5	97 ± 9
JWH-073	85 ± 11
JWH-073 3OH	91 ± 9
JWH-073 4OH	93 ± 9
JWH-073 3OH-d_5	93 ± 10

2.4.8 Selectivity and Matrix: The selectivity of the assay was determined using ten different lots of JWH-018 and JWH-073 free plasma. Each individual lot was analyzed with and without internal standard. No peaks were detected that co-eluted with JWH-018, JWH-073, their metabolites or with the ISTDs. This ensured that endogenous plasma components did not interfere with the assay.

2.4.9 Carryover: Sample carryover of the JWH-018, JWH-073 and metabolites was evaluated in each of the batches using two different procedures. First, immediately following the injection of the highest calibrator (100 ng/mL for JWH-018 and JWH-073; 10 ng/mL for their metabolites) a negative control (drug-free) was injected. Lack of carryover was confirmed as JWH-018, JWH-073 and metabolites were not detected in the negative control. Second, an injection of HQC (75 ng/mL of JWH-018 and JWH-073 and 7.5 ng/mL of the metabolites) was immediately followed by injection of the LQC (3 ng/mL JWH-018 and JWH-073 and 0.3 ng/mL of the metabolites). This procedure was routinely applied each time the high calibrator, HQC and LQC samples were analyzed. Lack of carryover was confirmed for JWH-018, JWH-073 and metabolites as no analytes were detected in the negative control and the analyses of the LQC samples did not demonstrate a significant quantitated bias of more than 20%.

2.4.10 Stability: Stability of the JWH-018, JWH-073 and metabolites in plasma was determined under several specific conditions and time intervals. The experiments were performed using three of the control specimens: LQC, MQC and HQC. All studies included three replicate analyses of each QC specimen. The “freeze/thaw” stability JWH-018, JWH-073 and their metabolites in plasma was assessed to evaluate the possible effects of specimen stored frozen and then thawed for re-analysis. QC specimens stored at -20ºC were put through three freeze/thaw cycles with the last freeze cycle lasting 24 hours. They were then extracted and quantitated against freshly prepared calibrators. The “bench-top” stability of the JWH-018, JWH-073 and their metabolites in plasma was assessed to evaluate the possible effects of specimen transportation and processing in the laboratory by having the QC specimens sit at room temperature for 72 hours. They were then extracted and quantitated against freshly prepared calibrators. The “post-preparative” stability of the JWH-018, JWH-073 and their metabolites in plasma was evaluated by having extracts sit in the UPLC/MS/MS’s auto-sampler. A batch of the extracted LQC, MQC and HQC were quantitated against a freshly prepared calibration. The extracted controls were then allowed to sit in the auto-sampler for 72 hours at 5°C after which they were re-injected and quantitated from the initial calibration. The results of the initial analysis were compared to those of the re-injected samples. The results of these stability tests demonstrated that JWH-018, JWH-073 JWH-018 4OH, JWH-018 5OH, JWH-073 3OH and JWH-073 4OH in plasma were stable under the conditions of the freeze-

thaw, bench-top and post-preparative studies. In each study, these analyites at the QC concentrations were within ± 20% of the target values.

2.4.11 Temperature and Time Study: Short-term temperature stability and long-term stability of JWH-018, and JWH-073 and respective metabolites in human plasma was assessed. Samples were stored at -80, -20, or 4°C for 24 hrs, 2 wks, or 4 wks. They were then extracted and quantitated against freshly prepared calibrators. Differences in concentration as a function of storage time and temperature were determined using two-way ANOVAs for the LQC, MQC, and HQCs for each compound and metabolite (GraphPad Prism, 6.0, La Jolla, CA).

3. RESULTS

3.1 Concentration-dependent SC cigarettes: Cigarettes prepared according to the described procedure with JWH-018 and JWH-073 were within 25% of their expected strengths across concentrations at all times tested as assessed by averaging two segments from each cigarette (**Table 3**). Some exceptions were lower strength cigarettes as observed with 0.040% JWH-018 one and 6 months after preparation. No differences were observed in JWH-018 and JWH-073 cigarette concentrations as a function of storage duration (JWH-018, F [2, 21] = 0.1240, p > 0.05; JWH-073, F [2, 21] = 0.0214, p > 0.05).

Table 3. SC concentrations in cigarettes as % (w/w of plant material of the cigarette)

1 MONTH

JWH-018 (n = 2)

Expected Concentration (mg/800mg)	Expected Strength (% JWH-018)	Actual Strength (% JWH-018)	Relative Difference From Expected Strength (%)
0.32	0.040	0.048 (0.008)	119 (19.8)
1.00	0.125	0.113 (0.014)	90 (11.3)
3.20	0.400	0.512 (0.013)	128 (2.8)
10.0	1.250	1.192 (0.033)	95 (2.8)

JWH-073 (n = 2)

Expected Concentration (mg/800mg)	Expected Strength (% JWH-073)	Actual Strength (% JWH-073)	Relative Difference From Expected Strength (%)
1.0	0.125	0.155 (0.004)	124 (2.8)

3.2	0.400	0.318 (0.040)	80 (9.9)
10.0	1.250	1.196 (0.074)	96 (6.4)
32.0	4.000	3.329 (0.293)	83 (7.1)

3 MONTHS

JWH-018 (n = 2)

Expected Concentration (mg/800mg)	Expected Strength (% JWH-018)	Actual Strength (% JWH-018)	Relative Difference From Expected Strength (%)
0.32	0.040	0.024 (0.001)	61 (1.4)
1.00	0.125	0.108 (0.006)	87 (4.9)
3.20	0.400	0.400 (0.051)	100 (12.7)
10.0	1.250	0.994 (0.055)	80 (4.9)

JWH-073 (n = 2)

Expected Concentration (mg/800mg)	Expected Strength (% JWH-073)	Actual Strength (% JWH-073)	Relative Difference From Expected Strength (%)
1.0	0.125	0.110 (0.012)	88 (9.9)
3.2	0.400	0.400 (0.055)	100 (14.1)
10.0	1.250	1.146 (0.024)	92 (2.1)
32.0	4.000	3.794 (0.351)	95 (8.5)

6 MONTHS

JWH-018 (n = 2)

Expected Concentration (mg/800mg)	Expected Strength (% JWH-018)	Actual Strength (% JWH-018)	Relative Difference From Expected Strength (%)
0.32	0.040	0.026 (0.002)	67 (4.9)
1.00	0.125	0.110 (0.004)	88 (2.8)
3.20	0.400	0.412 (0.013)	103 (3.5)
10.0	1.250	1.478 (0.074)	118 (5.7)

JWH-073 (n = 2)

Expected Concentration (mg/800mg)	Expected Strength (% JWH-073)	Actual Strength (% JWH-073)	Relative Difference from Expected Strength (%)
1.0	0.125	0.131 (0.009)	105 (7.1)
3.2	0.400	0.401 (0.028)	100 (7.1)
10.0	1.250	1.166 (0.002)	93 (0.0)
32.0	4.000	3.901 (0.140)	98 (3.5)

Two segments of one cigarette per condition (drug, concentration, and storage duration) were analyzed. Mean values (±SD) of the two segments are presented for *Actual Strength* and the *Relative Difference from Expected Strength*. Values surpassing 100% of expected strength indicate that the actual strength of the segments were higher than expected.

The procedure also yielded concentrations that were consistent throughout the cigarettes **(Table 4**). Specifically, the average difference between cigarettes segments was less than 20% for both JWH-018 and JWH-073 cigarettes across concentrations; no differences as a function of drug concentration were detected (JWH-018, $F [3, 8] = 0.4110$, $p > 0.05$; JWH-073, $F [3, 8] = 2.646$, $p > 0.05$).

Table 4. Concentration consistency between cigarette segments (n = 2)

JWH-018	**Percent Difference Between Segments**			
Strength (%)	1 month	3 month	6 month	Average (SEM)
0.040	23.4	5.1	9.5	12.7 (5.5)
0.125	17.8	8.1	4.5	10.1 (4.0)
0.400	3.7	18.1	4.6	8.8 (4.7)
1.250	3.9	7.8	7.1	6.2 (1.2)

JWH-073	**Percent Difference Between Segments**			
Strength (%)	1 month	3 month	6 month	Average (SEM)
0.125	3.2	15.9	9.5	9.6 (3.7)
0.400	17.7	19.4	10.0	15.7 (2.9)
1.250	8.8	2.9	0.2	4.0 (2.5)
4.000	12.4	13.1	5.1	10.2 (2.6)

Two segments of one cigarette per condition (drug, concentration, and storage duration) were analyzed.

3.2 Stability of compounds and metabolites in plasma: Stability of JWH-018, JWH-073 and metabolites in plasma were determined under several specific conditions and time intervals. The results of these stability tests demonstrated that JWH-018, JWH-073 JWH-018 4OH, JWH-018 5OH, JWH-073 3OH and JWH-073 4OH in plasma were stable under the conditions of the freeze-thaw, bench-top and post-preparative studies (**Table 5**). In each study, these analytes at the QC concentrations were within ± 20% of the target values, a commonly recommended acceptable positive control result (SWGTOX, 2013**)**. Statistical analyses revealed changes in stability of parent compounds and some metabolites were detected as a function of storage time and temperature (see **Table 5** for details), with storage time affecting stability of JWH-018 (HQC, $F [2, 18] = 5.368$, $p < 0.05$), JWH-018 4OH (LQC, $F [2, 18] = 5.421$, $p < 0.05$), JWH-073 (MQC, $F [2, 18] = 3.593$, $p <$

0.05; HQC, F [2, 18] = 7.797, p < 0.01), JWH-073 3OH (LQC, F [2, 18] = 4.582, p < 0.05; HQC, F [2, 18] = 3.837, p < 0.05) and 4OH (MQC, F [2, 18] = 13.68, p < 0.001; HQC, F [2, 18] = 7.342, p < 0.01) and storage temperature affecting stability of JWH-018 (HQC, F [2, 18] = 6.478, p < 0.01) and JWH-073 (MQC, F [2, 18] = 6.481, p < 0.01). Although the direction of effect was not consistent across compounds, storage duration negatively impacted stability (i.e., JWH-018 HQC, F [2, 18] = 5.368, p < 0.05, JWH-073 MQC, F [2, 18] = 3.593, p < 0.05 and HQC, F [2, 18] = 7.797, p < 0.01).

Table 5. Stability of parent compounds and metabolites in plasma

			Low Control (3 ng/mL) ‡			
JWH-018 (n = 3)	**(-4°C)**		**(-24°C)**		**(-80°C)**	
Time/Temp	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD
24 hr	2.8	0.6	3.1	0.2	3.0	0.4
2 weeks	3.2	0.6	2.4	0.3	2.5	0.4
1 month	2.5	0.1	2.8	0.3	3.1	0.3
			Mid Control (30 ng/mL)			
24 hr	26.0	0.9	31.7	1.5	32.5	2.2
2 weeks	25.0	1.5	26.2	3.6	25.1	2.2
1 month	28.4	4.5	27.7	10.0	27.8	4.8
			High Control (75 ng/mL) *, ##			
24 hr	75.3	6.2	81.8	0.7	80.3	5.4
2 weeks	66.5	6.9	79.7	2.4	70.4	10.6
1 month	62.8	4.3	78.2	3.4	63.9	13.8
			Low Control (0.3 ng/mL) *			
JWH-018 4OH (n = 3)	(-4°C)		(-24°C)		(-80°C)	
Time/Temp	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD
24 hr	0.32	0.05	0.27	0.01	0.29	0.01
2 weeks	0.27	0.01	0.27	0.01	0.25	0.02
1 month	0.30	0.02	0.31	0.02	0.29	0.04
			Mid Control (3.0 ng/mL)			
24 hr	3.1	0.2	3.0	0.2	3.2	0.7
2 weeks	2.9	0.1	2.9	0.1	3.0	0.1
1 month	3.3	0.1	3.4	0.5	3.1	0.2
			High Control (7.5 ng/mL)			
24 hr	7.8	0.4	7.5	0.4	7.5	1.5
2 weeks	7.9	0.4	6.7	0.3	7.4	0.5
1 month	7.7	0.1	7.7	0.4	7.5	0.2
			Low Control (0.3 ng/mL)			
JWH-018 5OH (n = 3)	(-4°C)		(-24°C)		(-80°C)	
Time/Temp	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD

24 hr	0.31	0.06	0.28	0.03	0.29	0.01
2 weeks	0.28	0.01	0.27	0.02	0.32	0.04
1 month	0.28	0.02	0.28	0.02	0.27	0.02
Mid Control (3.0 ng/mL)						
24 hr	3.0	0.1	2.9	0.2	3.1	0.10
2 weeks	3.1	0.1	3.2	0.2	3.1	0.20
1 month	3.0	0.4	3.0	0.5	2.9	0.10
High Control (7.5 ng/mL)						
24 hr	7.4	0.8	7.3	0.5	7.3	0.20
2 weeks	7.4	0.3	7.5	0.7	6.7	0.60
1 month	7.9	1.7	7.9	1.6	6.7	0.50

Low Control (3.0 ng/mL)

JWH-073 (n = 3)	(-4°C)		(-24°C)		(-80°C)	
Time/Temp	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD
24 hr	3.2	0.6	2.7	0.1	3.0	0.1
2 weeks	2.9	0.6	3.3	0.3	3.3	0.3
1 month	3.5	0.1	2.8	0.4	2.9	0.4
Mid Control (30 ng/mL) *, ##						
24 hr	28.1	1.0	28.0	2.3	29.4	1.3
2 weeks	32.3	1.4	35.9	2.4	28.4	1.3
1 month	28.3	3.9	33.7	6.6	25.7	2.0
High Control (75 ng/mL) **						
24 hr	73.4	3.4	64.0	4.0	64.0	4.6
2 weeks	82.0	9.4	81.3	6.6	69.4	10.7
1 month	60.8	2.2	73.5	2.2	62.7	10.4

Low Control (0.3 ng/mL) *

JWH-073 3OH (n = 3)	(-4°C)		(-24°C)		(-80°C)	
Time/Temp	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD
24 hr	0.34	0.05	0.28	0.01	0.33	0.02
2 weeks	0.29	0.01	0.27	0.01	0.31	0.03
1 month	0.29	0.02	0.29	0.03	0.27	0.01
Mid Control (3.0 ng/mL)						
24 hr	3.3	0.1	3.2	0.3	3.2	0.10
2 weeks	3.0	0.1	3.1	0.1	3.0	0.20
1 month	3.1	0.1	3.2	0.4	3.1	0.20
High Control (7.5 ng/mL) ‡						
24 hr	8.0	0.4	7.4	0.1	7.5	0.30
2 weeks	6.8	0.4	7.6	0.2	7.3	0.60
1 month	7.2	0.3	7.3	0.2	7.3	0.30

Low Control (0.3 ng/mL) ‡

JWH-073 4OH (n = 3)	(-4°C)		(-24°C)		(-80°C)	
Time/Temp	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD
24 hr	0.29	0.03	0.30	0.01	0.32	0.01
2 weeks	0.31	0.03	0.26	0.01	0.31	0.02
1 month	0.29	0.01	0.31	0.03	0.30	0.02
Mid Control (3.0 ng/mL) **						
24 hr	3.4	0.1	3.2	0.20	3.4	0.20
2 weeks	2.9	0.1	3.0	0.01	2.9	0.10
1 month	3.2	0.3	3.4	0.30	3.3	0.04
High Control (7.5 ng/mL) *						
24 hr	8.2	0.6	7.6	0.6	7.8	0.3
2 weeks	6.7	0.2	7.5	0.5	7.2	0.1
1 month	7.4	0.3	7.5	0.4	7.9	0.4

Significant differences in concentration as a function of storage time are indicated by * = $p < 0.05$, ** = $p < 0.01$, storage temperature $^{\#}$ = $p < 0.05$, $^{\#\#}$ = $p < 0.01$, and interaction between storage time and temperature ‡ = $p < 0.05$.

4. DISCUSSION

Although synthetic cannabinoids continue to be abused worldwide, no studies to date have assessed their direct effects on behavior and physiology. As such, much of what we know about these compounds is from preclinical research, surveys to understand demographics of users, patterns of use, and motivation for use, and from case-report literature regarding their significant adverse effects (Cooper, 2016). The discrepancy between the widespread use of SCs and the data pertaining to the direct behavioral and physiological effects of these drugs results in a significant gap in the understanding of how to treat both acute intoxication and SC use disorders. For instance, a recent survey found that 80% of physicians in urban emergency departments reported feeling unprepared to treat patients presenting with Spice intoxication, and 99% reported that more education on emerging drugs and abuse patterns is needed (Lank et al., 2013). Controlled drug-administration studies of these compounds are necessary in order to understand their acute and long-term effects including their abuse liability, cognitive, psychotomimetic, and physiological effects. The methods described herein assessed cigarettes (one per drug concentration and time point tested) to establish a preparation to administer concentration-dependent smoked JWH-018 and JWH-073. These concentration-dependent cigarettes will be instrumental in assessing the dose-dependent effects of synthetic cannabinoids relative to those of cannabis in the human laboratory. The preparation yielded uniform distribution of synthetic cannabinoids across the majority of the cigarettes with some samples differing by more than 20% between the two cigarette sections.

In order to ensure superior uniformity, future preparations should mix the plant material with drug solution for more than 15 seconds to ensure equal saturation prior to preparing the cigarettes. The optimal conditions to assess parent compounds and respective metabolites in human plasma using UPLC/MS/MS were also validated in order to study the pharmacokinetics of the parent compounds and active metabolites after smoked SC administration. The analytes were within ± 20% of the target values, a common standard for considering a control as accurate (SWGTOX, 2013**)**, yet findings suggest that storage duration may play a role in stability of the parent compounds and metabolites in plasma.

While recent reports of SC use include oral and vaporized routes of administration, smoking plant matter adulterated with the compounds is the most popular method (Brents and Prather, 2014). As such, the described preparation of SC-laced cigarettes reflects how they are most commonly used in the natural ecology. Furthermore, the design allows for direct comparison to cannabis, capitalizing on the administration methods that have been used for over two decades to establish the its effects in the human laboratory (i.e., 800 mg cannabis cigarettes with increasing concentrations of THC; Cooper and Haney, 2009a; Haney et al., 1999; Haney, 2009). One of the only reports on controlled administration of JWH-018 utilized a glass pipe to administer the compound to volunteers. For that preparation, the compound was added to a 'small' amount of plant matter and smoked (Toennes et al., 2017).The method yielded reliable plasma levels of the parent compounds and metabolites as assessed using LC-MS/MS. However, residue in the pipe revealed that the amount of drug left over after each session varied significantly (0.09 - 2.10 mg) suggesting that participants were not exposed to the complete dose of 2-3 mg. The preparation for smokable SCs described herein may provide a more standard, reliable way to administer concentration-dependent smokable JWH-018 and JWH-073.

Human laboratory studies are a powerful means of studying behavioral and pharmacological effects of drugs of abuse (Cooper and Haney, 2009b; Haney, 2009). Only under laboratory conditions with continuous monitoring can the precise nature of the dose- and time-dependent physiological risks and behavioral effects of synthetic cannabinoids be determined systematically. Such a study would provide fundamental data relating the onset and duration of the psychoactive and physiological effects of JWH-018 and JWH-073 to the pharmacokinetics of the parent compounds and respective metabolites, an analysis possible only under controlled conditions. Given the extreme adverse effects associated with SC use (Ford et al., 2017) controlled studies of the

compounds require extraordinary control and methods in place to minimize risks. Two small studies to date have administered JWH-018 to a total of 8 participants (Teske et al., 2010; Toennes et al., 2017) and reported that the drug was well tolerated suggesting limited risk in future studies. In order to further minimize risks, only current healthy SC users with no previous serious adverse effects should be recruited for such study. Furthermore, when first establishing the acute effects of the SCs, doses should be administered in ascending order under single-blind conditions. Vital signs should be continuously monitored and participants should be fully informed of the side effects that they might experience. While compounds in SC products are shifting (i.e., Castaneto et al., 2014; Wiley et al., 2015) the methods for assessing the effects of these early SCs will provide the foundation for assessing other compounds in the future as they emerge.

5. CONCLUSION:

These series of studies provide methods to investigate concentration-dependent physiological and behavioral effects and pharmacokinetics of smoked SCs under carefully controlled conditions and to compare their effects to cannabis. Such studies are integral in the understanding of the risks associated with SC use and will be central in investigating potential treatments for both acute intoxication and the negative consequences of long-term exposure including synthetic cannabinoid use disorders.

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Figure 1

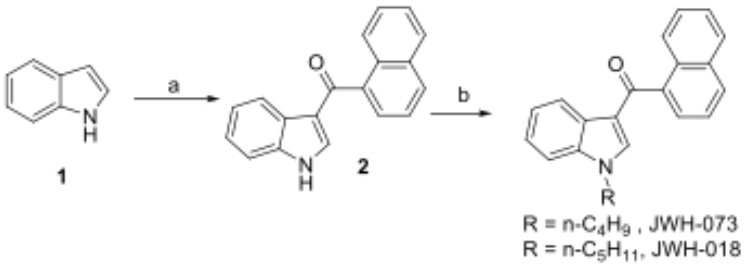

Figure 1. Reagents and conditions: a) 1-Naphthoyl chloride, Et_2AlCl, 0 ^oC to RT, 24h; b).1-bromopentane or 1-bromobutane, KOH/DMF, 0 ^oC to 40 ^oC, 4h.

HIGHLIGHTS

1. Synthetic cannabinoids (SCs) are a serious public health concern.
2. There are no controlled, dose-dependent studies on their behavioral and physiological effects in volunteers.
3. A validated method to prepare concentration-specific SC cigarettes for smoked study is described
4. Optimized methods to assess the compounds and metabolites in plasma after administration were investigated.