Synthetic Cannabinoids in Oral Fluid

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Abstract

At the end of 2010, the U.S. Drug Enforcement Administration (DEA) used its emergency scheduling authority to temporarily control five chemicals, JWH-018, JWH-073, JWH-200, CP-47497, and cannabicyclohexanol (CP-47497 C8), often referred to as “Spice”, K2, or “synthetic cannabinoids” because of their reported cannabis-like effects. JWH-250 is commonly encountered, and HU-210 was already controlled, so these were also included in the research. We report the first analytical procedure for the simultaneous determination of these compounds in oral fluid specimens collected with the Quantisal™ device using solid-phase extraction and liquid chromatography with tandem mass spectrometry. The method was validated and applied to specimens taken from two individuals who had purchased the synthetic compounds while still legally available in the U.S. After a single session of smoking “Blueberry Posh”, the peak concentration of JWH-018 detected was 35 μg/L 20 min after smoking; JWH-018 was still detectable 12 h after a single intake. After a single session of smoking “Black Mamba”, JWH-018 was detected with a peak concentration of 5 μg/L after 20 min. In this subject, the compound was not detectable after 12 h.

Introduction

Notice was given at the end of 2010 by the Drug Enforcement Agency (DEA) that five members of the “synthetic cannabinoids” group or “Spice” compounds were to be banned in the U.S. The substances were 1-pentyl-3-(1-naphthoyl)-indole (JWH-018), 1-butyl-3-(1-naphthoyl)-indole (JWH-073), 1-(2-(4-morpholinyl)ethyl)-3-(1-naphthoyl)-indole (JWH-200), 5-(1,1-dimethylheptyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (CP-47497), and 5-(1,1-dimethyloctyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol (cannabicyclohexanol; CP-47497 C8 homologue). The drugs have been described by users as having cannabis-like effects, and some of these compounds show strong binding to cannabinoid receptors. In general, plant materials are sprayed with one or a combination of these synthetic compounds before sale. The chemical analysis of various herbal products has predominantly shown the presence of CP-47497 or its homologue CP-47497 C8, JWH-018, and/or JWH-073 (1). The (+)-1,1-dimethylheptyl analogue of 11-hydroxy-Δ9-tetrahydrocannabinol, (1,1-dimethylheptyl-11-hydroxytetrahydrocannabinol) is known as HU-210 and has been reportedly found in seizures of “Spice Gold”, “Spice Silver”, and “Spice Diamond” made by the U.S. Customs and Border Protection in 2009. HU-210 is considered to be over 100 times more potent than Δ9-tetrahydrocannabinol (Δ9-THC) present in marijuana, and it was already classified as a controlled substance as an analogue of marijuana.

In recent articles, Spice drugs have been described as “a new trend” (2) as “new designer drugs” (3), and as a “never ending story” (4). Emergency room physicians in San Diego, CA reported a case of two patients admitted following an intake of JWH-018 and JWH-073; the doctors suspect that cases will increase (5). A comprehensive paper was recently published which used high-resolution accurate mass spectrometry (MS) to produce a database of over 140 compounds associated with these products (6). In 2009, Möller et al. (7) described screening for JWH-018 and its major metabolites in urine for anti-doping purposes using liquid chromatography (LC)–MS–MS. Although no intact JWH-018 was detectable, the procedure described the monitoring of hydroxylated metabolites. In 2010, Sobolevsky et al. (8) also identified JWH-018 metabolites in urine using both gas chromatography (GC) and LC–MS after smoking of synthetic cannabinoids. Teske et al. (9) described the determination of JWH-018 in serum, and more recently, Dresen et al. (10) published an article on the determination of several Spice compounds, also in serum: JWH-015, JWH-018, JWH-019, JWH-020, JWH-073, JWH-081, JWH-200, JWH-250, WIN-55212-2, and methanandamide. To date the identification of any of these compounds in oral fluid has not been published.

Oral fluid is becoming increasingly popular as a specimen for the detection of drugs at the roadside, and in workplace testing. It is easy to collect, non-invasive and can give information on recent drug intake. Several publications have reported the presence of Δ9-THC and its metabolite 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid in oral fluid using the Quantisal
oral fluid collection device (11–13). These publications have included information on the recovery of cannabinoids from the collection pads as well as concentrations detected in marijuana smokers or Marinol® users. Further, a recent presentation at the Society of Forensic Toxicologists annual conference (2010) showed that subjects taking Marinol preferred oral fluid collections using the Quantisal device compared to expectoration. The authors noted: “Even with a higher LOQ, Quantisal oral fluid collection provided specimens with better detection rates than expectoration. These results highlight the importance of collection method in oral fluid drug tests and aid in the interpretation of drug test results” (14).

In the work described here, the Quantisal device was used for oral fluid collection, and the detection of Spice components in oral fluid is described for the first time.

Materials and Methods

Collection devices, reagents, and standards

Quantisal devices for the collection of oral fluid specimens were obtained from Immunalysis (Pomona, CA). The devices contain a cotton collection pad which is placed in the mouth. The incorporated volume adequacy indicator turns blue when 1 mL of oral fluid (±10%) has been collected. Fifty subjects (25 female; 25 male) were asked to use the device following the collection procedure instructions. Prior to collection, each device, including the pad, was weighed. After the volume adequacy indicator turned blue on the collector stem, the subject placed the collector with the collection pad into the transport tube, capped the tube, and returned it to the observer. The tubes were weighed, and the difference in weight prior to the collection process was noted. Within a confidence interval of 99%, the volume collected was between 0.907 and 1.079 mL. The claim of 1 mL ±10% of saliva collected with the volume adequacy indicator was determined.

Under routine collection conditions, after the volume adequacy indicator has turned blue, the pad is placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). Drug concentrations detected are adjusted accordingly.

HPLC-grade solvents were obtained from Spectrum Chemicals (Gardena, CA). The positive pressure extraction manifold and the solid-phase columns (Trace-N TN-315) were purchased from SPEWare (Baldwin Park, CA). LC columns were obtained from Agilent Technologies (Palo Alto, CA).

The standard compounds JWH-018, JWH-073, JWH-200, JWH-250, HU-210, CP-47497, and CP-47497 C8 homologue as well as deuterated JWH-018-d₉ and JWH-073-d₇ were purchased from Cayman Chemicals (Ann Arbor, MI).

Calibrators and controls

The deuterated internal standards (JWH-018-d₉ and JWH-073-d₇) and the unlabeled drug standards were prepared in methanol at a concentration of 100 μg/mL. The working solutions were diluted from the stock to a concentration of 10 μg/mL in methanol. The solutions were stored at –20°C when not in use. Controls were prepared by fortifying drug-free synthetic oral fluid with various concentrations of cannabinoids. Drug-free negative specimens and positive controls at 4 and 40 μg/L were included in every batch.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition*</th>
<th>Fragment Voltage (V)</th>
<th>Collision Energy (eV)</th>
<th>Polarity</th>
<th>Ratio of Quantifying to Qualifying Transition (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JWH-018-d₉</td>
<td>351.3 &gt; 223.4</td>
<td>140</td>
<td>20</td>
<td>positive</td>
<td>n/a†</td>
</tr>
<tr>
<td>JWH-018</td>
<td>342.2 &gt; 155.1</td>
<td>120</td>
<td>20</td>
<td>positive</td>
<td>11.4-17.1</td>
</tr>
<tr>
<td>JWH-250</td>
<td>336.3 &gt; 188.2</td>
<td>120</td>
<td>12</td>
<td>positive</td>
<td>74.5-111</td>
</tr>
<tr>
<td>JWH-073-d₇</td>
<td>335.3 &gt; 207.2</td>
<td>120</td>
<td>20</td>
<td>positive</td>
<td>n/a</td>
</tr>
<tr>
<td>JWH-073</td>
<td>328.2 &gt; 155.1</td>
<td>120</td>
<td>20</td>
<td>positive</td>
<td>45.2-67.9</td>
</tr>
<tr>
<td>CP 47497</td>
<td>331.3 &gt; 313.3</td>
<td>160</td>
<td>25</td>
<td>negative</td>
<td>64.2-96.3</td>
</tr>
<tr>
<td>CP 47497 C8</td>
<td>331.3 &gt; 259.3</td>
<td>160</td>
<td>35</td>
<td>negative</td>
<td>74.7-112</td>
</tr>
<tr>
<td>HU-210</td>
<td>385.3 &gt; 367.4</td>
<td>120</td>
<td>30</td>
<td>negative</td>
<td>33.2-49.8</td>
</tr>
<tr>
<td>CP 47497</td>
<td>317.3 &gt; 299.2</td>
<td>160</td>
<td>20</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>CP 47497</td>
<td>317.3 &gt; 245.2</td>
<td>160</td>
<td>30</td>
<td>negative</td>
<td></td>
</tr>
</tbody>
</table>

* Quantitation transitions are underlined.
† n/a = not applicable for internal standard.
Sample preparation

Seven calibration standards were prepared in oral fluid at concentrations of 0.5, 2, 5, 10, 20, 50, and 100 μg/L for all analytes. Both deuterated internal standards were added (10 μg/L). For authentic specimens, the Quantisal devices were vortex mixed for 15 min prior to extraction. Trace-N solid-phase extraction columns were conditioned with methanol (0.5 mL) and 0.1 M acetic acid (0.1 mL). To each 1-mL aliquot of calibrator, control or specimen, acetic acid (0.1 M, pH 4, 1 mL) was added and the samples loaded onto the columns. The columns were washed with deionized water (DI); glacial acetic acid/water (80:20, v/v, 1 mL); and DI water/methanol (40:60, v/v, 1 mL). The columns were allowed to dry under nitrogen for 5 min. The compounds were eluted with hexane/glacial acetic acid (98:2, v/v, 2 mL). The entire extraction procedure was carried out using a positive pressure manifold, which allows uniform flow rate through the columns. The eluent was evaporated to dryness under nitrogen at 40°C and reconstituted in methanol (50 μL). The samples were injected into the LC–MS–MS system.

For quantitation JWH-018-d₉ was used as the internal standard for JWH-018, CP-47497, and CP-47497 C₈. Deuterated JWH-073-d₇ was used as the internal standard for JWH-073, JWH-250, and HU-210.

LC–MS–MS

An Agilent Technologies 1200 series LC pump coupled to a 6430 triple-quadrupole MS, operating in electrospray ionization mode (ESI) with either positive or negative polarity depending on the compound. The LC column was an Agilent Extend C₁₈ (2.1 × 50 mm × 1.8 μm), the column temperature was held at 60°C and the injection volume was 5 μL. The mobile phase consisted of Solvent A: 0.2% acetic acid and Solvent B: acetonitrile. At time 0, the solvent composition was 95% A; 5% B. After 5 min the ratio had changed to 100% B; after 7 min it was returned to 1% B. The run time was 9.2 min and a post-time of 3 min was required for equilibration. The flow rate was set to 0.5 mL/min. The gas temperature was 350°C, the gas flow was 10 L/min, and the nebulizer pressure was 55 psi. Nitrogen was used as the collision gas and the capillary voltage was +4000 V in positive mode; −4000 V in negative mode. Two transitions were selected and optimized for each drug. Table I shows the optimized fragment voltages for the parent ion (M+1; M–1) and the collision energy for fragmentation of the product ions. Each subsequent analysis required the ratio between the quantitative ion and the qualifier ion to be within ±20% in order to meet the criterion for a positive result. Figure 1 shows a chromatogram for the primary transitions of the compound at a concentration of 10 μg/L; the ratio of primary to secondary transition for each compound was also determined at the calibration point of 10 μg/L.

Method Validation

Recovery from the collection pad

Six synthetic oral fluid specimens fortified with the synthetic cannabinoids at concentrations of 4 and 40 μg/L were prepared. The collection pad was placed into the samples until 1 mL (+10%) had been collected, as evidenced by the blue volume adequacy indicator incorporated into the stem of the collector, then the pad was transferred to the Quantisal buffer, capped, and stored overnight to simulate transportation to the laboratory. The following day, the tubes were vortex mixed for 15 min, pads were compressed, and an aliquot of the specimen was analyzed as described. The amount recovered from the pad was compared to an absolute concentration (100%) where drug was added to the buffer and left overnight at room temperature without the pad, then subjected to extraction and analysis.

Data analysis

Calibration was carried out using linear regression analysis over a concentration range of 0.5–100 μg/L. Peak-area ratios
of target analytes and the internal standard were calculated for each concentration using Agilent MSD software. The data were fit to a linear least-squares regression curve, not forced through the origin, and with equal weighting. For confirmation, two transitions were monitored for each of the compounds; one for the internal standard. The ratio of the qualifying transition was required to be within 20% of that established using the known calibration standards (at 10 μg/L) in order to be acceptable.

**Linearity and sensitivity**

The limit of quantitation (LOQ) of the method was determined using serial dilutions to the lowest point where the acceptable criteria for the quantitation of a compound were met, that is, the chromatographic peak shape, retention time (within 2% of calibration standard) and qualifier transition ratio (±20%) compared to the 10 μg/L calibration standard was acceptable. The quantitative value of the LOQ had to be within ±20% of the target concentration and replicate analyses were required to have low variation in response (n = 5; CV, 15%). Because all specimens were to be quantitative, the limit of detection (LOD) was not determined.

**Selectivity**

Five drug-free oral fluid specimens were collected using the Quantisal device. An aliquot of each was taken and subjected to extraction and analysis as described, in order to assess potential interferences associated with endogenous compounds or the transportation buffer.

Additionally, to other aliquots of the drug-free fluid, common drugs of abuse were added at concentrations of 2000 μg/L. Δ⁹-THC, 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid, 11-hydroxy-THC, cannabinol, cannabidiol, cocaine, benzoylecgonine, norcocaine, cocaethylene, codeine, morphine, 6-acetylmorphine, 6-acetylcocaine, oxycodone, oxymorphone, hydrocodone, hydromorphone, amphetamine, methamphetamine, methylenedioxyamphetamine (MDMA), methylenedioxy-3,4-methylenedioxyamphetamine (MDMA), benzphetamine, methylenedioxyamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), phentermine, fentanyl, phencyclidine, tramadol, carisoprodol, meprobamate, citalopram, venlafaxine, amitriptyline, cyclobenzaprine, imipramine, dothiepin, dexamfetamine, methimazole, and methadone were added to drug-free oral fluid, extracted, and analyzed as described.

**Imprecision and ion suppression**

The imprecision of the assay at two control levels was evaluated. Specimens were fortified with all the compounds simultaneously at concentrations of 4 and 40 μg/L. Each concentration was analyzed according to the described procedure (n = 6; intraday imprecision) for five consecutive days (n = 30; interday imprecision).

**Matrix effects**

Procedures involving LC–MS–MS may be subject to changes in analyte response due to the effect of biological matrices. The published protocol from Matzuweski et al. (15) was used for the assessment of matrix effects and process efficiency. Briefly, a non-extracted drug standard at a concentration of 10 μg/L was prepared as well as drug-free matrix extracts and negative controls (extracts containing only internal standard).

The recovery of the compounds from the oral fluid was determined by first assessing the response of the extracted samples (n = 3) at a concentration of 10 μg/L (R \text{ES}). Next, oral fluid was extracted and drug was added post-extraction at a concentration of 10 μg/L (n = 3) (R \text{PES}). The percentage recovery was then calculated from the equation (R \text{ES}/R \text{PES}) \times 100.

The reduction in response due to matrix effects (ion suppression) was determined by assessing the peak-area response of a non-extracted neat drug standard (n = 3) at a concentration of 10 μg/L (R \text{NES}). The non-extracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The % matrix effect due to ion suppression was then calculated using the equation (R \text{PES}/R \text{NES}) \times 1\times 100. The overall efficiency of the process was calculated as (R \text{ES}/R \text{NES}) \times 100.

It has been reported that reduction or elimination of matrix effects is best achieved by utility of deuterated internal standards where possible, extensive matrix clean-up before injection and optimized chromatographic and mass spectral conditions.
Stability of the synthetic cannabinoids in oral fluid

The stability of the synthetic cannabinoids in Quantisal buffer-oral fluid was determined over 7 days at both room temperature and at 4°C. Oral fluid samples were fortified with 40 μg/L of each compound. The samples were allowed to remain at room temperature and at 4°C for a week. On days 1, 4, and 7, an aliquot was removed and analyzed as described.

The stability of the extracts was also investigated. Autosampler vials, after analysis, were re-capped and stored at 7°C overnight, before being re-analyzed after 24 and 48 h; the concentration change between the days was noted.

Authentic samples

Specimens were collected from two naïve volunteers who had purchased the compounds while they were still legally available in the U.S. Subject #1 smoked “Blueberry Posh”, and subject #2 smoked “Black Mamba”. Using Quantisal oral fluid collection devices, specimens were collected prior to the start of smoking, then at the various time points after smoking. Subject 1 gave specimens after 20 min, 40 min, 1 h, 2 h, and 12 h; Subject 2 gave samples after 20 min, 40 min, 1 h, 5 h, and 12 h. The specimens were analyzed the day after collection, then were stored at 4°C for one month and re-analyzed according to the described procedure.

Results and Discussion

Method validation

The extraction method was based on our previous publication for the determination of cannabinoids in oral fluid, including marijuana plant constituents and the pyrolytic precursor to THC, 2-carboxy-THC (16). No endogenous interference was noted from drug-free extracts, nor was there exogenous interference from any of the commonly encountered drugs, including THC and its main metabolites, which were analyzed at high concentrations. The limit of quantitation for all the analytes was 0.5 μg/L. Because of its basic nature, JWH-200 did not extract efficiently using the described procedure which was optimized for acidic/neutral drugs. Changing the method to a cation exchange extraction allowed JWH-200 to extract, but HU-210 and CP-47497 and its C8 homologue were diminished. Therefore, JWH-200 was removed from the procedure, even though its response on the LC–MS–MS was excellent, and it should be easily analyzed in urine or other biological specimens that do not require extraction before analysis.

Linearity was acceptable from the LOQ to 100 μg/L ($r^2 > 0.99; n = 5$) for all compounds. Interday and intraday imprecision, percentage recovery, matrix effect, and process efficiency as determined with ion suppression experiments are shown in Table II. The intraday imprecision of the assays for all drugs was < 6% at both concentrations; interday < 10% at both concentrations. The overall process efficiency was > 70% for all compounds, and ion suppression effects were limited by the use of solid-phase extraction and deuterated internal standards, particularly for JWH-018 and JWH-073, which produced the lowest amount of signal suppression (11%).

Extraction from the pad and stability of the analytes

The percentage recovery from the pad for JWH-018, JWH-073, JXH-250, HU-210, CP-47497, and CP-47497 C8 at concentrations of 4 and 40 μg/L ($n = 6$) were approximately 60% for all compounds at both levels. The highest recovery was 74% (CV 0.5%) for CP-47497 at 4 μg/L; the lowest was 55% (CV 0.5%) for JWH-073 at 4 μg/L. Stability of the compounds at room temperature and at 4°C over a period of 7 days: JWH compounds (A) and CP47497, CP47497 analogue, and HU-210 (B).

Figure 2. Stability of the compounds at room temperature and at 4°C over a period of 7 days: JWH compounds (A) and CP47497, CP47497 analogue, and HU-210 (B).

Figure 3. JWH-018 in oral fluid in specimens collected after a single session of smoking herbal products. Analysis was carried out one day after collection and one month after collection following refrigerated storage (4°C).
0.3%) for JWH-018 at 4 μg/L. The recoveries were essentially equivalent at both levels, but as a general rule the efficiency of the PAD recovery diminishes with increasing concentration due to saturation which may account for low percentage recovery reports in some publications. Other variables include residence time in the buffer, which for most drugs should be a minimum of 4 h; the experiments here were designed to mimic overnight transportation to a test facility.

The stability of the compounds at room temperature and at 4°C over a period of 7 days is presented in Figure 2. JWH-073 declined by 25%, and JWH-073 and JWH-018 showed losses of 16% at room temperature. However, when refrigerated, JWH-073 lost only 10%, and the other two compounds showed no degradation. The CP-47497, CP-47497 C8, and HU-210 compounds showed similar losses whether at room temperature or refrigerated ranging from 9 to 14%. In order to maintain all the compounds with approximately 10% degradation, collected specimens should be stored at refrigerated temperatures prior to extraction. The extracts themselves were extremely stable when left in the autosampler of the instrument at 7°C for 48 h, with a maximum variation of 5% from the target value at 4, 10, and 40 μg/L for all compounds.

Authentic specimens

The procedure was applied to specimens collected from naïve synthetic marijuana smokers following a single smoking session. Subjects reported that “Black Mamba” appeared to have a greater euphoric effect than the “Blueberry Posh”. The specimens were stored at 4°C for one month then reanalyzed; both sets of results are shown in Figure 3.

Following intake of “Blueberry Posh”, JWH-018 was identified in the first specimen collected after 20 min at a peak concentration of 35 μg/L; it was also detected in subsequent oral fluid samples, except for the one taken prior to smoking, and was still present 12 h after a single smoking session at a concentration of 0.5 μg/L. In the subject who smoked “Black Mamba”, JWH-018 was present in much lower concentrations than Subject #1, showing a peak concentration of only 3 μg/L 20 min after smoking; however, JWH-018 was detectable for 5 h in very low concentration. After storage at 4°C for one month, the samples were reanalyzed and found to be extremely stable with almost identical concentrations detected (Figure 3); these data are consistent with the stability experiments.

An extracted ion chromatogram showing the transitions and ±20% acceptability band around the intensity of the qualifying transition from the sample collected 40 min after smoking (Subject #1) is presented in Figure 4; the concentration of JWH-018 was 10 μg/L.

According to the Drug Enforcement Agency, K2 or Spice has street names such as “Bliss”, “Black Mamba”, “Bombay Blue”, “Fake Weed”, “Genie”, and “Yucatan Fire” among others, and compounds typically detected in the mixes include JWH-018, JWH-073, and HU-210 and its non-pharmacologically active enantiomer HU-211. The presence of JWH-018 in both of these herbal varieties was confirmed using full scan GC–MS. Major ions were present at m/z 341, 284, 324, and 214. Tocopherol (m/z 430, 165, and 205) was also identified in the material, which has been reported by other researchers (17, 18).

Oral fluid concentrations may more readily correlate to plasma or serum concentrations than urine. Two publications have reported the determination of various Spice components in serum. Dresen et al. (10) successfully applied their procedure to over 100 serum samples from subjects in Germany. The most prevalent compound was JWH-081 detected within a concentration range of 0.11–16.9 μg/L; mean 2.35 followed by JWH-250 (0.14–18.1 μg/L; mean 2.49), JWH-018 (0.3–8.1 μg/L; mean 1.84) and JWH-073 (0.23–0.6 μg/L; mean 0.42). JWH-019, JWH-020, JWH-200, and methanandamide were not detected in any of the specimens. At the time of the experiments, JWH-018 and JWH-073 were controlled in Germany; JWH-081 and JWH-250 were not. Teske et al. (9) reported on two subjects who smoked approximately 50 μg/kg of JWH-018. Their serum concentrations reached values of approximately 10 μg/L, dropping rapidly within 3 h to less than 1% of their maximum concentration. This is a similar pattern to that observed in the smokers in our study, where concentrations dropped rapidly in the first few hours after intake, having reached an observed maximum at the first collected time point (20 min).

Conclusions

The simultaneous determination of several Spice compounds in oral fluid is reported for the first time. The procedure is applicable to the analysis of specimens collected using the Quantisal device for the presence of synthetic cannabinoids, which were recovered from the pad > 60% at two concentrations. Following a single smoking session of two different herbal product brands, JWH-018 was detected in oral fluid, with the highest concentrations appearing 20 min after a single smoking session. JWH-018 was detectable 5 h after smoking in one individual and 12 h after smoking in the other.
References


