HHS Public Access

Author manuscript

Ther Drug Monit. Author manuscript; available in PMC 2016 October 01.

Published in final edited form as:

Ther Drug Monit. 2015 October ; 37(5): 661–669. doi:10.1097/FTD.0000000000000201.

Validation of an ELISA Synthetic Cannabinoids Urine Assay

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Abstract

Background—Synthetic cannabinoids are touted as legal alternatives to cannabis, at least when first released, and routine urine cannabinoid screening methods do not detect these novel psychoactive substances. Synthetic cannabinoids are widely available, are a major public health and safety problem, and a difficult challenge for drug testing laboratories. We evaluated performance of the NMS JWH-018 direct ELISA kit to sensitively, selectively, and rapidly screen urinary synthetic cannabinoids.

Materials/ Methods—The NMS ELISA kit targeting the JWH-018 N-(5-hydroxypentyl) metabolite was utilized to screen 2492 urine samples with 5 and 10μg/L cutoffs. A fully validated LC-MS/MS method for 29 synthetic cannabinoids markers confirmed all presumptive positive and negative results. Performance challenges at ±25 and ±50% of cutoffs determined intra- and inter-plate imprecision around proposed cutoffs.

Result—The immunoassay was linear from 1–500μg/L with intra- and inter-plate imprecision of ≤8.2% and <14.0%, respectively. No interferences were present from 93 common drugs of abuse, metabolites, co-administered drugs, over-the-counter medications or structurally similar compounds, and 19 of 73 individual, synthetic cannabinoids (26%) exhibited moderate to high cross-reactivity to JWH-018 N-(5-hydroxypentyl) metabolite. Sensitivity, specificity, and efficiency results were 83.7%, 99.4% and 97.6% and 71.6%, 99.7% and 96.4%, with the 5 and 10μg/L urine cutoffs, respectively.

Conclusion—This high throughput immunoassay exhibited good diagnostic efficiency and documented that the NMS JWH-018 direct ELISA is a viable method for screening synthetic cannabinoids in urine targeting the JWH-018 N-(5-hydroxypentyl) and related analytes. Optimal performance was achieved with a matrix-matched 5μg/L urine cutoff.

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Keywords

Synthetic cannabinoids; ELISA; immunoassay

1. Introduction

Synthetic cannabinoids are marketed as natural, herbal mixtures not for human consumption, but are abused as novel psychoactive substances. Synthetic cannabinoids are touted as legal alternatives to cannabis, at least when first released, and are not detected in routine urine drug screening methods, making them attractive to those subject to urine drug tests[1]. Synthetic cannabinoids bind to the same CB_1 and CB_2 cannabinoid receptors as Δ^9-tetrahydrocannabinol (THC)[2]. CB_1 agonists primarily located in the central nervous system are responsible for psychotropic effects, while CB_2 receptor agonists are important to immune function and analgesia[3]. Some synthetic cannabinoids have greater affinity for CB_1 receptors as compared to THC, resulting in more intense and prolonged reactions[4,5]. The 2012 DAWN Report, U.S. Substance Abuse and Mental Health Services Administration (SAMHSA), stated that synthetic cannabinoids were responsible for 28,500 emergency room visits in 2011[6]. The inconsistent composition and potency of synthetic cannabinoids herbal mixtures contributes to reported unpredictable effects. Reported adverse effects include seizures, psychosis, paranoia, altered mental status, intense anxiety, hallucinations, increased blood pressure and heart rates, panic attacks, nausea and vomiting[7–11].

In response to these adverse effects and high abuse potential, the Synthetic Drug Abuse Prevention Act was passed in July 2012. While 26 synthetic compounds are currently classified as Schedule I drugs under the Controlled Substances Act, cannabimimetic agents, any chemical that mimics cannabis' effects via CB_1 receptors, also are banned[12]. This led to the rapid development and introduction of novel psychoactive substances with modified chemical structures.

Synthetic cannabinoids are an increasingly popular recreational drug[13]. According to the 2012 Monitoring The Future survey, 11.4% of 12th graders used synthetic cannabinoids in the last year; making it the second most popular illegal drug among American teenagers, after cannabis[14]. A similar trend also was noted among young adults. In a sampling of students (n=852) from a large American university, 8% reported smoking synthetic cannabinoids at least once in their lifetime[15].

As a result of this recent popularity, several qualitative[16–21] and quantitative[22–26] methods were developed utilizing liquid chromatography tandem mass spectrometry (LC-MS/MS) to detect one or more synthetic cannabinoids in urine. Gas chromatography mass spectrometry (GC-MS)[17, 18] and tandem MS (GC-MS/MS)[19] methods also were published.

Identification and quantification of synthetic cannabinoids and their metabolites in biological specimens is an ongoing challenge for laboratories, as their constantly changing structures require new antibody production for commercial immunoassays to stay current. Mass spectrometric analytical methods for identification and confirmation are hampered by lack of commercially available reference standards. Furthermore, these methods must be

updated and validated to include the latest synthetic cannabinoids, creating a cycle where drug detection lags behind the newly emerging synthetic cannabinoids. Griffiths et al. commented that synthetic cannabinoids are a transient product that is challenging the existing models of drug control[27].

A rapid, inexpensive screening procedure for synthetic cannabinoids in multiple biological matrices is important for clinical, forensic, drug treatment, driving under the influence, and workplace drug testing programs. Enzyme Linked Immunosorbent Assays (ELISA) screening techniques are flexible, easily automated and have the required sensitivity and specificity that has made this an increasingly popular option for drug testing laboratories[28–30].

The aim of this study was to evaluate the National Medical Services (NMS) JWH-018 direct ELISA kit as a screening method for the detection of synthetic cannabinoids in 2492 urine samples. All presumptive positive and negative results were confirmed by a validated LC-MS/MS method for 29 synthetic cannabinoid markers to determine sensitivity, specificity and efficiency.

2. Materials and Methods

2.1 ELISA Instrumentation

The qualitative, automated analysis of synthetic cannabinoids was performed on a Freedom EVO 100 platform configured with a microtiter plate washer and reader. (Tecan Group, San Jose, CA). The Freedom EVO 100 is a flexible liquid handling system for low to medium throughput applications. The 8-channel liquid handling arm and robotic manipulator arm enhance workstation capabilities allowing for fully automated ELISA plate processing.

2.2 NMS Synthetic Cannabinoids Microplate ELISA Kit

The NMS JWH-018 direct ELISA kit contained all components required for analysis with the exception of calibrators and controls. Each kit contained coated 96-well microtiter plates, drug enzyme conjugate, tetramethylbenzidine (TMB) substrate, 0.2N HCl stop solution, and Phosphate Buffered Saline (PBS). The assay was performed without modification according to the manufacturer’s instructions. All kit components were stored at 4°C until analysis.

Briefly, a 20μL aliquot (blank, cutoff, control or unknown) and 100μL drug enzyme conjugate were added to the coated microtiter plate and incubated at room temperature for 60min. After incubation, the plate was washed five times with 300μL PBS using an automatic plate washer. After washing, plates were manually inverted and slapped dry to remove residual liquid from the wells. The liquid handling arm added 100μL TMB substrate to initiate a colorimetric reaction. After 30 min, 100μL acid stop solution was added and the absorbance (450nm) immediately measured with a plate reader. Total analysis time was approximately 2h.

2.3 Calibrators, Controls and Performance Challenges

JWH-018 N-(5-hydroxypentyl) metabolite was purchased from Cayman Chemicals (Ann Arbor, MI). Stock standard solutions of cutoff calibrators and controls were prepared from different ampoules by diluting with appropriate volumes of methanol. Cutoff calibrators (5 and 10μg/L) were prepared by fortifying drug-free urine and PBS with stock calibrator solution. Working performance challenges at ±25% and ±50% of each cutoff were prepared by fortifying drug-free urine with stock control solution. Positive controls at 10 and 20μg/L were prepared by fortifying drug-free urine with stock control solution to evaluate assay performance at 5 and 10μg/L.

2.4 Specimens

We tested 20,017 authentic anonymous urine specimens from the Department of Defense (DoD) drug testing laboratories, which previously screened negative for cannabinoids, cocaine, amphetamines, phencyclidine and opiates, during routine urinalysis testing. Specimens were collected from all around the world from July 2011 through June 2012 and were stored at room temperature before initial immunoassay analyses, as per DoD protocol. Specimens were analyzed with the Randox Drugs of Abuse V biochip array technology for synthetic cannabinoids. Presumptive positive (1,424) and randomly selected negative (1,068) specimens were stored at 4 – 7°C before this ELISA determination and LC-MS/MS confirmation. This project was funded under an inter-agency agreement between the DoD Drug Demand Reduction Initiative and the National Institute on Drug Abuse, National Institutes of Health.

2.5 Method Development

Analysis was performed according to the manufacturer's instructions, although additional experiments were performed to optimize assay performance. The recommended cutoff calibrator was 5μg/L JWH-018 N-(5-hydroxypentyl) metabolite in PBS. Our plate configuration allowed evaluation of two cutoff concentrations (5 and 10μg/L) in two matrices, PBS and urine. Negative and positive (10 and 20μg/L) matrix matched urine controls were always run at the beginning and end of each plate; with urine performance challenges at ±25 and ±50% of each cutoff included in the middle to evaluate the assay's ability to correctly classify concentrations near decision points (Figure 1). Single absorbances of each cutoff at the end of the plate were used to assess drift. Specimens were presumptive positive when absorbance at 450nm was less than or equal to that of the averaged cutoff calibrators in the first column of the plate.

2.6 Analytical Validation and Acceptance Criteria

The method was validated by determining limit of detection (LOD), linearity, intra- and inter-plate imprecision, inter-read imprecision, plate drift, cross-reactivity, interference, carryover and matrix effect.

The LOD was determined from absorbances of 9 negative urine samples from 9 drug-free volunteers. Mean absorbances and standard deviations were evaluated and LOD calculated by subtracting 3 times absorbance SD from the mean absorbance of the 9 negative urine samples (A_0).

Linearity was investigated using a non-linear regression model and expressed as the coefficient of determination (r^2). We initially evaluated linearity with triplicate analysis of the following JWH-018 N-(5-hydroxypentyl) concentrations: 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 25, 50, 100, 250 and 500μg/L in pooled negative urine on three different days. Absorbances were plotted against concentration using an exponential (two phase decay) function. To characterize linearity, the natural logarithms of concentration and absorbance also were plotted.

In-house controls, prepared in pooled negative urine, were used to evaluate intra-plate imprecision at 1, 2.5, 5, 7.5 and 10μg/L JWH-018 N-(5-hydroxypentyl). Seven replicates of each control level were assayed on a single plate. For inter-plate imprecision, in-house performance challenges at ±25 (3.75 and 6.25μg/L) and ±50% (2.5 and 7.5μg/L) of the cutoff level (5μg/L) were assayed in singlicate on 35 plates over 2 weeks. Similarly, inter-plate absorbances also were monitored at ±25 and ±50% (5, 7.5, 12.5 and 15μg/L) of the 10μg/L cutoff. To monitor inter-read imprecision, one plate consisting of a full calibration curve (1–500μg/L) was read 7 times over 30 min to determine any variation in absorbance after addition of stop solution. Mean absorbances and standard deviations were calculated and imprecision expressed as percent coefficient of variation (%CV).

Variations in absorbance as a function of physical location on the 96-well plate (drift) were monitored across all plates (n=35). Duplicate absorbances for the 5 and 10μg/L cutoffs in PBS (column 1) and duplicate 10μg/L urine cutoff calibrators in column 1 were averaged and compared to singlicate absorbance determinations from column 12. Average absorbances for the 5μg/L urine cutoff (column 6) were compared to single absorbance determinations in column 12 using the formula; % Drift = [(Absorbance column 12 − Average cutoff absorbance)/ Average cutoff absorbance]*100.

Negative pooled urine samples were individually fortified with each available synthetic cannabinoid at 500μg/L and analyzed (Tables 1a & 1b). Single absorbances from 73 individual synthetic cannabinoids were compared with blank, negative and averaged 5μg/L urine calibrator (n=2) absorbances. Initial estimates of cross-reactivity were based upon these preliminary absorbances. Absorbances ≥ the negative sample were classified as null, while absorbances between the blank and the 5μg/L urine calibrator were said to have <1% cross reactivity. No further testing was performed on these compounds. However, if absorbances were <5μg/L calibrator, samples were diluted with negative urine and re-analyzed in duplicate for comparison against a calibration curve (1 – 250μg/L). Cross-reactivity (%) was calculated as 100*(apparent concentration from the calibration curve) / (analyte concentration).

Immunoassay interferences can alter antibody binding and affect concentrations by increasing or decreasing signal response. Methanolic stock solutions of 93 common drugs of abuse, metabolites, co-administered drugs, over-the-counter medications and structurally similar compounds (Table 2) were added to conical centrifuge tubes and evaporated to dryness at 37°C under nitrogen. Analytes were reconstituted with 5mL blank urine for a final concentration of 1000μg/L. Additional challenges including; ethanol (5mg/mL), NaCl (40g/L), ascorbic acid (4g/L) and urine specimens at different pH values (<4 and >8) also

were evaluated. Interference samples (1000μg/L in urine) were aliquotted into duplicate tubes and one set fortified with JWH-018 N-(5-hydroxypentyl) metabolite (5μg/L) to evaluate signal suppression. Drug-free urine was also fortified at 5μg/L, and analyzed in triplicate in columns 1 and 6. Mean absorbances (n=6) and associated standard deviations were calculated to determine normal absorbance range for samples containing 5μg/L of JWH-018 N-(5-hydroxypentyl). Signal suppression was considered present when absorbances of interference challenges were outside of this normal range.

To investigate carryover, pooled negative urine samples (n=4) were fortified with JWH-018 N-(5-hydroxypentyl) metabolite at 750μg/L. Samples from the same pool of blank urine were analyzed before and after each carryover sample. Carryover was evaluated by determining if the blanks before and after were statistically different.

Absorbances of cutoff calibrators prepared in urine and PBS were assessed to evaluate matrix effects. Blank urine samples (pH 5.0 – 6.0) and PBS (pH 7.4) were fortified with JWH-018 N-(5-hydroxypentyl) metabolite at 5 and 10μg/L and analyzed in duplicate at the beginning of each plate. (n=35 plates)

2.7 LC-MS/MS Analysis

The qualitative LC-MS/MS synthetic cannabinoid method was fully validated and previously published[16]. Briefly, authentic urine (100μL) was fortified with internal standards and ammonium acetate buffer to adjust pH, prior to hydrolysis with beta-glucuronidase. Samples were extracted/precipitated with acetonitrile, vortexed and centrifuged at 15000g and 4°C to produce a supernatant suitable for LC-MS/MS analysis. The method was fully validated with good analytical recovery (53–95%), low matrix effect (95–122%) and LOD's between 0.5 and 10μg/L for all analytes.

2.8 Diagnostic Efficiency

Urine specimens (n=2492) were analyzed by immunoassay and the reference LC-MS/MS method to evaluate ELISA performance. True-positive (TP), true-negative (TN), false-positive (FP), and false-negative (FN) results were determined by comparing ELISA and LC-MS/MS results. A sample was considered positive if the analytical result was greater than or equal to the specified LC-MS/MS cutoff. A sample was considered TP if the immunoassay and LC-MS/MS were positive, and if both results were negative, the sample was TN. A positive immunoassay result and a negative LC-MS/MS result for all synthetic cannabinoids was considered a FP. A negative immunoassay result and an LC-MS/MS positive result for at least one of the synthetic cannabinoid analytes was considered a FN. Sensitivity of the immunoassay at a specific cutoff was calculated as TP/(TP + FN) × 100 and specificity as TN/(TN + FP) × 100. Efficiency was calculated as (TP +TN)/total number × 100.

3. Results

We present assay performance criteria for the NMS JWH-018 direct ELISA kit as a screening method for the detection of synthetic cannabinoids in urine. Additional

performance characteristics (sensitivity, specificity and efficiency) were determined by comparing ELISA results to the LC-MS/MS reference method.

Detection limits were determined after three separate experiments with drug-free urine. Daily LODs of 0.7, 0.5 and 0.8μg/L were empirically obtained (n=9); an average LOD of 0.7μg/L was reported. The calibration curve (0.25 – 500μg/L) was constructed by plotting absorbances of each calibrator (n=3) against concentration. The non-linear regression model using an exponential (two phase decay) function is shown in Figure 2a. The natural logarithms of mean absorbance vs. concentration exhibited linearity from 1–500μg/L, with a coefficient of determination of 0.993 (Figure 2b).

Of the 73 synthetic cannabinoids analyzed, 29 showed zero or low (<1%) cross-reactivity at 500μg/L (Table 1a), while 26% (19 compounds) presented moderate to high cross-reactivity at 10μg/L (Table 1b). The NMS JWH-018 direct ELISA kit showed significant cross-reactivity with metabolites of other synthetic cannabinoids including JWH-200, JWH-073 N-(3-hydroxybutyl) metabolite, JWH-073 N-(4-hydroxybutyl) metabolite, JWH-019 N-(6-hydroxyhexyl) metabolite and AM-2201 N-(4-hydroxypentyl) metabolite.

Interference was evaluated by analyzing 93 common drugs of abuse, metabolites, co-administered drugs, over-the-counter medications and structurally similar compounds (Table 2). No samples fortified at 1000μg/L exhibited a positive result. Likewise, interference samples fortified with JWH-018 N-(5-hydroxypentyl) metabolite (5μg/L) exhibited absorbances similar to mean (n=6) absorbances for the 5μg/L cutoff. There was no significant difference (p=0.27) between the absorbances of blank urine specimens (n=4) analyzed before and after 750μg/L carryover samples (n=4).

Intra-plate imprecision (n=7) was ≤8.2% CV from 1–10μg/L (Table 3). Inter-plate imprecision was evaluated using single absorbances (n=35) from performance challenges at ±25 and ±50% of each cutoff (5 and 10μg/L). Inter-plate imprecision was between 9.0 – 14.0% CV (Table 3). Inter-read imprecision was evaluated with absorbance readings from a single plate containing one replicate of each level of the calibration curve (1, 2.5, 5, 7.5, 10, 25, 50, 100, 250 and 500μg/L). Absorbance readings were taken immediately after adding the stop solution and at 5 min intervals. For all calibrators, imprecision was ≤4% and absolute % difference from initial absorbance was between 4.9 to 10.5%.

For replicates (n=2) positioned in the same column (in consecutive wells) the median % agreement (range) for 5 and 10μg/L PBS cutoff calibrator absorbances was 8 (−18 to 35) and 10 (−11to 39) respectively. Several absorbances (n=6) had differences >23%. Similarly, median % differences (range) for fortified urine calibrators (5 and 10μg/L) were 5 (−25 to 23) and 8 (−7 to 31), respectively. Again, the highest value in these ranges occurred only once. This highlights the necessity of using duplicate cutoff samples and monitoring expected absorbances and separation rates of calibrators and controls. Absorbances in the last column were almost always higher than initial averaged values, but despite these differences, positive urine controls (10 and 20μg/L) at the end of the plate were always positive for both cutoffs (5 and 10μg/L) in urine and PBS.

A significant difference was observed between JWH-018 N-(5-hydroxypentyl) metabolite solutions prepared in urine and PBS at 5 and 10μg/L. At the same concentration, solutions prepared in PBS had higher absorbances, and greater Δ absorbance differences than those prepared in urine (Table 4). Despite similar mean absorbances for the 5μg/L urine (0.583 ± 0.066) and 10μg/L PBS cutoffs (0.486 ± 0.090), the differences were significant (p=1.63e-06, n=35).

The frequency of each averaged cutoff to classify these urine performance challenge concentrations as positive or negative based on a single absorbance from each plate (n=35) are presented in Table 5. Initial testing found significant differences (p<0.05) between all challenge concentrations (n=6) and the 5μg/L urine cutoff. However, further evaluation of performance challenges (n=35) around the 5μg/L cutoff showed significant differences between the negative challenges (2.5 and 3.75μg/L), but not for positive challenges (6.25 and 7.5μg/L) above the cutoff. Based on similar absorbances and the response of the calibration curve, these results are more typical and reflect daily differences in assay sensitivity. Interestingly, the 5μg/L urine cutoff showed better performance below the cutoff, while the 5μg/L PBS cutoff was better for concentrations above. The best agreement between performance challenge results obtained and expected was for the 10μg/L urine cutoff.

4. Diagnostic Assay Performance

The designation of individual specimens as TP, TN, FP, and FN and the performance characteristics of the NMS JWH-018 direct ELISA kit immunoassay versus LC–MS/MS cutoffs are summarized in Table 6. As expected, the lower 5μg/L cutoffs (PBS and urine) exhibited higher overall sensitivity (90.7% and 83.7%), yet produced the greatest numbers (29 and 14) of FP results. Specificity and efficiency for the 5μg/L cutoffs in PBS and urine were similar, with both greater than 97.6%. Raising the ELISA cutoff from 5 to 10μg/L resulted in an 12.5% and 12.1% loss of assay sensitivity for PBS and urine, respectively. FP results could be due to cross-reactivities with low concentrations of multiple synthetic cannabinoid metabolites, or of analytes not included in the confirmatory method, while FN results may be explained by differences in ELISA cutoff concentrations and LC-MS/MS LOD’s (0.5 – 10μg/L).

5. Discussion

A major source of drift across the plate is the different timing for reactions between antibody and antigens (drug-free and conjugate). In an effort to minimize this effect, we optimized the liquid handling parameters to include a pause step during the addition of the conjugate. This ensured that each sample was in contact with the antibodies for a similar time. However, plate drift was still present but reduced and generally more pronounced for lower drug concentrations. Inter-plate variability also was observed, suggesting the need to calibrate each plate when monitoring forensic urine tests. When comparing the 5μg/L cutoffs prepared in urine and PBS, we observed higher absorbances in buffer than in urine. It appears that the conjugate binds better with the antibodies in the presence of buffer, or in the absence of matrix elements.

Cross-reactivity is advantageous in drug screening assays as it enhances the detection of compounds with similar chemical structures making the screening process more practical and less expensive, an important characteristic for synthetic cannabinoids analysis. We observed some common structural characteristics when reviewing the cross-reactivity of 73 synthetic cannabinoids in the NMS JWH-018 direct ELISA kit. It appears that hydroxyl substituents on the alkyl side chain increased cross-reactivity. For compounds tested, the general order of reactivity was: pentanoic acid > 5-hydroxypentyl > 4-hydroxypentyl > 5-hydroxypentyl glucuronide > parent compound. However, reactivity decreased significantly when the hydroxyl substituent was on the indole moiety. These data may be useful for predicting cross-reactivity of new synthetic cannabinoids. However, it is important to emphasize that these conclusions are restricted to this assay and this targeted metabolite. However, more detailed experiments with a complete database of synthetic cannabinoids would be necessary to definitively document these effects, and allow the prediction of cross-reactivity results based on chemical structures. This highlights a limitation of all synthetic cannabinoid immunoassays, as newer compounds (PB-22, RCS-4, RCS-8, XRL-11 and AKB48) may not react with current ELISA kit antibodies. These FN results are a reality, especially when laboratories are continually faced with newly emerging abused synthetic cannabinoids.

If we consider that FN results are of greater concern in a screening assay, then the 5μg/L PBS cutoff had the best performance (Table 6). The higher sensitivity of the 5μg/L PBS may be explained by the absence of matrix effect, as cutoff solutions were prepared in buffer. While there are advantages of preparing calibrators in buffer or synthetic urine (stability, separation) these solutions only approximate matrix effects. The urine performance challenge classifications of samples at ±25% of the cutoff showed correct identification more frequently for matrix matched cutoffs than buffered prepared cutoffs; with the 10μg/L urine cutoff performing best. However, increasing the cutoff reduced sensitivity and yielded the most FN samples. Based on our results, we recommend the use of a 5μg/L cutoff in urine when using the NMS JWH-018 direct ELISA kit as a screening method for the detection of synthetic cannabinoids in urine.

ACKNOWLEGMENTS

This work was supported by the Intramural Research Program of the National Institute on Drug Abuse, National Institutes of Health and the Department of Defense through an interagency agreement. We thank National Medical Services for generously providing the immunoassay analysis kits.

References

1. Dresen S, Kneisel S, Weinmann W, et al. Development and validation of a liquid chromatography-tandem mass spectrometry method for the quantitation of synthetic cannabinoids of the aminoalkylindole type and methanandamide in serum and its application to forensic samples. J Mass Spectrom. 2011; 46:163–171. [PubMed: 21259394]
2. Pertwee RG. Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. Curr Med Chem. 2010; 17:1360–1381. [PubMed: 20166927]
3. Porter AC, Felder CC. The endocannabinoid nervous system: unique opportunities for therapeutic intervention. Pharmacol Ther. 2001; 90:45–60. [PubMed: 11448725]

4. Brents LK, Reichard EE, Zimmerman SM, et al. Phase I hydroxylated metabolites of the K2 synthetic cannabinoid JWH-018 retain in vitro and in vivo cannabinoid 1 receptor affinity and activity. PLoS One. 2011; 6:e21917. [PubMed: 21755008]
5. Devane WA, Breuer A, Sheskin T, et al. A novel probe for the cannabinoid receptor. J Med Chem. 1992; 35:2065–2069. [PubMed: 1317925]
6. Substance Abuse and Mental Health Services Administration. HHS Publication No. (SMA) 13-4760, DAWN Series D-39. Rockville, MD: Substance Abuse and Mental Health Services Administration; 2013. Drug Abuse Warning Network, 2011: National Estimates of Drug-Related Emergency Department Visits.
7. Harris CR, Brown A. Synthetic cannabinoid intoxication: a case series and review. J Emerg Med. 2013; 44:360–366. [PubMed: 22989695]
8. Castellanos D, Singh S, Thornton G, et al. Synthetic cannabinoid use: a case series of adolescents. J Adolesc Health. 2011; 49:347–349. [PubMed: 21939863]
9. Every-Palmer S. Synthetic cannabinoid JWH-018 and psychosis: an explorative study. Drug Alcohol Depend. 2011; 117:152–157. [PubMed: 21316162]
10. Simmons J, Cookman L, Kang C, et al. Three cases of "spice" exposure. Clin Toxicol (Phila). 2011; 49:431–433. [PubMed: 21740143]
11. Simmons JR, Skinner CG, Williams J, et al. Intoxication from smoking "spice". Ann Emerg Med. 2011; 57:187–188. [PubMed: 21251535]
12. U.S. Government. Synthetic Drug Abuse Prevention Act. 2012. 1152b,S.31871-139.
13. European Monitoring Centre for Drugs, Drug Addiction (EMCDDA). Understanding the 'Spice' phenomenon. Lisbon: EMCDDA; 2009. Available at: http://www.emcdda.europa.eu/html.cfm/index90917EN.html.
14. Johnston, LD.; O'Malley, PM.; Bachman, JG.; Schulenberg, JE. Monitoring the Future National Results on Drug Use: 2012 Overview Key Findings on Adolescent Drug Use. Ann Arbor: Institute for Social Research, The University of Michigan; 2012.
15. Hu X, Primack BA, Barnett TE, et al. College students and use of K2: an emerging drug of abuse in young persons. Subst Abuse Treat Prev Policy. 2011; 6:16. [PubMed: 21745369]
16. Wohlfarth A, Scheidweiler KB, Chen X, et al. Qualitative confirmation of 9 synthetic cannabinoids and 20 metabolites in human urine using LC-MS/MS and library search. Anal Chem. 2013; 85:3730–3738. [PubMed: 23458260]
17. Grigoryev A, Melnik A, Savchuk S, et al. Gas and liquid chromatography-mass spectrometry studies on the metabolism of the synthetic phenylacetylindole cannabimimetic JWH-250, the psychoactive component of smoking mixtures. J Chromatogr B Analyt Technol Biomed Life Sci. 2011; 879:2519–2526.
18. Grigoryev A, Savchuk S, Melnik A, et al. Chromatography-mass spectrometry studies on the metabolism of synthetic cannabinoids JWH-018 and JWH-073, psychoactive components of smoking mixtures. J Chromatogr B Analyt Technol Biomed Life Sci. 2011; 879:1126–1136.
19. Sobolevsky T, Prasolov I, Rodchenkov G. Detection of JWH-018 metabolites in smoking mixture post-administration urine. Forensic Sci Int. 2010; 200:141–147. [PubMed: 20430547]
20. Hutter M, Broecker S, Kneisel S, et al. Identification of the major urinary metabolites in man of seven synthetic cannabinoids of the aminoalkylindole type present as adulterants in 'herbal mixtures' using LC-MS/MS techniques. J Mass Spectrom. 2012; 47:54–65. [PubMed: 22282090]
21. Scheidweiler KB, Jarvis MJ, Huestis MA. Nontargeted SWATH acquisition for identifying 47 synthetic cannabinoid metabolites in human urine by liquid chromatography-high-resolution tandem mass spectrometry. Anal Bioanal Chem. 2014
22. Moran CL, Le VH, Chimalakonda KC, et al. Quantitative measurement of JWH-018 and JWH-073 metabolites excreted in human urine. Anal Chem. 2011; 83:4228–4236. [PubMed: 21506519]
23. de Jager AD, Warner JV, Henman M, et al. LC-MS/MS method for the quantitation of metabolites of eight commonly-used synthetic cannabinoids in human urine--an Australian perspective. J Chromatogr B Analyt Technol Biomed Life Sci. 2012; 897:22–31.
24. Yanes EG, Lovett DP. High-throughput bioanalytical method for analysis of synthetic cannabinoid metabolites in urine using salting-out sample preparation and LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2012; 909:42–50.

25. Chimalakonda KC, Moran CL, Kennedy PD, et al. Solid-phase extraction and quantitative measurement of omega and omega-1 metabolites of JWH-018 and JWH-073 in human urine. Anal Chem. 2011; 83:6381–6388. [PubMed: 21740038]

26. Scheidweiler KB, Huestis MA. Simultaneous quantification of 20 synthetic cannabinoids and 21 metabolites, and semi-quantification of 12 alkyl hydroxy metabolites in human urine by liquid chromatography-tandem mass spectrometry. J Chromatogr A. 2014; 1327:105–117. [PubMed: 24418231]

27. Griffiths P, Sedefov R, Gallegos A, et al. How globalization and market innovation challenge how we think about and respond to drug use: 'Spice' a case study. Addiction. 2010; 105:951–953. [PubMed: 20659053]

28. Barnes AJ, Young S, Spinelli E, et al. Evaluation of a homogenous enzyme immunoassay for the detection of synthetic cannabinoids in urine. Forensic Sci Int. 2014; 241:27–34. [PubMed: 24845968]

29. Spinelli E, Barnes AJ, Young S, et al. Performance characteristics of an ELISA screening assay for urinary synthetic cannabinoids. Drug Test Anal. 2014

30. Arntson A, Ofsa B, Lancaster D, et al. Validation of a novel immunoassay for the detection of synthetic cannabinoids and metabolites in urine specimens. J Anal Toxicol. 2013; 37:284–290. [PubMed: 23625703]

PBS	20-Ur	U	U	U	3.75	U	U	U	U	U	U
5-PBS	20-Ur	U	U	U	6.25	U	U	U	U	U	U
5-PBS	U	U	U	U	2.5	U	U	U	U	U	U
NEG	U	U	U	U	7.5	U	U	U	U	U	5-PBS
10-Ur	U	U	U	U	12.5	U	U	U	U	U	5-Ur
10-Ur	U	U	U	U	15	U	U	U	U	U	10-PBS
10-PBS	U	U	U	U	5-Ur	U	U	U	U	U	10-Ur
10-PBS	U	U	U	U	5-Ur	U	U	U	U	U	20-Ur

PBS - Phosphate Buffered Saline 0.1M (pH 7.4)

NEG - Blank Urine (Drug-free pooled urine)

5-Ur – Calibrator (Drug-free urine fortified at 5μg/L)

5-PBS – Calibrator (PBS fortified at 5μg/L)

10-Ur – Calibrator (Drug-free urine fortified at 10μg/L)

10-PBS – Calibrator (PBS fortified at 10μg/L)

U - Urine samples

20-Ur – Positive QC (Drug-free urine fortified at 20μg/L)

2.5 – Performance challenge control (-50% of 5μg/L cutoff)

3.75 – Performance challenge control (-25% of 5μg/L cutoff)

6.25 – Performance challenge control (+25% of 5μg/L cutoff)

7.5 – Performance challenge control (+50% of 10μg/L &-25% of 10μg/L cutoff)

12.5 – Performance challenge control (+25% of 10μg/L cutoff)

15 – Performance challenge control (+50% of 10μg/L cutoff)

Figure 1. Representative 96-well plate layout utilized in the validation of NMS direct ELISA kit for screening synthetic cannabinoids in urine targeting the JWH-018 N-(5-hydroxypentyl) metabolite

PBS - Phosphate Buffered Saline 0.1M (pH 7.4)
NEG - Blank Urine (Drug-free pooled urine)
5-Ur – Calibrator (Drug-free urine fortified at 5μg/L)
5-PBS – Calibrator (PBS fortified at 5μg/L)
10-Ur – Calibrator (Drug-free urine fortified at 10μg/L)
10-PBS – Calibrator (PBS fortified at 10μg/L)

U - Urine samples

20-Ur – Positive QC (Drug-free urine fortified at 20μg/L)

2.5 – Performance challenge control (−50% of 5μg/L cutoff)

3.75 – Performance challenge control (−25% of 5μg/L cutoff)

6.25 – Performance challenge control (+25% of 5μg/L cutoff)

7.5 – Performance challenge control (+50% of 10μg/L &−25% of 10μg/L cutoff)

12.5 – Performance challenge control (+25% of 10μg/L cutoff)

15 – Performance challenge control (+50% of 10μg/L cutoff

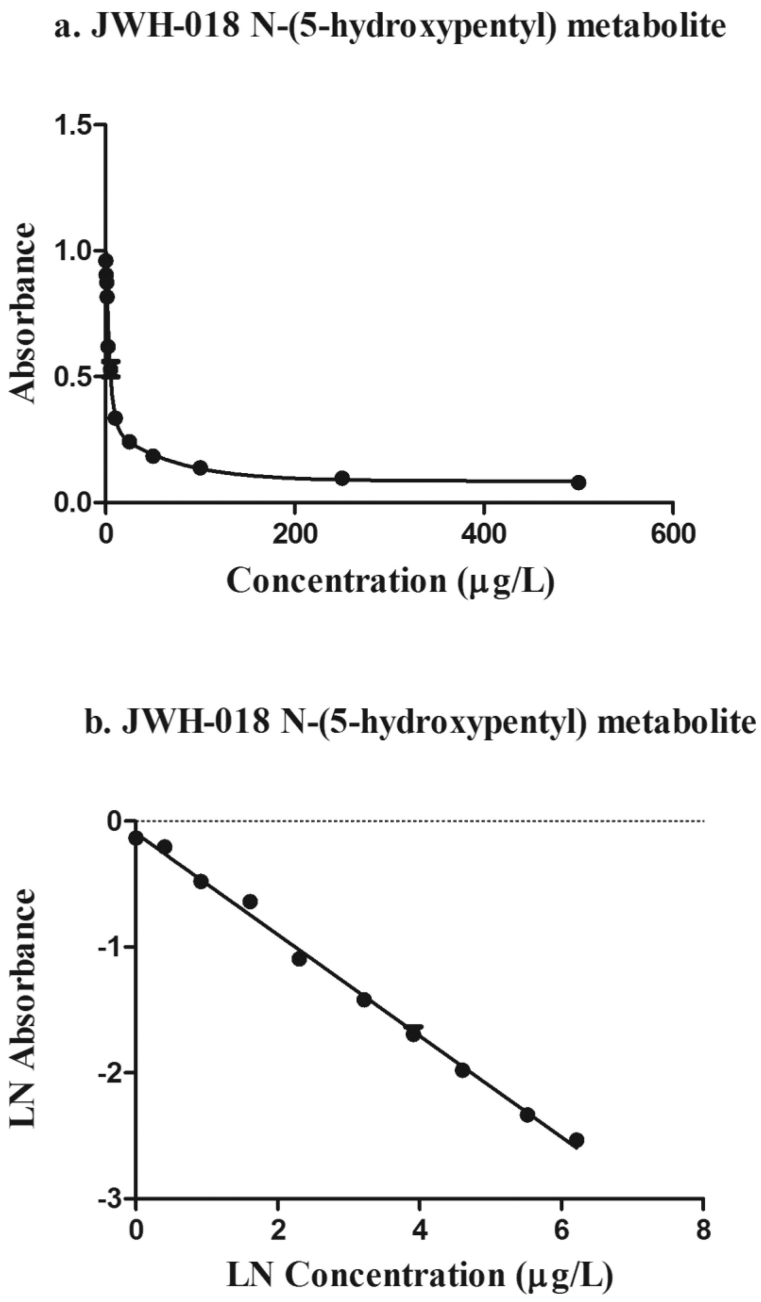

Figure 2. Linearity evaluation

a) Non-linear calibration curve of JWH-018 N-(5-hydroxypentyl) metabolite in urine (0.25–500µg/L)

b) Linear regression line of JWH-018 N-(5-hydroxypentyl) metabolite in urine (1–500µg/L) r^2=0.993

Table 1

a. Synthetic cannabinoids (n=42) with <0.4% cross-reactivity to the NMS JWH-018 N-(5-hydroxypentyl) metabolite ELISA kit

Synthetic Cannabinoid	Concentration (μg/L)	Cross-reactivity %
JWH-203	500	Negative
JWH-203 2-hydroxyindole metabolite	500	Negative
CP 47,497	500	Negative
CP 47, 497 C7-hydroxy metabolite	500	Negative
CP 47, 497 C8 homolog	500	Negative
CP 47, 497 C8 homolog-C8 hydroxy metabolite	500	Negative
JWH-250 5-hydroxyindole metabolite	500	Negative
URB754	500	Negative
JWH-018 2-hydroxyindole metabolite	500	Negative
AKB848	500	Negative
JWH-007	500	Negative
STS-135	500	Negative
JWH-251	500	Negative
JWH-018 adamantyl analog	500	Negative
JWH-018 adamantyl carboxamide	500	Negative
XRL-11	500	Negative
HU-210	500	<1
UR-144	500	<1
UR-144 N-pentanoic acid metabolite	500	<1
UR-144 N-(5-hydroxypentyl) metabolite	500	<1
JWH-210 5-hydroxyindole metabolite	500	<1
JWH-250	500	<1
JWH-250 N-pentanoic acid metabolite	500	<1
JWH-250 N-(5-hydroxypentyl) metabolite	500	<1
JWH-250 N-(4-hydroxypentyl) metabolite	500	<1
JWH-210	500	<1
RCS-4	500	<1
RCS-4 N-pentanoic acid metabolite	500	<1
RCS-8	500	<1
JWH-019 5-hydroxyindole metabolite	100	0.1
JWH-018 7-hydroxyindole metabolite	100	0.1
JWH-073 4-hydroxyindole metabolite	100	0.1
JWH-019	100	0.1
JWH-398	100	0.1
JWH-022	100	0.1
AM2201 N-(4-fluoropentyl) isomer	100	0.2
JWH-081	100	0.2

a. Synthetic cannabinoids (n=42) with <0.4% cross-reactivity to the NMS JWH-018 N-(5-hydroxypentyl) metabolite ELISA kit

Synthetic Cannabinoid	Concentration (μg/L)	Cross-reactivity %
JWH-122	100	0.2
RCS-4 N-(5-hydroxypentyl) metabolite	100	0.3
RCS-4 2 methoxy isomer	100	0.3
JWH-073	100	0.3
JWH-015	100	0.4

b. Synthetic cannabinoids (n=31) with >0.5% cross-reactivity to the NMS JWH-018 N-(5-hydroxypentyl) metabolite ELISA kit at 10–100μg/L.

Synthetic Cannabinoid	Concentration (μg/L)	Cross-reactivity %
AM694	100	0.5
JWH-073 7-hydroxyindole metabolite	100	0.5
WIN 55,212-2	100	1
JWH-210 N-(4-hydroxypentyl) metabolite	100	1.2
JWH-073 5-hydroxyindole metabolite	100	1.3
JWH-210 N-(5-hydroxypentyl) acid metabolite	100	2
JWH-073 6-hydroxyindole metabolite	100	2.5
AM2201 6-hydroxyindole metabolite	100	3.4
JWH-081 N-(5-hydroxypentyl) metabolite	100	5.8
JWH-210 N-pentanoic acid metabolite	100	7.1
JWH-200 5-hydroxyindole metabolite	100	11
JWH-200 6-hydroxyindole metabolite	100	12.3
JWH-018 5-hydroxyindole metabolite	10	2
JWH-018	10	2
JWH-018 6-hydroxyindole metabolite	10	2
MAM2201	10	2
AM2201	10	6
JWH-398 N-(5-hydroxypentyl) metabolite	10	11
JWH-122 N-(5-hydroxypentyl) metabolite	10	11
MAM2201 N-pentanoic acid metabolite	10	13
JWH-398 N-pentanoic acid metabolite	10	14
AM1220	10	21
JWH-073 N-butanoic acid	10	53
JWH-018 N-(5-hydroxypentyl)-β-D-glucuronide	10	56
AM2201 N-(4-hydroxypentyl) metabolite	10	60
JWH-018 N-(4-hydroxypentyl) metabolite	10	79
JWH-073 N-(4-hydroxybutyl) metabolite	10	130
JWH-073 N-(3-hydroxybutyl) metabolite	10	133
JWH-019 N-(6-hydroxyhexyl) metabolite	10	136

b. Synthetic cannabinoids (n=31) with >0.5% cross-reactivity to the NMS JWH-018 N-(5-hydroxypentyl) metabolite ELISA kit at 10–100μg/L.

Synthetic Cannabinoid	Concentration (μg/L)	Cross-reactivity %
JWH-018 N-pentanoic acid	10	249
JWH-200	10	271

Table 2

Exogenous compounds (n=93) fortified in blank urine at 1000μg/L to investigate interferences and fortified into a low JWH-018 N-(5-hydroxypentyl) cutoff (5μg/L) to investigate potential signal suppression.

2C-B	**diazepam**	**nicotine**
11-OH-THC	diphenhydramine	nitrazepam
6-acetylcodeine	ecgonine	norbenzoylecgonine
6-acetylmorphine	ecgonine ethyl ester	norbuprenorphine
7-aminoclonazepam	ecgonine methyl ester	norcocaethylene
7-aminoflunitrazepam	EDDP	norcocaine
7-aminonitrazepam	EMDP	norcodeine
acetaminophen	ephedrine	norcotinine
acetylsalicylic acid	ethylamphetamine	nordiazepam
alprazolam	flunitrazepam	norfluoxetine
amphetamine	fluoxetine	normorphine
anhydroecgonine methyl ester	flurazepam	noroxycodone
BDB	HMA	noroxymorphone
benzoylecgonine	HMMA	oxazepam
bromazepam	hydrocodone	oxycodone
brompheniramine	hydromorphine	oxymorphone
buprenorphine	ibuprofen	paroxetine
caffeine	imipramine	pentazocine
cannabigerol	ketamine	phentermine
cannabidiol	lorazepam	p-hydroxyamphetamine
cannabinol	MBDB	p-hydroxybenzoylecgonine
cathinone	MDA	p-hydroxycocaine
chlorpheniramine	MDEA	p-hydroxymethamphetamine
clomipramine	MDMA	p-methoxyamphetamine
clonazepam	methadone	p-methoxymethamphetamine
clonidine	methaphetamine	propoxyphene
cocaethylene	m-hydroxybenzoylecgonine	pseudoephedrine
cocaine	m-hydroxycocaine	temazepam
codeine	morphine	THC
cotinine	morphine-3-glucuronide	THCCOOH
dextromethorphan	morphine-6-glucuronide	trans-3’-hydroxycotinine

2C-B: 4-bromo-2,5-dimethoxyphenethylamine
11-OH-THC: 11-hydroxy-Δ9-tetrahydrocannabinol
BDB: 3,4-(methylenedioxyphenyl)-2-butanamine
EDDP: 2-ethylidene-1,5-dimethyl-3-3-diphenylpyrrolidine
EMDP: 2-ethylidene-5-methyl-3-3-diphenylpyraline
HMA: 4-hydroxy-3-methoxyamphetamine
HMMA: 4-hydroxy-3-methoxymethamphetamine
MBDB: n-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine
MDA: 3,4-methylenedioxyamphetamine
MDEA: 3,4- methylenedioxyethylamphetamine
MDMA: 3,4-methylenedioxymethamphetamine

THC: Δ9-tetrahydrocannabinol
THCCOOH: 11-nor-9-carboxy-Δ9-tetrahydrocannabinol

Table 3

Intra-plate imprecision (n=7) for JWH-018 N-(5-hydroxypentyl) concentrations (1–10 μg/L). Inter-plate imprecision (n=35) for JWH-018 metabolite using positive controls (10 & 20μg/L) and performance challenge concentrations at ±25% and ±50% of each cutoff. Inter-read imprecision for a representative calibration curve (1–500μg/L) for JWH-018 N-(5-hydroxypentyl) in urine.

Urine JWH-018 metabolite μg/L	Intra-plate (n=7) Absorbance %		Inter-plate (n=35) Absorbance %		Inter-read (n=7) Absorbance %	
	Mean (SD)	CV	Mean (SD)	CV	Mean (SD)	CV
1	0.827 (0.051)	6.2	-	-	0.902 (0.024)	2.7
2.5	0.613 (0.027)	4.2	0.787 (0.092)	11.6	0.727 (0.020)	2.8
3.75	-	-	0.630 (0.079)	12.5	-	-
5	0.511 (0.036)	7.0	0.594 (0.067)	11.2	0.474 (0.015)	3.2
6.25	-	-	0.557 (0.067)	12.0	-	-
7.5	0.469 (0.038)	8.2	0.555 (0.078)	14.0	0.407 (0.014)	3.5
10	0.327 (0.020)	5.5	0.388 (0.035)	9.0	0.374 (0.011)	3.1
12.5	-	-	0.351 (0.035)	10.0	-	-
15	-	-	0.321 (0.031)	9.8	-	-
20	-	-	0.284 (0.037)	13.0	-	-
25	-	-	-	-	0.222 (0.007)	3.3
50	-	-	-	-	0.157 (0.006)	3.7
100	-	-	-	-	0.118 (0.005)	4.0
250	-	-	-	-	0.087 (0.003)	3.7
500	-	-	-	-	0.070 (0.001)	2.1

Table 4

Matrix effect of NMS JWH-018 N-(5-hydroxypentyl) metabolite direct ELISA cutoffs prepared in urine and phosphate buffered saline (PBS), evaluated in duplicate across 35 plates.

Matrix	Cutoff µg/L	Absorbance (Mean ± SD)	C.V. (%)
PBS	5	0.752 ± 0.083	11.0
	10	0.486 ± 0.090	18.5
Urine	5	0.583 ± 0.066	11.2
	10	0.402 ± 0.045	11.3

Table 5

Performance challenge classifications (n=35) at ±25% and ±50% of each evaluated cutoff (5 & 10 μg/L) in each evaluated matrix (Urine & Phosphate Buffered Saline) across 35 plates.

Cutoffs (μg/L)	JWH-018 N-(5-hydroxypentyl) metabolite Performance Challenge concentration (μg/L) in urine							Correct classification (%)			
								Negative		Positive	
	2.5	3.75	5.0	6.25	7.5	12.5	15	−50%	−25%	+25%	+50%
5-PBS	25/35 (−)	5/35 (−)	-	34/35 (+)	32/35 (+)	-	-	71	14	97	91
5-UR	35/35 (−)	30/35 (−)	-	24/35 (+)	23/35 (+)	-	-	100	86	69	66
10-PBS	-	-	29/35 (−)	-	27/35 (−)	33/35 (+)	35/35 (+)	83	77	94	100
10-UR	-	-	35/35 (−)	-	35/35 (−)	34/35 (+)	34/35 (+)	100	100	97	97

(−) = Negative result
(+) = Positive result
PBS = Phosphate Buffered Saline
UR= Urine

Table 6

Diagnostic performance of NMS JWH-018 N-(5-hydroxypentyl) metabolite direct ELISA for 2492 urine samples. All samples confirmed by LC-MS/MS.

Cutoff	5 μg/L in PBS	5 μg/L in Urine	10 μg/L in PBS	10 μg/L in Urine
True Positive	262	242	226	207
True Negative	2174	2189	2195	2196
False Positive	29	14	8	7
False Negative	27	47	63	82
Sensitivity %	90.7	83.7	78.2	71.6
Specificity %	98.7	99.4	99.6	99.7
Efficiency %	97.8	97.6	97.2	96.4