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Quantitative urine confirmatory testing for synthetic cannabinoids in randomly collected urine specimens

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Synthetic cannabinoid intake is an ongoing health issue worldwide, with new compounds continually emerging, making drug testing complex. Parent synthetic cannabinoids are rarely detected in urine, the most common matrix employed in workplace drug testing. Optimal identification of synthetic cannabinoid markers in authentic urine specimens and correlation of metabolite concentrations and toxicities would improve synthetic cannabinoid result interpretation. We screened 20 017 randomly collected US military urine specimens between July 2011 and June 2012 with a synthetic cannabinoid immunoassay yielding 1432 presumptive positive specimens. We analyzed all presumptive positive and 1069 negative specimens with our qualitative synthetic cannabinoid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, which confirmed 290 positive specimens. All 290 positive and 487 randomly selected negative specimens were quantified with the most comprehensive urine quantitative LC-MS/MS method published to date; 290 specimens confirmed positive for 22 metabolites from 11 parent synthetic cannabinoids. The five most predominant metabolites were JWH-018 pentanoic acid (93%), JWH-*N*-hydroxypentyl (84%), AM2201 *N*-hydroxypentyl (69%), JWH-073 butanoic acid (69%), and JWH-122 *N*-hydroxypentyl (45%) with 11.1 (0.1-2,434), 5.1 (0.1-1,239), 2.0 (0.1-321), 1.1 (0.1-48.6), and 1.1 (0.1-250) μg/L median (range) concentrations, respectively.

Alkyl hydroxy and carboxy metabolites provided suitable biomarkers for 11 parent synthetic cannabinoids; although hydroxyindoles were also observed. This is by far the largest data set of synthetic cannabinoid metabolites urine concentrations from randomly collected workplace drug testing specimens rather than acute intoxications or driving under the influence of drugs. These data improve the interpretation of synthetic cannabinoid urine test results and suggest suitable urine markers of synthetic cannabinoid intake. This article is a U.S. Government work and is in the public domain in the USA.

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Keywords: synthetic cannabinoids; immunoassay; LC-MS/MS; urine drug testing

Introduction

Synthetic cannabinoids bind to cannabinoid receptors and were developed for studying endocannabinoid pharmacology and potential therapeutic administration; although no drugs are currently available for clinical use to date.[1–5] Synthetic cannabinoids were introduced as 'legal' cannabis alternatives and became popular worldwide for their psychoactive effects and lack of detectability by routine cannabinoid testing.[6] Synthetic cannabinoids, sold on the Internet, in gas stations and head shops, are labelled 'not for human consumption'. Acute adverse effects from synthetic cannabinoid intake include agitation, anxiety, psychosis/hallucinations, seizures, loss of consciousness, headache, reddened conjunctivae, nausea, vomiting, dry mouth, shortness of breath, tachycardia, chest pain, hypertension, stroke, and in rare cases, acute kidney injury and death.[7] Calls to poison control centres declined since the peak of 6698 in 2011 to 2643 in 2013[8]; however, it is unknown if this is a reflection of decreased synthetic cannabinoid intake, physicians' experience in dealing with synthetic cannabinoid cases, or other reasons.

New synthetic cannabinoids constantly emerge in response to legislative efforts worldwide. The Synthetic Drug Abuse Prevention Act of 2012, enacted on 9 July 2012, permanently placed JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-203, JWH-250, JWH-398, AM694, AM2201, CP 47,497, CP 47,497 C8-homolog, RCS-4, RCS-8 and their analogues under US Drug Enforcement Administration Schedule I regulation.[9] Synthetic cannabinoids also are controlled in Europe,[10] Japan,[11] Australia,[12] and New Zealand[13] and also are banned by the World Anti-Doping Agency (WADA).[14]

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Identification of synthetic cannabinoids in biological matrices is critical for documenting intake in clinical and forensic laboratories. Synthetic cannabinoid parent analytes were identified via gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) in blood,[15–17] serum,[18,19] oral fluid,[20,21] and hair,[22–24] while mostly metabolites were found in urine.[12,25–35] Although parent synthetic cannabinoids identification in blood, serum, and oral fluid can document acute intoxication and impairment,[17,36,37] the window of drug detection in these matrices generally is short. Longer detection may occur after chronic frequent intake,[38] similar to what has been documented following chronic frequent cannabis intake.[39] Urine drug testing is less invasive and offers a wider window of detection, making urine the preferred matrix for workplace drug testing. However, urine requires monitoring metabolites, which are initially unknown for new synthetic cannabinoids. Identifying these targets is critical as new compounds emerge, and reference standards must be synthesized before emerging compounds can be effectively monitored. Moreover, various synthetic cannabinoids can produce common metabolites[25,28,30] further complicating urine test interpretation.

Synthetic cannabinoids and their metabolites do not cross-react with routine cannabinoid immunoassays, making them desirable for circumventing drug detection efforts. Urine drug testing programmes typically employ immunoassay screening for identifying presumptive positive specimens, and confirmation by GC-MS or LC-MS/MS.[40] Immunoassay screening for synthetic cannabinoids is challenging due to the time required to raise antibodies to metabolites of the constantly new drugs on the market and to develop, validate, and commercialize new assays. Therefore, many laboratories are developing mass spectrometric screening methods for synthetic cannabinoids.[41–45] These assays target multiple metabolites in a single assay and additional analytes are readily added; however, metabolite standard availability limits the expediency with which this can be accomplished. Many available LC-MS/MS assays are qualitative, but quantifying synthetic cannabinoid urinary metabolites improves results interpretation allowing metabolite concentration correlations with toxicity and adverse outcomes, understanding the drug's pharmacokinetics, and comparing relative concentrations of metabolites to suggest optimal markers.

Limited synthetic cannabinoid urinary metabolites' concentrations are available following self-administration,[12,28] emergency room visits,[46] and forensic cases.[12,25,28–30,32] In addition, there is only one report of JWH-018 and JWH-073 metabolites concentrations in US Air Force personnel (n = 49) following random or probable cause drug testing.[31] Most urine concentration data are for only a few analytes following acute intoxications. Knowing synthetic cannabinoid urinary analytes' concentrations in individuals without apparent signs and symptoms of acute intoxication can assist workplace and human performance drug testing programmes in interpreting synthetic cannabinoid test results. To assess personnel readiness and deter illicit drug intake among US service members, the Department of Defense (DOD) established mandatory random and for cause workplace drug testing programmes. Urine specimens, under chain-of-custody, are collected from service members worldwide and shipped at room temperature to any of the six designated DOD forensic toxicology drug testing laboratories for initial immunoassay screening for common drugs of abuse followed by GC-MS confirmation. In conjunction with routine drug testing, the Armed Forces Medical Examiner System conducts prevalence testing by analyzing previously screened negative specimens for emerging drugs of abuse. This enables DOD to assess the severity and impact of illicit drug intake, supporting addition of new drugs into the drug testing panel.

We investigated the suitability of different synthetic cannabinoid urinary markers and their concentrations in US military personnel, with specimens collected in their routine random urine drug testing program. We measured synthetic cannabinoid metabolite concentrations in 777 urine specimens with the most comprehensive published quantitative LC-MS/MS method targeting 53 synthetic cannabinoid analytes consisting of 20 parents and 33 metabolites.

Materials and methods

Reagents and supplies

Standards and deuterated internal standards were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Acetonitrile, ammonium acetate, ethyl acetate, and formic acid were obtained from Sigma-Aldrich (St Louis, MO, USA) and methanol from Fisher Scientific (Fairlawn, NJ, USA). Solvents utilized in the analysis were HPLC grade or better. Abalone beta-glucuronidase containing 1 500 000 units/g beta-glucuronidase and 150 000 units/g sulfatase was obtained from Campbell Science (Rockton, IL, USA). One mL Isolute supported liquid extraction (SLE+) columns (Biotage, Inc., Charlotte, NC, USA) were utilized in the sample preparation. Ultra Biphenyl HPLC column was purchased from Restek, Inc. (Bellefonte, PA, USA).

Authentic urine specimens

Urine specimens in this study are a subset of 20 017 de-identified, randomly collected US service members' specimens from around the world. Specimens were analyzed for routine drugs of abuse by five DOD forensic toxicology drug-testing laboratories between July 2011 and June 2012. No preservatives were added to the urine per US military protocol. These 20 017 specimens screened negative for cocaine, delta-9-tetrahydrocannabinol (THC), amphetamines, opioids, and phencyclidine (PCP). Presumptive THC positive specimens were not available for our testing because they are confirmed and stored for a minimum of 12 months at DOD testing laboratories, as per chain of custody requirements. Specimens were shipped and stored at room temperature prior to our synthetic cannabinoid immunoassay screening and were subsequently stored at 4 °C. Positive specimens were stored at room temperature for 6-152 days (63 mean, 69 median) after collection, and subsequently stored at 4 °C for 248-505 (330 mean, 330 median) days before quantitative analysis.

The Randox Drugs of Abuse V immunoassay has four distinct synthetic cannabinoid antibodies: three (SCI, II, and III) primarily target JWH-018 and one (SCIV) targets JWH-250 metabolites. Manufacturer recommended screening cutoff concentrations were: SCI 10, SCII 20, SCIII 5, and SCIV 5 μg/L. Cross-reactivity with synthetic cannabinoids and metabolites included in the qualitative and quantitative LC-MS/MS methods were evaluated and results are summarized in Supplementary Material Table 1. Limits of detection (LOD) and lower limits of quantification (LLOQ) are listed in Table 1 for the LC-MS/MS qualitative and quantitative assays, respectively. Specimens were screened and confirmed as illustrated in Figure 1. We included 324 true negative (TN), 5 false negative (FN), 163 false positive (FP), and 285 true positive (TP) specimens based on the Randox immunoassay screen and LC-MS/MS qualitative confirmation to rigorously assess synthetic cannabinoid

Table 1. Limits of detection (LOD) and lower limits of quantification (LLOQ) for the qualitative[45] and quantitative[47] confirmatory assays for synthetic cannabinoids, respectively

Analyte	Parent/ Metabolite	Qualitative LOD (μg/L)	Quantitative LLOQ (μg/L)
JWH-018	P	1.0	0.2
JWH-018 pentanoic acid	M	2.5	0.1
JWH-018 *N*-hydroxypentyl[a]	M	0.5	0.1
JWH-018 5-hydroxyindole[b]	M	1.0	0.1
JWH-018 6-hydroxyindole[b]	M	1.0	0.1
JWH-019	P	—	0.1
JWH-019 *N*-hydroxyhexyl[a]	M	—	0.1
JWH-019 5-hydroxyindole	M	—	0.1
JWH-073	P	5.0	0.1
JWH-073 butanoic acid	M	5.0	0.1
JWH-073 *N*-hydroxybutyl[a]	M	2.5	0.1
JWH-073 5-hydroxyindole[b]	M	2.5	0.1
JWH-073 6-hydroxyindole[b]	M	2.5	0.1
JWH-081	P	10.0	0.1
JWH-081 *N*-hydroxypentyl[a]	M	2.5	0.1
JWH-122	P	2.5	0.1
JWH-122 pentanoic acid	M	—	0.2
JWH-122 *N*-hydroxypentyl[a]	M	0.5	0.1
JWH-200	P	—	0.1
JWH-200 5-hydroxyindole	M	5.0	1.0
JWH-200 6-hydroxyindole	M	2.5	0.1
JWH-203	P	—	0.5
JWH-210	P	10.0	0.1
JWH-210 pentanoic acid	M	2.5	0.1
JWH-210 *N*-hydroxypentyl[a,b]	M	1.0	0.1
JWH-210 5-hydroxyindole	M	10.0	0.2
JWH-250	P	5.0	0.2
JWH-250 pentanoic acid	M	0.5	0.2
JWH-250 *N*-hydroxypentyl[a,b]	M	1.0	0.2
JWH-250 5-hydroxyindole	M	2.5	0.1
JWH-398	P	—	0.5
JWH-398 pentanoic acid	M	—	1.0
JWH-398 *N*-hydroxypentyl[a]	M	—	0.5
AM694	P	—	0.1
AM2201	P	1.0	0.1
AM2201 *N*-hydroxypentyl[a]	M	2.5	0.1
AM2201 6-hydroxyindole	M	5.0	1.0
CP 47,497	P	—	0.5
CP 47,497 C7 hydroxy	M	—	0.5
CP 47,497 C8	P	—	0.5
CP 47,497 C8 hydroxy	M	—	0.5
HU-210	P	—	0.5
MAM2201	P	2.5	0.1
MAM2201 *N*-hydroxypentyl[a]	M	—	0.1
RCS-4	P	2.5	0.1
RCS-4 pentanoic acid	M	10.0	0.2
RCS-4 *N*-hydroxypentyl[a]	M	5.0	0.2
RCS-4 4-hydroxypentyl 4′-hydroxyphenyl (M9)	M	—	0.2
RCS-4 5-hydroxypentyl 4′-hydroxyphenyl (M10)	M	—	0.2
RCS-8	P	—	0.1
UR-144 pentanoic acid	M	—	0.2
UR-144 *N*-hydroxypentyl[a]	M	—	0.2
XLR-11	P	—	0.2

[a]Analytes isomeric chromatographic separation unattainable; LLOQs determined for a single alkyl hydroxy isomer; analyzed semi-quantitatively.
[b]Analytes not chromatographically separated, LOD determined together.

prevalence and concentrations in our dataset. When we screened these 20 017 specimens with the Randox Drugs of Abuse V synthetic cannabinoid immunoassay, 1 432 were identified as presumptive positive, and 290 confirmed positive via our 29 analyte qualitative LC-MS/MS method.[45] We qualitatively identified 16 synthetic cannabinoid metabolites and 1 parent compound (AM2201). Subsequently, we re-analyzed all 290 qualitatively confirmed positive and 487 negative specimens by our published quantitative LC-MS/MS method[47] targeting 53 synthetic cannabinoid analytes with 0.1–1 μg/L lower limits of quantification (LLOQ) and 50-100 μg/L upper limits of quantification (ULOQs). Specimens with analyte concentrations exceeding ULOQ were diluted with blank urine and re-analyzed. Additional synthetic cannabinoid analytes not included in the qualitative method are listed in Table 1. All commercially available synthetic cannabinoid standards and internal standards (n = 24) available at the time of method development were included.

Sample preparation and quantitative LC-MS/MS analysis

We analyzed specimens with our published method.[47] Briefly, 200 μL urine specimens were hydrolyzed with β-glucuronidase, and proteins precipitated with acetonitrile prior to SLE. Analytes were eluted with ethyl acetate and dried completely under nitrogen. Specimens were reconstituted in mobile phase (A:B, 50:50, *v/v*), transferred to autosampler vials, and analyzed on an AB SCIEX 5500 QTRAP® triple quadrupole linear ion trap mass spectrometer. Each specimen was injected twice on an Ultra Biphenyl high performance liquid chromatography (HPLC) column obtaining sensitive positive and negative ionization mode results; two multiple reaction monitoring transitions were examined for all analytes. Total analysis time was 30.9 min (19.5 and 11.4 min for positive and negative mode injections, respectively). Baseline chromatographic separation of 12 isomeric alkyl hydroxy metabolites (Table 1) was unattainable, yielding semi-quantitative results. Inter-day analytical recovery (bias) and imprecision (N = 20) were 88.3-112.2% and 4.3-13.5% coefficient of variation, respectively.

Molar ratios of metabolites within each parent synthetic cannabinoid were determined by dividing molar concentration of each metabolite by molar concentration of the most frequently observed metabolite for each parent synthetic cannabinoid. The qualitative LC-MS/MS assay's performance was evaluated by comparing qualitative and quantitative results. Sensitivity, specificity, and efficiency were determined per Ellefsen *et al.*[48] Sensitivity was calculated as the number of TP specimens divided by the sum of TP and FN specimens multiplied by 100. Specificity was calculated as the number of TN specimens divided by the sum of TN and FP specimens multiplied by 100. Efficiency is equal to the sum of TP and TN specimens divided by the total number of samples (n = 777) multiplied by 100.

Results

Of 777 urine specimens analyzed, 290 contained one or more of 22 synthetic cannabinoid metabolites from 11 parent synthetic cannabinoids. No parent compounds were identified. Mean, median, range of concentrations and number of confirmed specimens for each synthetic cannabinoid metabolite are listed in Table 2. The following 11 parent synthetic cannabinoids were identified in various combinations (JWH-018, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, JWH-398, AM2201, MAM2201, RCS-4,

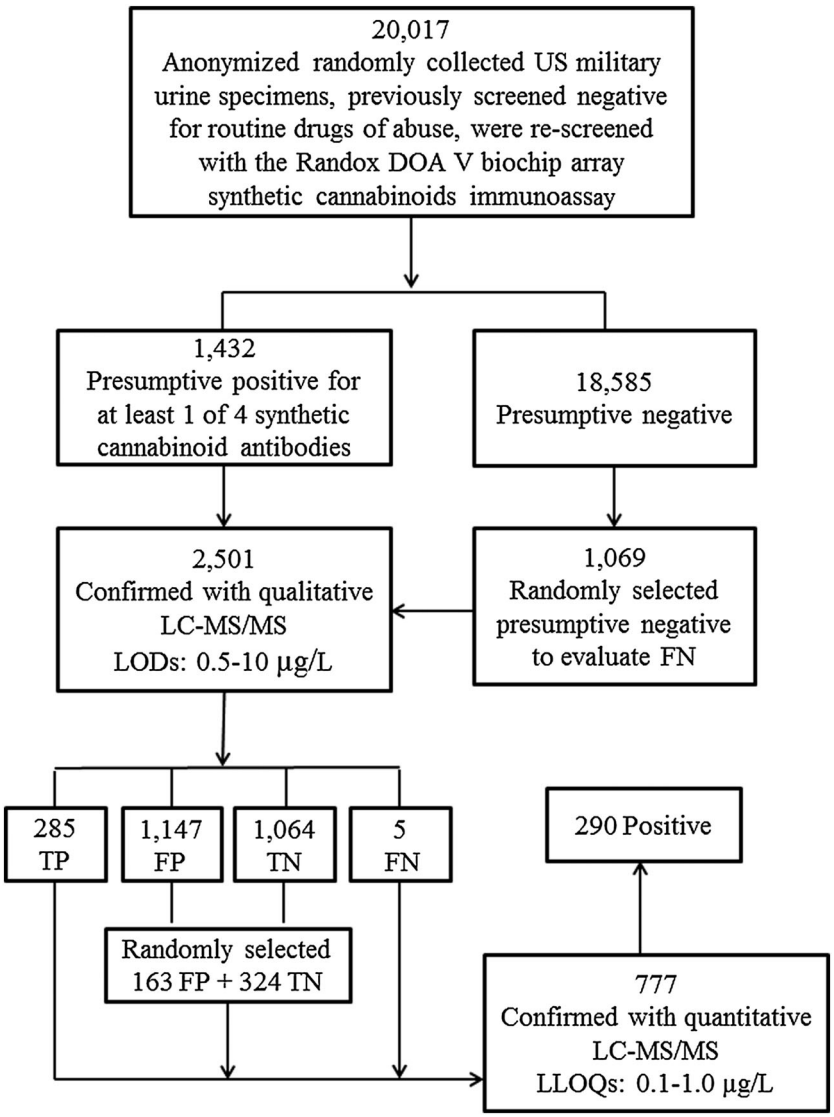

Figure 1. Flowchart for the analyses of 20,017 authentic urine specimens for synthetic cannabinoids by immunoassay and liquid chromatography tandem mass spectrometry (LC-MS/MS). Presumptive positive and randomly selected negative specimens were first confirmed by qualitative LC-MS/MS to determine true positive (TP), true negative (TN), false positive (FP), and false negative (FN). All TP, FN and selected FP and TN were subsequently analyzed by the quantitative LC-MS/MS. Limits of detection (LOD), lower limits of quantification (LLOQ).

and UR-144); JWH-018, JWH-073, JWH-122, and AM2201 were the most frequently found (Table 3). Among 22 metabolites, the most commonly observed were JWH-018 pentanoic acid (mean 121, median 11.1, range 0.1–2,434 µg/L), JWH-018 *N*-hydroxypentyl (mean 59.7, median 5.1, range 0.1–1,239 µg/L), JWH-073 butanoic acid (mean 4.6, median 1.1, range 0.1–48.6 µg/L), JWH-122 *N*-hydroxypentyl (mean 11.3, median 1.1, range 0.1–250 µg/L), AM2201 *N*-hydroxypentyl (mean 16.4, median 2.0, range 0.1–321 µg/L), UR-144 pentanoic acid (mean 19.8, median 3.0, range 0.2–183 µg/L), and UR-144 *N*-hydroxypentyl (mean 7.7, median 1.9, range 0.2–83.2 µg/L).

Metabolite distributions from each parent synthetic cannabinoid are presented in Table 4. Of the specimens positive for JWH-018 metabolites (233/290), most contained JWH-018 pentanoic acid and JWH-018 *N*-hydroxypentyl. For JWH-073 metabolites positive specimens (199/290), all contained JWH-073 butanoic acid. Only one specimen contained JWH-081 *N*-hydroxypentyl. For JWH-122 metabolites positive specimens (130/290), 129 contained JWH-122 *N*-hydroxypentyl, and for JWH-210 metabolites positive specimens (13/290), all contained JWH-210 *N*-hydroxypentyl. JWH-250 pentanoic acid was identified in 19/20 specimens containing JWH-250 metabolites. Three specimens contained JWH-398 *N*-hydroxypentyl. All 201 specimens positive for AM2201 metabolites contained AM2201 *N*-hydroxypentyl. Sixteen specimens contained MAM2201 *N*-hydroxypentyl. All eight specimens positive for RCS-4 contained the RCS 4 M9 metabolite, while 65 of 73 containing UR-144 metabolites were positive for UR-144 N-hydroxypentyl.

Comparing qualitative and quantitative confirmation assay results revealed 14 discordant results. Our LC-MS/MS qualitative analysis found 6 specimens containing single analytes: JWH-018 pentanoic acid (1), JWH-018 *N*-hydroxypentyl (3), JWH-073 *N*-hydroxybutyl (1), and AM2201 N-hydroxypentyl (1); and one specimen contained both JWH-018 pentanoic acid and JWH-018 N-hydroxypentyl, but none of these analytes exceeded LLOQ when specimens were analyzed by quantitative LC-MS/MS. In addition, seven qualitatively negative specimens quantified > LLOQ; 3 contained JWH-018 pentanoic acid (0.1–0.2 µg/L) and/or JWH-018 N-hydroxypentyl (0.3 µg/L), 3 contained UR-144 pentanoic

Table 2. Concentrations of synthetic cannabinoid metabolites quantified by liquid chromatography tandem mass spectrometry in 290 positive authentic urine specimens. Median molar ratio between metabolite and most frequently identified metabolite for each parent synthetic cannabinoid was calculated

Metabolite	N	μg/L			%
		Mean	Median	Range	Median Metabolite Ratio (Range)
JWH-018 pentanoic acid	271	121	11.1	0.1–2434	—
JWH-018 *N*-hydroxypentyl	243	59.7	5.1	0.1–1239	44.1 (0.1–671)
JWH-018 5-hydroxyindole	2	0.2	0.2	0.2–0.2	0.4 (0.1–0.7)
JWH-018 6-hydroxyindole	7	0.5	0.4	0.1–1.1	0.1 (0.1–4.0)
JWH-073 butanoic acid	199	4.6	1.1	0.1–48.6	—
JWH-073 *N*-hydroxybutyl	77	1.3	0.5	0.1–13.2	10.3 (0.4–76.5)
JWH-081 *N*-hydroxypentyl	1	0.1	0.1	0.1	—
JWH-122 pentanoic acid	43	3.0	1.0	0.2–17.0	11.3 (0.8–607.9)
JWH-122 *N*-hydroxypentyl	129	11.3	1.1	0.1–250	—
JWH-210 *N*-hydroxypentyl	13	0.4	0.2	0.1–1.7	—
JWH-210 5-hydroxyindole	3	1.0	0.7	0.3–2.2	126.2 (37.5–242.9)
JWH-250 pentanoic acid	19	17.2	1.3	0.2–275	—
JWH-250 *N*-hydroxypentyl	6	0.7	0.3	0.3–1.4	30.1 (25.4–54.9)
JWH-250 5-hydroxyindole	6	5.3	0.3	0.1–30.4	5.2 (0.6–12.7)
JWH-398 *N*-hydroxypentyl	3	0.9	0.9	0.8–1.0	—
AM2201 *N*-hydroxypentyl	201	16.4	2.0	0.1–321	—
AM2201 6-hydroxyindole	40	3.5	2.0	1.2–18.6	5.7 (2.3–13.2)
MAM2201 *N*-hydroxypentyl	16	0.7	0.5	0.1–2.1	—
RCS-4 pentanoic acid	2	0.3	0.3	0.3–0.3	2.7(0.7–4.7)
RCS-4 M9 metabolite	8	0.4	1.5	0.4–36.4	—
UR-144 pentanoic acid	63	19.8	3.0	0.2–183	185 (8.8–3992)
UR-144 *N*-hydroxypentyl	65	7.7	1.9	0.2–83.2	—

(1.1–11.9 μg/) and/or UR-144 *N*-hydroxypentyl (9.34–29.0 μg/L), and one contained JWH-018 pentanoic acid (0.4 μg/L), JWH-018 *N*-hydroxypentyl (0.1 μg/L), JWH-122 N-hydroxypentyl (0.1 μg/L), UR-144 pentanoic acid (0.9 μg/L) and UR-144 *N*-hydroxypentyl (2.4 μg/L). Thus, qualitative LC-MS/MS sensitivity and specificity were 97.6% and 98.5%, respectively, compared to the quantitative LC-MS/MS LLOQs. The overall positivity rate in the US military between July 2011 and June 2012 for synthetic cannabinoid intake was 1.4%, based on analysis of 20 017 randomly collected urine specimens.

Discussion

Based on our extensive method validation of the Randox Drugs of Abuse V immunoassay, we documented that two of the four manufacturers' recommended screening cutoffs were inappropriately low, producing a high FP rate for our authentic specimens. We also examined potential FN immunoassay samples and only identified 5 FN with our qualitative LC-MS/MS from 1 069 presumptive negative samples (Figure 1). For evaluation of the quantitative LC-MS/MS performance, we reanalyzed all 285 TP, and 5 FN specimens, and randomly selected 487 additional samples to analyze including 163 FP and 324 TN specimens to specifically investigate potential FN tests. Our qualitative LC-MS/MS assay identified 480/487 TN when compared to our quantitative LC-MS/MS method. The small inconsistency is explained by the addition of newly available analytes not included in the qualitative LC-MS/MS and the quantitative LC-MS/MS method's lower LOQs for some analytes (Table 1).

Only eight reports detail synthetic cannabinoid metabolite concentrations in urine, primarily in small datasets of 1-49 individuals,[12,25,28–32,49] while we present the largest dataset of 290 from 777 randomly collected specimens reporting quantitative synthetic cannabinoid results determined with the most comprehensive LC-MS/MS quantitative method published to date. Our main objectives were determining suitable synthetic cannabinoid urinary targets and concentration ranges in randomly collected specimens under a workplace drug-testing program. Study limitations include limited immunoassay cross-reactivity, potential synthetic cannabinoid metabolites' instabilities, semi-quantitative concentrations for 12 alkyl hydroxy analytes due to unattainable isomeric baseline chromatographic resolution, and lack of commercially available standards precluding quantification of additional urinary synthetic cannabinoid metabolites that were possibly present in our urine specimens. Metabolites for 5 parent synthetic cannabinoids (AM694, HU-210, JWH-203, RCS-8, and XRL11; though the latter shares UR-144 pentanoic acid metabolite with UR-144) included in the quantitative method were unavailable at the time of method validation, which also limited our ability to detect intake.

Most specimens collected from service members are shipped to their designated forensic drug testing laboratories within 24-72 h of collection. These specimens are stored at room temperature during shipment and up to the point of immunoassay screening. Only presumptive positive specimens are stored at 4-7 °C; confirmed positive specimens are subsequently stored at -20 °C for one year. Specimens from military units located outside of United States (e.g. Europe, Asia, and Middle East) are often stored for weeks to months at room temperature prior to shipment. Therefore, we also assessed the feasibility of detecting synthetic cannabinoids in these urine specimens stored in non-ideal, yet realistic conditions encountered during DOD testing. To our knowledge, there are no published data regarding synthetic cannabinoid analytes' stability in authentic urine specimens. During our quantitative method

Table 3. Distribution of synthetic cannabinoid metabolites quantified in 290 positive authentic urine specimens

JWH-018	JWH-073	JWH-081	JWH-122	JWH-210	JWH-250	JWH-398	AM2201	MAM2201	RCS-4	UR-144	# Synthetic cannabinoid parent	# Positive (%)
+	+						+				3	62 (21.4)
+	+		+				+				4	44 (15.2)
+											1	30 (10.3)
+	+						+			+	4	18 (6.2)
+	+		+				+			+	5	16 (5.5)
+							+				2	13 (4.5)
+			+								2	8 (2.8)
+										+	2	8 (2.8)
			+								1	7 (2.4)
+			+							+	3	7 (2.4)
+	+										2	6 (2.1)
+	+				+		+				4	6 (2.1)
+	+		+	+			+				5	6 (2.1)
+							+			+	3	5 (1.7)
+	+		+				+	+		+	6	5 (1.7)
+	+		+		+		+				5	4 (1.4)
	+										1	3 (1.0)
+	+		+								3	3 (1.0)
										+	1	3 (1.0)
+	+		+				+	+			5	3 (1.0)
+	+		+				+		+		5	3 (1.0)
	+		+								2	2 (0.7)
+			+		+						3	2 (0.7)
+			+				+				3	2 (0.7)
+			+					+			3	2 (0.7)
+	+		+					+			4	2 (0.7)
+	+		+	+			+			+	6	2 (0.7)
+	+									+	3	1 (0.3)
			+							+	2	1 (0.3)
+	+			+			+				4	1 (0.3)
+	+		+							+	4	1 (0.3)
+			+		+				+		4	1 (0.3)
+	+	+			+		+				5	1 (0.3)
+	+			+	+		+				5	1 (0.3)
+			+					+		+	4	1 (0.3)
+					+		+			+	4	1 (0.3)
+	+		+	+						+	5	1 (0.3)
+	+				+		+		+		5	1 (0.3)
+	+		+	+		+	+				6	1 (0.3)
+	+		+		+	+	+				6	1 (0.3)
+	+		+		+		+	+			6	1 (0.3)
+	+		+				+	+	+		6	1 (0.3)
+	+		+				+		+	+	6	1 (0.3)
+	+		+		+		+		+	+	7	1 (0.3)
+	+		+	+		+	+	+		+	8	1 (0.3)

Note: Several synthetic cannabinoids can produce common metabolites. For example, AM2201 intake can produce JWH-018 and JWH-073 metabolites or MAM2201 can produce JWH-122 metabolites. However, metabolites in this table were grouped under their respective parent synthetic cannabinoid based on structure.

validation, synthetic cannabinoid metabolites fortified in urine were stable for 72 h at 4 °C and 16 h at room temperature; however, parent analytes degraded after 16 h at room temperature except for JWH-200, CP 47,497-C7, CP 47,497-C8, and HU-210.[47] This short-term parent synthetic cannabinoid instability suggests that instability may be one reason that parent analytes are rarely detected in urine. Because our samples were collected from random routine drug testing and stored at room temperature for prolonged periods of time (mean 62 days, median 64 days, range 6–236 days) prior to LC-MS/MS analysis, it is possible metabolite instability occurred, reducing urinary metabolite concentrations. However, some synthetic cannabinoid metabolites were detected in specimens kept at room temperature for up to 152 days and 505 days at 4 °C, suggesting longer urinary analyte stability. Our reported 1.4% positivity rate could be an underestimation due to analyte instability.

Table 4. Distribution of synthetic cannabinoid metabolites identified in 290 of 777 authentic urine specimens by liquid chromatography-tandem mass spectrometry

Parent Compound	Metabolites identified (limits of quantification, μg/L)				# Positive	% Positive
JWH-018	pentanoic acid (0.1)	*N*-OH-pentyl (0.1)	5-OH-indole (0.1)	6-OH-indole (0.1)		
	+	+	+	+	2	0.7
	+	+		+	5	1.7
	+	+			233	80.3
	+				31	10.7
		+			3	1.0
					274	94.5
JWH-073	butanoic acid (0.1)	*N*-OH-butyl (0.1)	5-OH-indole (0.1)	6-OH-indole (0.1)		
	+	+			77	26.5
	+				122	42.1
					199	68.6
JWH-081	*N*-OH-pentyl (0.1)					
	+				1	0.3
JWH-122	pentanoic acid (0.1)	*N*-OH-pentyl (0.1)				
	+	+			42	14.5
		+			87	30.0
	+				1	0.3
					130	44.8
JWH-210	pentanoic acid (0.1)	*N*-OH-pentyl (0.1)	5-OH-indole (0.2)			
		+			10	3.4
		+	+		3	1.0
					13	4.5
JWH-250	pentanoic acid (0.2)	*N*-OH-pentyl (0.2)	5-OH-indole (0.1)			
	+	+	+		2	0.7
	+	+			3	1.0
	+		+		4	1.4
	+				10	3.4
		+			1	0.3
					20	6.9
JWH-398	pentanoic acid (1.0)	*N*-OH-pentyl (0.5)				
		+			3	1.0
AM2201	*N*-OH-pentyl (0.1)	6-OH-indole (1.0)				
	+	+			40	13.8
	+				161	55.5
					201	69.3
MAM2201	*N*-OH-pentyl (0.1)					
	+				16	5.5
RCS-4	pentanoic acid (0.2)	*N*-OH-pentyl (0.2)	M9 (0.2)	M10 (0.2)		
	+		+		2	0.7
			+		6	2.1
					8	2.8
UR-144	pentanoic acid (0.2)	*N*-OH-pentyl (0.2)				
	+	+			55	19.0
		+			10	3.4
	+				8	2.8
					73	25.2

Note: Several synthetic cannabinoids can produce common metabolites. For example, AM2201 intake can produce JWH-018 and JWH-073 metabolites or MAM2201 can produce JWH-122 metabolites. However, metabolites in this table were grouped under their respective parent synthetic cannabinoid based on structure.

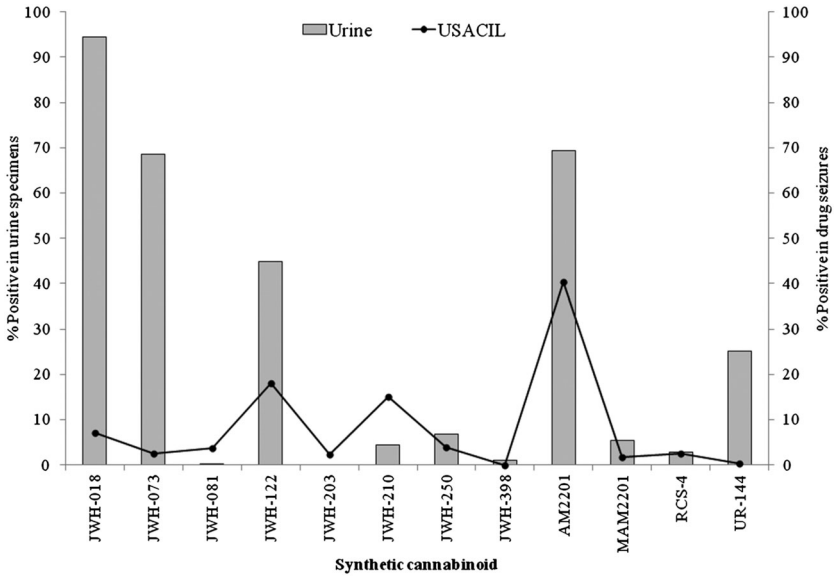

Figure 2. Distribution of synthetic cannabinoid metabolites assigned to their respective parent analyte, regardless of shared metabolic pathways with other parents, identified in 290 authentic urine specimens collected between November 2011 and June 2012. Positivity rates in authentic urine specimens (left y-axis) compared with the United States Army Criminal Investigation Laboratory (USACIL) drug seizures (n = 1144) positivity rate (right y-axis) from military bases analyzed within the same period for synthetic cannabinoids. 1-Pentyl-3-(1-adamantoyl)indole, 2NE1, AM2233, CP 47,497 C8 homologue, RCS-4 methoxy isomer, RCS-4 butyl homologue, RCS-8, and XLR-11 also were identified (≤0.3%) in seizures, but not included in the graph.

Urine specimens were collected from July 2011 to June 2012. Of 1 144 seized materials analyzed for synthetic cannabinoids by the United States Army Criminal Investigation Laboratory (USACIL) between November 2011 and June 2012 (report received by personal communication), the top four most prevalent were AM2201, JWH-122, JWH-210, and JWH-018 at 40.3%, 18.1%, 15.1%, and 7.1%, respectively (Figure 2). JWH-073, JWH-081, JWH-250, MAM2201, RCS-8, UR-144, and XLR-11 were identified in less than 5% of total seizures while JWH-398 was not detected at all. Overall, our trend mirrored USACIL seizures considering AM2201 metabolism yields JWH-018 and JWH-073 metabolites.[28,30,50] Although JWH-210 had a higher rate of prevalence in seized materials than our confirmed specimens, the compound's prevalence peaked in August 2011 but steadily declined by January 2012. Most of our specimens were collected between February 2012 and June 2012, which coincided with the period of JWH-210 decline and AM2201 emergence. Thus, JWH-018 metabolites presence in 201 specimens also containing AM2201 metabolites may have originated from AM2201 intake, or a mixture of AM2201 and JWH-018 consumption. Herbal mixtures also may contain one or more synthetic cannabinoids as identified by other laboratories.[51] Therefore, consumption can produce common urinary metabolites from different parent analytes and may impact immunoassay screening rates.

Of 22 metabolites from 11 parent synthetic cannabinoids (JWH-018, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, JWH-398, AM2201, MAM2201, RCS-4 and UR-144), JWH-018 pentanoic acid had the highest mean (121 μg/L) and median (11.1 μg/L) concentrations. Except for JWH-018 *N*-hydroxypentyl, median concentrations for other synthetic cannabinoid metabolites were <3 μg/L suggesting lower doses, a greater time between use and urine collection, or greater analyte instability. Many synthetic cannabinoids possess stronger CB_1 receptor binding affinity than THC,[7] requiring lower dosages for achieving desired cannabimimetic effects, and also producing lower urinary metabolite concentrations than observed for THC and metabolites. Brent *et al*. determined the CB_1 K_i for JWH-018 and its 4-hydroxyindole metabolite were 1.2 ± 0.3 nM and 2.7 ± 0.3 nM, respectively, which were 5 6 times lower than THC's K_i (15.3 ± 4.5 nM).[52] Moreover, JWH-018 and 4-hydroxindole also are full CB_1 agonists with EC_{50} values 6.8 ± 2.5 and 17.0 ± 9.6 nM, respectively, compared with THC's EC_{50} of 167.4 ± 85.7 nM.

Synthetic cannabinoid urinary concentration ranges found in our authentic specimens were compared to those reported in the literature. In 2010 ElSohly Laboratories analyzed 33 forensic urine samples, submitted for workplace drug testing programs, and identified JWH-018 pentanoic acid in 17 samples with concentrations between 11.6–27,256 μg/L and JWH-018 *N*-hydroxypentyl (21.9–5 530 μg/L), but only 35% contained JWH-018 6-hydroxyindole (3.1–35.7 μg/L).[32] No other metabolites were included in their LC-MS/MS assay. In contrast, our JWH-018 pentanoic acid and JWH-018 *N*-hydroxypentyl concentrations were lower, possibly suggesting lower exposures, longer times since administration or instability during storage for our specimens. Lovett *et al*. reported synthetic cannabinoid results for 49 urine specimens collected after March 2012 from US Air Force service members suspected of synthetic cannabinoid intake or subject to random drug testing.[31] Specimens contained JWH-018 pentanoic acid (0.7–458 μg/L), JWH-018 *N*-4-hydroxypentyl (0.1–152 μg/L), JWH-018 *N*-5-hydroxypentyl (0.4–706 μg/L), JWH-073 butanoic acid (0.8–305.5 μg/L), and JWH-072 propanoic acid (4.7–943.8 μg/L). All specimens contained JWH-018 pentanoic acid and JWH-072 propanoic acid metabolites; 46/49 and 33/49 contained JWH-073 butanoic acid and JWH-018 *N*-5 hydroxypentyl, respectively. JWH-072 propanoic acid concentrations were 1.6–16.6 times higher than those of JWH-018 pentanoic acid. The authors suggested that JWH-072 propanoic acid was a common biomarker for JWH-018, JWH-073, and AM2201. JWH-072 propanoic acid (or JWH-018 *N*-propanoic acid) was not commercially available prior to our method validation. Our observed JWH-018 pentanoic concentrations were similar to those reported by Lovett *et al*. Jang *et al*. investigated urinary AM2201- and JWH-018 metabolites in individuals arrested for suspected AM2201 (n = 9) and JWH-018 (n = 11) abuse.[30] AM2201

N-4-hydroxypentyl (5.1–38.2 μg/L) and AM2201 6-hydroxyindole (3.6–24.4 μg/L) were detected in 5 and 6 AM2201-exposed suspects, respectively. However, all specimens (n = 9) also contained JWH-018 pentanoic acid (9.6–401 μg/L) and JWH-018 *N*-5-hydroxypentyl (3.1–150 μg/L). Four specimens positive for AM2201 metabolites also contained JWH-073 butanoic acid (4.2–16.9 μg/L). None contained JWH-018 6-hydroxyindole. All urine samples collected from individuals who abused JWH-018 (n = 11) were positive for JWH-018 *N*-4-hydroxypentyl at 2.9– 604 μg/L; however, 8 also contained JWH-018 *N*-5-hydroxypentyl (2.5–213 μg/L) and JWH-018 pentanoic acid (2.7–241.8 μg/L), 6 contained JWH-073 butanoic acid (3.3–219.3 μg/L) while only 2 contained JWH-018 6-hydroxyindole (3.5–4.9 μg/L). We observed co-occurrence of JWH-018 pentanoic acid, JWH-018 *N*-hydroxypentyl, JWH-018 5-hydroxyindole, and JWH-018 6-hydroxyindole in 2 specimens. We also observed 179 (61.7%) positive specimens contained JWH-018 pentanoic acid, JWH-073 butanoic acid, and AM2201 *N*-hydroxypentyl. Metabolite concentrations of AM2201 4-hydroxypentyl reported by Jang *et al*. were similar to our mean and median concentrations of 16.4 and 2.0 μg/L, respectively. Although AM2201 *N*-hydroxypentyl is indicative of AM2201 intake, the median concentration for this metabolite in specimens also positive for JWH-018 metabolites was 2.2 μg/L compared to 11.1 μg/L JWH-018 *N*-hydroxypentyl and 35.1 μg/L JWH-018 pentanoic acid suggesting that the fluorinated metabolite undergoes different metabolic rate. These highlight the challenges of interpreting results and determining the most suitable urinary biomarker.

Converging metabolic pathways for similar synthetic cannabinoids produce common metabolites and most synthetic cannabinoid products contain one or more parent drugs, explaining the presence of multiple synthetic cannabinoid metabolites in individual specimens. Structurally similar synthetic cannabinoids, especially halogenated compounds, produce common metabolites to their non-halogenated analogs. Chimalakonda *et al*. published human liver microsomal AM2201 and JWH-018 metabolic studies and reported common metabolites (JWH-018 pentanoic acid, JWH-018 *N*-hydroxypentyl) from both.[50] Oral ingestion of 5 mg pure AM2201 produced JWH-018 pentanoic acid, JWH-018 *N*-5-hydroxypentyl, JWH-073 butanoic acid, JWH-073 *N*-4-hydroxybutyl, AM2201 *N*-4-hydroxypentyl, and AM2201 6-hydroxyindole.[28] JWH-073 butanoic acid also was detected in specimens from individuals who reported JWH-018 intake.[53] We also observed co-occurrence of AM2201 *N*-hydroxypentyl, JWH-018 pentanoic acid, and JWH-073 butanoic acid in 180 specimens. Either the JWH-018 pentanoic acid and/or JWH-073 butanoic acid were also derived from AM2201 or all three parent analytes were taken in any combination. Similar patterns can be expected for other fluorinated synthetic cannabinoids such as MAM2201 and XLR-11. MAM2201 intake can potentially produce JWH-122 pentanoic acid and JWH-122 *N*-hydroxypentyl metabolites. For this reason, we cannot assign JWH-122 metabolites from JWH-122 intake alone. We observed 16 positive specimens containing all three metabolites: MAM2201 *N*-hydroxypentyl (0.1-2.1 μg/L), JWH-122 pentanoic acid (0.5-17.0 μg/L), and JWH-122 *N*-hydroxypentyl (1.3-250 μg/L) and 87 positive specimens contained only JWH-122 pentanoic acid (0.1-19.1 μg/L). USACIL reported about 20% seized materials contained JWH-122 while MAM2201 was found in less than 5%. In comparison, 5.5% of our specimens contained MAM2201 *N*-hydroxypentyl and 30% contained JWH-122 metabolites. Others recommended including metabolites unique for the parent compound, i.e., AM2201 4-hydroxypentyl for AM2201[50] and JWH-018 4-hydroxypentyl for JWH-018.[28]

In evaluating the most suitable biomarkers for each synthetic cannabinoid, JWH-018, JWH-073, and JWH-250 carboxy (pentanoic or butanoic acid) metabolites were most frequently identified. Alkyl hydroxy metabolites occurred more frequently for JWH-122, JWH-210, JWH-398, and UR-144. However, median molar ratios between carboxy and alkyl hydroxy metabolites from each parent compound were highly variable (between 0.1 and 3 992%), making it difficult to distinguish optimal biomarkers but lead us to propose targeting carboxy and alkyl hydroxy metabolites. Median molar ratios of JWH-018, JWH-250 and AM2201 hydroxyindole metabolites were lower (<20%) than alkyl hydroxy metabolites and carboxy metabolites, highlighting that hydroxyindole metabolites are minor metabolites. Alkyl hydroxy and carboxy metabolites proved to be effective targets in our study, but other synthetic cannabinoids with different structures could produce different major metabolites. For instance, our hepatocyte studies with AKB48 and STS-135 identified monohydroxylated metabolites at the adamantane ring moiety as more predominant than monohydroxylated metabolites at the indazole or indole alkyl chain.[54,55] We also observed carboxy formation at the tetramethylcyclopropyl ring as the major metabolite for XLR-11 compared to UR-144 pentanoic acid for UR-144.[56] Adamowicz *et al*. also detected UR-144 mono-, dihydroxylated, and carboxy urinary metabolites in authentic specimens including metabolites generated from the opened-ring pyrolysis product.[57] These metabolites were unavailable during our method development. Metabolite molar concentration also can be affected by time since last intake, analyte stability, and inter-subject metabolism variability.

Conclusion

We measured concentrations of urinary synthetic cannabinoid metabolites in 290 urine specimens collected from November 2011 to June 2012, the largest dataset of authentic workplace drug testing urine specimens yet published. We quantified concentrations of 22 alkyl hydroxy and carboxy metabolites from 11 parent synthetic cannabinoids (JWH-018, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, JWH-398, AM2201, MAM2201, RCS-8, and UR-144) by the most comprehensive LC-MS/MS quantitative method to date. The highest synthetic cannabinoid metabolite concentration was 2 434 μg/L for JWH-018 pentanoic acid, although the majority (92.2%) of concentrations were less than 100 μg/L. We determined JWH-018 and AM2201 metabolites were the predominant analytes quantified in our urine specimens, a trend matching seized materials analyzed by USACIL. No parent synthetic cannabinoid was identified in urine, but in many cases, multiple metabolites were present from a single parent compound. Based on *in vitro* metabolic studies, structurally similar synthetic cannabinoids can produce common metabolites and can make data interpretation challenging. We observed that alkyl hydroxy and carboxy metabolites provided suitable urinary metabolite targets for most synthetic cannabinoids present in our specimens; however, this may vary for newly emerging compounds.

Disclaimer

The opinions or assertions herein are those of the authors and do not necessarily reflect the views of the National Institutes of Health, Department of Defense, Army, Navy, or Air Force.

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