Clinical, Forensic & Toxicology

LC/MS/MS Analysis of Metabolites of Synthetic Cannabinoids JWH-018 and JWH-073 in Urine

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Abstract

A liquid chromatographic method was developed to resolve a comprehensive set of metabolites of JWH-018 and JWH-073. In addition to the chromatographic analysis method, an extraction method was developed to recover a broad range of synthetic cannabinoid metabolites, including carboxylic acid metabolites that are not traditionally recovered using a high pH liquid/liquid extraction. The extraction and analysis methods were used to identify and quantify the significant metabolites in several authentic urine samples. In addition to the identification of known metabolites, two previously undocumented metabolites were detected.

The chromatographic method detailed in this application note employed a 5 µm Ultra Biphenyl 50 mm x 2.1 mm RP-HPLC column and MS/MS detection. The quantitative range validated for all metabolites was 1 ng/mL to 500 ng/mL in urine. Linearity for all metabolites was $r \geq 0.9982$. Accuracy for QC samples ranged from 89% to 100%. The extraction procedure gave metabolite recoveries from urine that ranged from 43% to 78%.

Based on the data shown here, this method is suitable for quantification of metabolites of JWH-018 and JWH-073 to support broader research studies that positively identify clinically significant metabolites and their concentrations in urine.

Introduction

Since 2008, synthetic cannabinoids such as JWH-018 and JWH-073 have gained popularity in the United States and Europe. These compounds are smoked as components of herbal incense mixtures that, until recently, were commercially available and legally sold. In early 2011, the US Drug Enforcement Agency (DEA) placed several of the most popular synthetic cannabinoids—including JWH-018 and JWH-073—on their Schedule 1 list, making the possession or consumption of these compounds illegal.

Because of the scheduling of JWH-018 and JWH-073, laboratories are now being tasked with developing methods to analyze urine for synthetic cannabinoids. Research has shown that the parent compounds are extensively metabolized prior to excretion and, therefore, are present at very low levels in urine samples [1]. The more abundant metabolites are better targets for screening assays; however, since the illicit use of synthetic cannabinoids is relatively recent, limited research has been completed to determine the exact metabolite profiles.

In previous work, several metabolites of JWH-018 and JWH-073 were identified using both in vivo and in vitro studies. The identified metabolites include mono- and di-hydroxylated metabolites, as well as carboxylated metabolites [1,2]. The hydroxylated and carboxylated metabolites are generally extracted separately due to differences in pKa values for these compounds. Both groups of metabolites present some chromatographic challenges; the hydroxylated analytes exist as multiple positional isomers that are indistinguishable by MS/MS, and the carboxylated compounds are hydrophilic, making them difficult to retain using RP-HPLC.

The purpose of this work was to develop an extraction and analysis method suitable for the identification and quantification of a wide range of known and unknown synthetic cannabinoid metabolites in urine. Our goal was to establish methodology that simplified extraction and provided reliable chromatographic resolution of clinically relevant metabolites.

Experimental

The overall experimental design included calibrators prepared in urine at 7 levels (1, 10, 25, 50, 100, 250, and 500 ng/mL), blanks from 6 separate sources (3 male, 3 female), 2 quality control (QC) samples fortified at 40 ng/mL, and 6 legally obtained authentic samples. Quantification and partial validation was performed using this set of samples. Extraction efficiency was determined by fortifying a post-extraction blank at 10 ng/mL and comparing the area of the resulting peak to the corresponding calibrator, which was fortified prior to extraction.

Sample Preparation

Urine samples were fortified with synthetic cannabinoid metabolites at the calibrator and QC levels defined above. Internal standard concentration was 40 ng/mL in urine for all samples, except the double blank.

Fortified and blank samples were hydrolyzed by adding 1 mL of sample to 1 mL of beta-glucuronidase from keyhole limpet (Sigma-Aldrich cat.# G8132) and incubating at 60 °C for 3 hours. The beta-glucuronidase solution was prepared at 5,000 Fishman units/mL in 100 mM ammonium acetate buffer (pH = 5.0).

Hydrolyzed samples were then extracted using 6 mL, 500 mg C18 high-load endcapped Resprep® SPE cartridges (cat.# 24052) according to the following procedure. Note that the cartridge should not be allowed to go dry until step 6.

1. Add 1 mL 5 mM ammonium acetate in 0.1% acetic acid (pH = 4.2) to hydrolyzed sample.
2. Condition cartridge with 3 rinses of 1 mL acetonitrile.
3. Condition cartridge with 3 rinses of 1 mL 5 mM ammonium acetate in 0.1% acetic acid.
4. Apply sample to cartridge and allow it to pass through under gravity.
5. Rinse cartridge with 3 portions of 1 mL 5 mM ammonium acetate in 0.1% acetic acid.
6. Dry cartridge under vacuum for 10 minutes.
7. Elute with 3 mL acetonitrile followed by 3 mL butyl chloride. Note that the use of butyl chloride is not necessary to obtain adequate recoveries. However, if it is used, add the first mL of butyl chloride to the last mL of acetonitrile to ensure the solvents are mixed.

Sample extracts were evaporated to dryness under nitrogen at 40 °C and then reconstituted in 0.5 mL (50:50) 0.05% acetic acid in water:0.05% acetic acid in acetonitrile.

Analysis

The instrument used for this analysis was a Shimadzu UFLCXR liquid chromatograph coupled to an AB SCIEX API 4000 LC/MS/MS detector. Instrument conditions are listed below, and MRM transitions are provided in Table I. Note that many of the analytes included here share common transitions. During compound optimization, transitions were chosen based on abundance as well as uniqueness, when possible.

Quantification was performed using the internal standards as specified in Table I. The choice of internal standard for quantification was based on both compound identification and retention time (e.g. JWH-073 4-hydroxybutyl was quantified using JWH-018 pentanoic acid-d4 rather than JWH-073 4-hydroxyindole-d7 due to the fact that its retention time was very early compared to the JWH-073 4-hydroxyindole-d7 internal standard).

LC Conditions

Instrument:	Shimadzu UFLCXR
Column:	5 µm Ultra Biphenyl 50 mm x 2.1 mm (cat.# 9109552)
Column Temperature:	25 °C
Mobile Phase A:	0.05% acetic acid in water (pH approx. 3.4)
Mobile Phase B:	0.05% acetic acid in acetonitrile
Injection Volume:	10 µL
Flow:	0.5 mL/min.

Gradient:

Time	%B
0.00	45
2.00	45
6.00	85
6.10	95
7.00	95
7.10	45
8.50	stop

MS/MS Conditions

Instrument:	API 4000 MS/MS
Ionization Mode:	ESI+
Data Acquisition Type:	MRM (non-scheduled)
Ion Spray Voltage:	3,000V
Source Temperature:	600 °C
Curtain Gas:	40 psi (275.8 kPa)
Gas 1:	40 psi (275.8 kPa)
Gas 2:	40 psi (275.8 kPa)
CAD Gas:	4 psi (27.6 kPa)

Table I MRM transitions and internal standards used for quantification.

Compound	MRM1 (Quant.)	MRM2 (Qual. 1)	MRM3 (Qual. 2)	Internal Standard Used for Quantification
JWH-073 4-hydroxybutyl	344.1/155.1	344.1/127.2	344.1/144.0	JWH-018 N-pentanoic acid-d4
JWH-073 N-butanoic acid	358.1/155.1	358.1/127.3	358.1/144.1	JWH-018 N-pentanoic acid-d4
JWH-018 N-pentanoic acid-d4 (IS)	376.1/155.2	376.1/230.4	376.1/248.3	NA
JWH-018 N-pentanoic acid	372.1/155.2	372.1/127.1	372.1/144.1	JWH-018 N-pentanoic acid-d4
JWH-018 5-hydroxypentyl	358.1/155.2	358.1/127.1	358.1/230.3	JWH-018 N-pentanoic acid-d4
JWH-073 6-hydroxyindole	344.1/155.2	344.1/127.1	344.1/145.1	JWH-073 4-hydroxyindole-d7
JWH-073 5-hydroxyindole	344.1/155.2	344.1/127.1	344.1/160.0	JWH-073 4-hydroxyindole-d7
JWH-073 7-hydroxyindole	344.2/155.1	344.2/127.1	344.2/216.3	JWH-073 4-hydroxyindole-d7
JWH-018 6-hydroxyindole	358.1/155.1	358.1/127.2	358.1/145.2	JWH-018 N-pentanoic acid-d4
JWH-018 5-hydroxyindole	358.1/155.1	358.1/127.2	358.1/160.2	JWH-073 4-hydroxyindole-d7
JWH-018 7-hydroxyindole	358.1/155.1	358.1/127.1	358.1/230.3	JWH-018 N-pentanoic acid-d4
JWH-073 4-hydroxyindole-d7 (IS)	351.3/127.0	351.3/223.0	351.3/155.0	NA
JWH-073 4-hydroxyindole	344.1/155.2	344.1/127.2	344.1/160.0	JWH-073 4-hydroxyindole-d7
JWH-018 4-hydroxyindole	358.1/155.2	358.1/230.2	358.1/127.1	JWH-073 4-hydroxyindole-d7

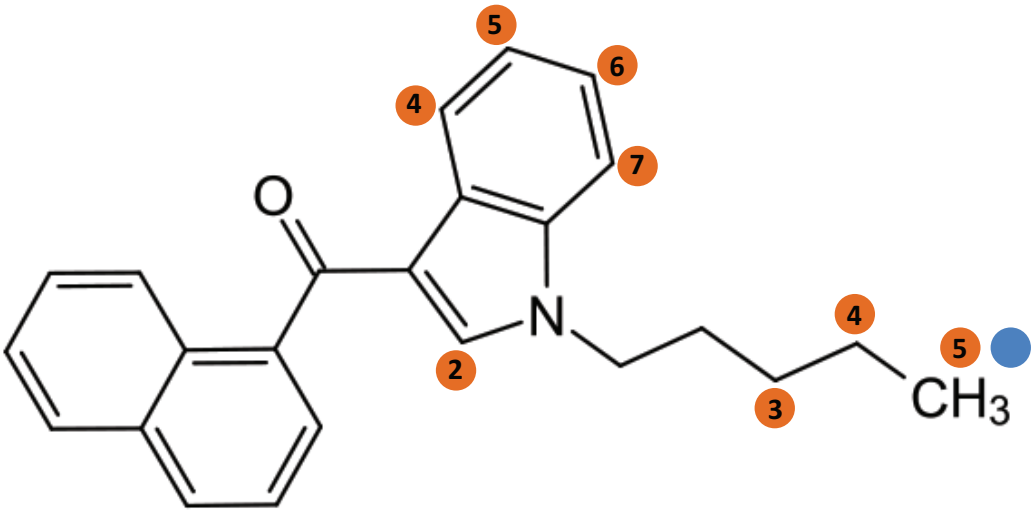
*NA=not applicable

Results and Discussion

Chromatography

Chromatographic separation is essential for analyzing JWH-018 and JWH-073 metabolites due to the presence of multiple positional isomers among the mono-hydroxylated metabolites. These isomers form because each parent compound has many sites available for hydroxylation (Figure 1). Since these positional isomers have identical molecular weights and very similar fragmentation patterns, they are indistinguishable by MS/MS detectors and chromatographic resolution is required for positive identification. Representative chromatograms of low and high calibrators are shown in Figures 2 and 3. All the isomeric analytes included in this method were resolved on the Ultra Biphenyl column. By chromatographically separating these isomers, the most abundant metabolites from a given parent compound can be identified in authentic samples and methodology can be further optimized specifically for metabolites of clinical significance.

Figure 1 Hydroxylation and carboxylation sites for JWH-018.



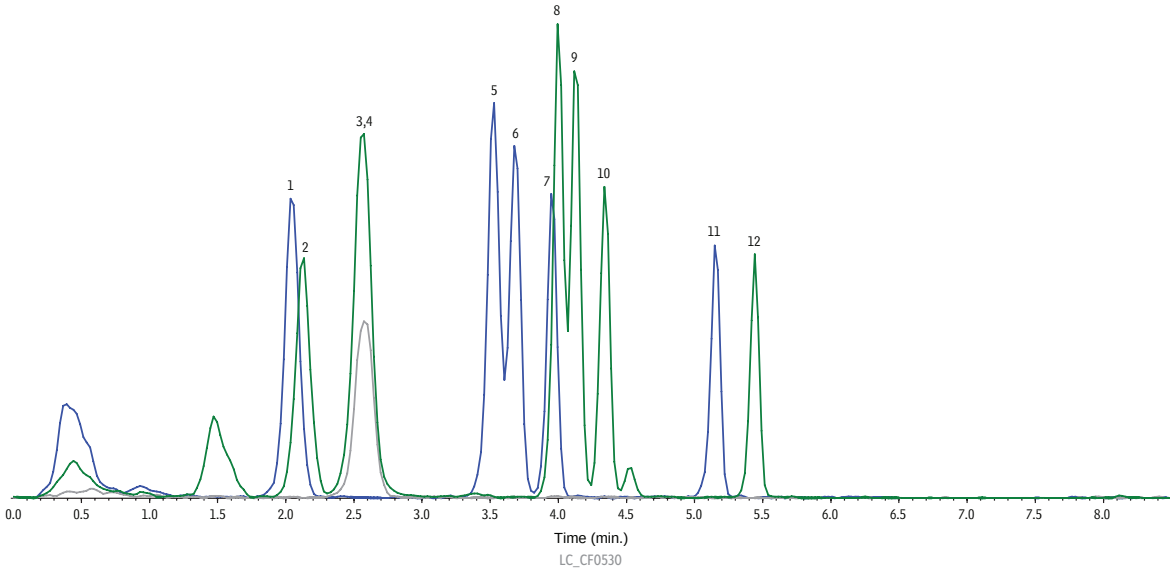
Hydroxylation site – numbers indicate position on indole ring or alkyl side chain.

Carboxylation site

Note: Possible hydroxylation and carboxylation sites are identical for JWH-073. JWH-018 2-hydroxyindole metabolite standard was not available and was not included in this method.

Figure 2 1 ng/mL calibration standard.

Peaks	RT (min.)	MRM1	MRM2	MRM3
1. JWH-073 4-hydroxybutyl	2.04	344.1/155.1	344.1/127.2	344.1/144.0
2. JWH-073 N-butanoic acid	2.13	358.1/155.1	358.1/127.3	358.1/144.1
3. JWH-018 N-pentanoic acid	2.59	372.1/155.2	372.1/127.1	372.1/144.1
4. JWH-018 5-hydroxypentyl	2.57	358.1/155.2	358.1/127.1	358.1/230.3
5. JWH-073 6-hydroxyindole	3.52	344.1/155.2	344.1/127.1	344.1/145.1
6. JWH-073 5-hydroxyindole	3.68	344.1/155.2	344.1/127.1	344.1/160.0
7. JWH-073 7-hydroxyindole	3.95	344.2/155.1	344.2/127.1	344.2/216.3
8. JWH-018 6-hydroxyindole	4.00	358.1/155.1	358.1/127.2	358.1/145.2
9. JWH-018 5-hydroxyindole	4.13	358.1/155.1	358.1/127.2	358.1/160.2
10. JWH-018 7-hydroxyindole	4.34	358.1/155.1	358.1/127.1	358.1/230.3
11. JWH-073 4-hydroxyindole	5.15	344.1/155.2	344.1/127.2	344.1/160.0
12. JWH-018 4-hydroxyindole	5.44	358.1/155.2	358.1/230.2	358.1/127.1



Column	Ultra Biphenyl (cat.# 9109552)
Dimensions:	50 mm x 2.1 mm ID
Particle Size:	5 µm
Pore Size:	100 Å
Temp.:	25 °C
Sample	
Diluent:	50:50 mobile phase
Conc.:	1 ng/mL extracted spiked sample
Inj. Vol.:	10 µL

Mobile Phase

A: water + 0.05% acetic acid (pH approx. 3.4)
B: acetonitrile + 0.05% acetic acid

Time (min.)	Flow (mL/min.)	%A	%B
0	0.5	55	45
2.00	0.5	55	45
6.00	0.5	15	85
6.10	0.5	5	95
7.00	0.5	5	95
7.10	0.5	55	45
8.50	stop		

Detector	API 4000
Model #:	API 4000
Ion Source:	TurboIonSpray®
Ion Mode:	ESI+
Ion Spray Voltage:	3 kV
Curtain Gas:	40 psi (275.8 kPa)
Gas 1:	40 psi (275.8 kPa)
Gas 2:	40 psi (275.8 kPa)
Interface Temp.:	600 °C
Mode:	MRM
Dwell Time:	30 ms
Instrument	API LC MS-MS

Notes

Since multiple transitions are shared between analytes, only 3 transitions are shown to simplify viewing. The transitions shown are: 344.1/155.1 (blue trace), 358.1/155.1 (green trace), 372.1/155.2 (gray trace). To show analytes in full scale, internal standards are not shown.

CAD Gas was set to 4 psi.

For 50 ng/mL calibration level, see chromatogram LC_CF0531.
For 500 ng/mL calibration level, see chromatogram LC_CF0532.

Sample was prepared according to the following method:
1) Spike 1 mL blank urine sample with analytes and internal standards.

2) *Hydrolyze sample*:
- Add 1 mL solution of beta-glucuronidase from keyhole limpet (Sigma-Aldrich cat.# G8132). Solution is prepared at a concentration of 5,000 Fishman units/mL in 100 mM ammonium acetate buffer (pH = 5.0).
- Incubate at 60 °C for 3 hours.

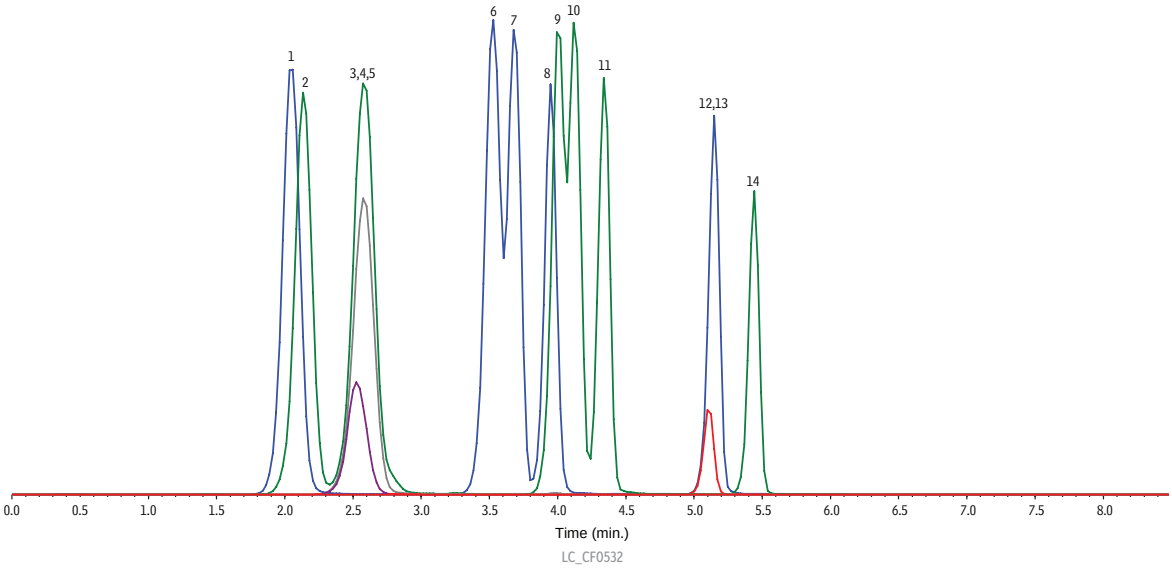
3) *Extract sample on 6 mL, 500 mg C18 high-load endcapped Resprep® SPE cartridge (cat.# 24052)*:
- Add 1 mL 5 mM ammonium acetate + 0.1% acetic acid (pH = 4.2) to sample.
- Condition cartridge with 3x 1 mL acetonitrile.
- Condition cartridge with 3x 1 mL 5 mM ammonium acetate + 0.1% acetic acid.
- Apply sample and allow to pass through under gravity.
- Rinse with 3x 1 mL 5 mM ammonium acetate + 0.1% acetic acid.
- Dry cartridge with vacuum for 10 minutes.
- Elute with 3 mL acetonitrile followed by 3 mL butyl chloride.

4) *Concentrate sample*:
- Evaporate sample to dryness under nitrogen at 40 °C.
- Reconstitute in 0.5 mL water + 0.05% acetic acid:acetonitrile + 0.05% acetic acid (50:50).

Acknowledgement Special thanks to Cayman Chemical for reference standards

Figure 3 500 ng/mL calibration standard.

Peaks	RT (min.)	MRM1	MRM2	MRM3
1. JWH-073 4-hydroxybutyl	2.05	344.1/155.1	344.1/127.2	344.1/144.0
2. JWH-073 N-butanoic acid	2.14	358.1/155.1	358.1/127.3	358.1/144.1
3. JWH-018 N-pentanoic acid-d4 (IS)	2.52	376.1/155.2	376.1/230.4	376.1/248.3
4. JWH-018 N-pentanoic acid	2.58	372.1/155.2	372.1/127.1	372.1/144.1
5. JWH-018 5-hydroxypentyl	2.58	358.1/155.2	358.1/127.1	358.1/230.3
6. JWH-073 6-hydroxyindole	3.53	344.1/155.2	344.1/127.1	344.1/145.1
7. JWH-073 5-hydroxyindole	3.69	344.1/155.2	344.1/127.1	344.1/160.0
8. JWH-073 7-hydroxyindole	3.95	344.2/155.1	344.2/127.1	344.2/216.3
9. JWH-018 6-hydroxyindole	4.00	358.1/155.1	358.1/127.2	358.1/145.2
10. JWH-018 5-hydroxyindole	4.13	358.1/155.1	358.1/127.2	358.1/160.2
11. JWH-018 7-hydroxyindole	4.34	358.1/155.1	358.1/127.1	358.1/230.3
12. JWH-073 4-hydroxyindole-d7 (IS)	5.11	351.3/127.0	351.3/223.0	351.3/155.0
13. JWH-073 4-hydroxyindole	5.15	344.1/155.2	344.1/127.2	344.1/160.0
14. JWH-018 4-hydroxyindole	5.44	358.1/155.2	358.1/230.2	358.1/127.1



LC_CF0532

Column
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 5 µm
Pore Size: 100 Å
Temp.: 25 °C

Sample
Diluent: 50:50 mobile phase
Conc.: 500 ng/mL extracted spiked sample
Inj. Vol.: 10 µL

Mobile Phase

A: water + 0.05% acetic acid (pH approx. 3.4)
B: acetonitrile + 0.05% acetic acid

Time (min.)	Flow (mL/min.)	%A	%B
0	0.5	55	45
2.00	0.5	55	45
6.00	0.5	15	85
6.10	0.5	5	95
7.00	0.5	5	95
7.10	0.5	55	45
8.50	stop		

Detector
Model #: API 4000
Ion Source: TurboIonSpray®
Ion Mode: ESI+
Ion Spray Voltage: 3 kV
Curtain Gas: 40 psi (275.8 kPa)
Gas 1: 40 psi (275.8 kPa)
Gas 2: 40 psi (275.8 kPa)
Interface Temp.: 600 °C
Mode: MRM
Dwell Time: 30 ms
Instrument API LC MS-MS

Notes

Since multiple transitions are shared between analytes, only 5 transitions are shown to simplify viewing. The transitions shown are: 344.1/155.1 (blue trace), 358.1/155.1 (green trace), 372.1/155.2 (grey trace), 376.1/155.2 (purple trace - internal standard), 351.3/127.0 (red trace - internal standard)

CAD Gas was set to 4 psi.

For 1 ng/mL calibration level, see chromatogram LC_CF0530.
For 50 ng/mL calibration level, see chromatogram LC_CF0531.

Sample was prepared according to the following method:
1) Spike 1 mL blank urine sample with analytes and internal standards.

2) *Hydrolyze sample*:
- Add 1 mL solution of beta-glucuronidase from keyhole limpet (Sigma-Aldrich cat.# G8132). Solution is prepared at a concentration of 5,000 Fishman units/ mL in 100 mM ammonium acetate buffer (pH = 5.0).
- Incubate at 60 °C for 3 hours.

3) *Extract sample on 6 mL, 500 mg C18 high-load endcapped Resprep® SPE cartridge (cat.# 24052)*:
- Add 1 mL 5 mM ammonium acetate + 0.1% acetic acid (pH = 4.2) to sample.
- Condition cartridge with 3x 1 mL acetonitrile.
- Condition cartridge with 3x 1 mL 5 mM ammonium acetate + 0.1% acetic acid.
- Apply sample and allow to pass through under gravity.
- Rinse with 3x 1 mL 5 mM ammonium acetate + 0.1% acetic acid.
- Dry cartridge with vacuum for 10 minutes.
- Elute with 3 mL acetonitrile followed by 3 mL butyl chloride.

4) *Concentrate sample*:
- Evaporate sample to dryness under nitrogen at 40 °C.
- Reconstitute in 0.5 mL water + 0.05% acetic acid:acetonitrile + 0.05% acetic acid (50:50).

Acknowledgement Special thanks to Cayman Chemical for reference standards

Partial Validation Data

The performance of the chromatographic method was evaluated based on signal-to-noise, linearity, back-calculated accuracy for calibrators, and ion suppression measurement (Table II). Adequate low level responses were obtained and good linearity ($r \geq 0.9982$) was observed for all metabolites over a quantitative range of 1 ng/mL to 500 ng/mL in urine. Comparison of back-calculated calibrator concentrations to prepared values showed accuracy was achieved at both ends of the linear range for all compounds.

Ion suppression was determined by peak area comparison of a blank sample spiked after extraction to a solvent standard. Based on the results gathered in this project, no suppression was observed, but significant enhancement occurred for several analytes.

Interferences and carryover were also evaluated in this study. A double blank sample was injected immediately after the high calibrator and no carryover or interference peaks were observed with areas greater than 5% of the low calibrator. In addition, 5 other blank samples from independent sources were analyzed and no interferences were detected in any of these samples.

Table II Chromatographic performance results.

Compound	S/N (Quant. Ion) 1 ng/mL*	Linearity (r) (Quant. Ion)	Back-Calculated Accuracy 1 ng/mL**	Back-Calculated Accuracy 500 ng/mL **	Ion Suppression (Quant. Ion) 10 ng/mL
JWH-018 N-pentanoic acid	307	0.9993	1.08	0.99	1.04
JWH-018 5-hydroxypentyl	176	0.9990	1.06	0.98	1.16
JWH-073 4-hydroxybutyl	755	0.9982	1.10	0.97	1.13
JWH-073 N-butanoic acid	181	0.9986	1.10	0.97	1.07
JWH-018 4-hydroxyindole	221	0.9997	1.03	0.99	1.40
JWH-018 5-hydroxyindole	370	0.9988	0.78	1.03	1.38
JWH-018 6-hydroxyindole	458	0.9987	0.88	0.81	1.17
JWH-018 7-hydroxyindole	259	0.9997	0.92	1.00	1.17
JWH-073 4-hydroxyindole	490	0.9996	0.97	0.99	1.33
JWH-073 5-hydroxyindole	1,590	0.9995	0.90	1.00	1.20
JWH-073 6-hydroxyindole	1,410	0.9999	0.97	1.00	1.09
JWH-073 7-hydroxyindole	489	0.9995	0.85	1.02	1.14

* Signal-to-noise ratio for all MRM transitions at the limit of quantitation was greater than 10:1.

** Reported accuracy, based on the quantification ion, was 77.9%–119% for all calibrators and all transitions.

Extraction recovery was assessed by peak area comparison of a post-extraction blank fortified at 10 ng/mL with the corresponding calibrator, which was fortified prior to extraction. QC samples were also analyzed to verify method performance. As shown in Table III, the extraction procedure recovered all the metabolites with results ranging from 43% to 78%. Although some of the recoveries were low, results were acceptable for the first 4 compounds, which were later identified in the authentic samples as being clinically significant metabolites. Subsequent experimentation determined that the low recoveries were due to incomplete elution of the compounds from the SPE cartridge, rather than to poor retention on the analytical column. The elution with butyl chloride, in addition to acetonitrile, increased recoveries by only a small amount and may be eliminated if desired.

Although recoveries for some compounds were relatively low, the use of a mid-pH extraction (pH = 4.2) as opposed to a high pH extraction allowed for the recovery of the carboxylic acid metabolites as well as the mono-hydroxy metabolites. Recoveries for clinically significant metabolites ranged from 70% to 78%. Previously published methods describe the use of a high pH liquid/liquid extraction for the analysis of synthetic cannabinoid metabolites [1]. While the extraction method used by Sobolevsky et al. was suitable for hydroxylated metabolites, the recoveries of carboxylated metabolites are very low at high pH. While a second liquid/liquid extraction at low pH is required to adequately recover carboxylated metabolites, the SPE extraction method developed for this analysis is suitable for both mono-hydroxylated and carboxylated metabolites of JWH-018 and JWH-073.

Table III Extraction performance results.

Compound	Avg. Accuracy 40 ng/mL QCs (n = 2)*	Extraction Recovery (Quant Ion) 10ng/mL
JWH-018 N-pentanoic acid	98%	78%
JWH-018 5-hydroxypentyl	97%	70%
JWH-073 4-hydroxybutyl	99%	72%
JWH-073 N-butanoic acid	97%	78%
JWH-018 4-hydroxyindole	98%	68%
JWH-018 5-hydroxyindole	93%	60%
JWH-018 6-hydroxyindole	91%	65%
JWH-018 7-hydroxyindole	89%	43%
JWH-073 4-hydroxyindole	100%	58%
JWH-073 5-hydroxyindole	94%	66%
JWH-073 6-hydroxyindole	97%	69%
JWH-073 7-hydroxyindole	91%	47%

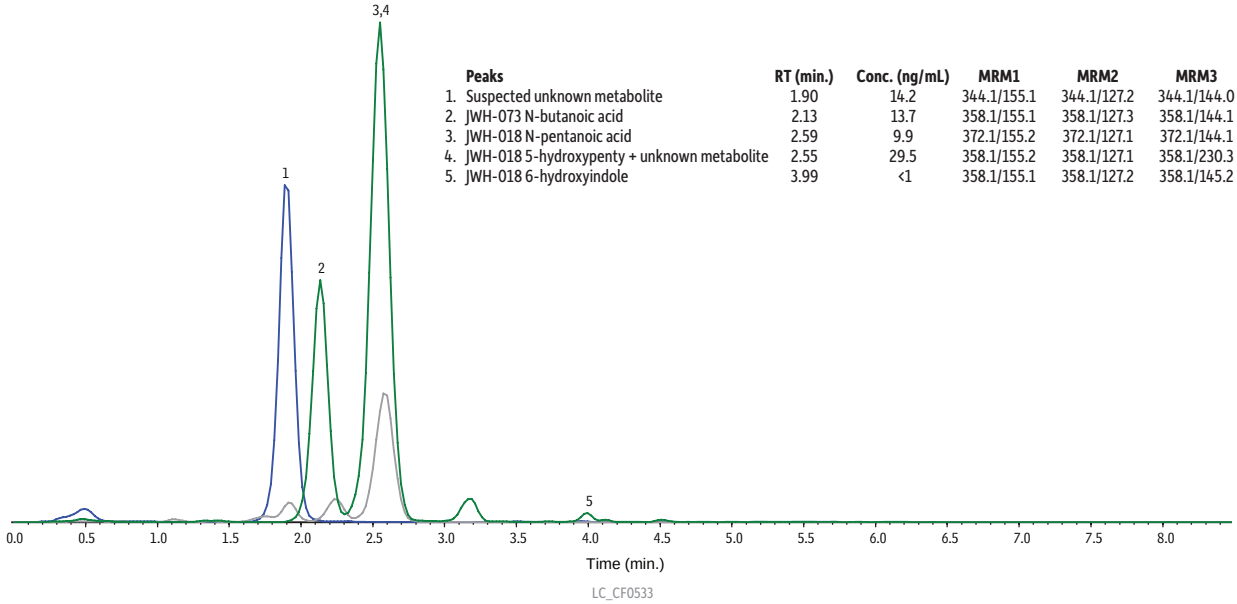
* Reported accuracy for quantification ion.

Authentic Sample Analysis

After validation, 6 authentic samples were prepared and analyzed according to the method established here (Table IV). All reported values met ion ratio criteria for the first qualifier MRM transition; however, most results for JWH-018 5-hydroxypentyl did not meet the ion ratio criteria for the second qualifier. As shown in Figure 4, the peak for this analyte was slightly broader than expected. To determine if these results were due to co-eluting interferences, samples and calibrators were re-analyzed using a longer column and a 60 minute isocratic method. The results from the isocratic analyses revealed a co-eluting peak with the same transitions as JWH-018 5-hydroxypentyl. This peak was not present in any of the blank samples, and based on this work, is most likely an undocumented metabolite of JWH-018. Based on recent work by NMS Labs, this unknown is most probably JWH-018 4-hydroxypentyl [3]. Chromatography for the isocratic method was not suitable for quantitation, so no quantitative results can be reported for this compound. Results shown for JWH-018 5-hydroxypentyl are the sum of JWH-018 5-hydroxypentyl and the unknown metabolite.

Although JWH-073 n-butanoic acid is present in several samples, no JWH-073 4-hydroxybutyl was detected. A large peak with the same transitions as JWH-073 4-hydroxybutyl was present in the authentic samples at a slightly earlier retention time than JWH-073 4-hydroxybutyl. Post-extraction spiking of sample #1 with 2 ng of JWH-073 4-hydroxybutyl confirmed that the observed peak was not due to JWH-073 4-hydroxybutyl. The unknown peak was not observed in any blank samples, suggesting that it is also an unknown metabolite of either JWH-018 or JWH-073. Based on recent work by NMS labs, this unknown is probably JWH-073 3-hydroxybutyl [3]. The chromatographic method used here was sufficient to partially resolve the unknown compound from the standard, and the unknown peak was quantitated using JWH-073 4-hydroxybutyl. The results for this unknown metabolite should be considered semi-quantitative.

Figure 4 Authentic sample #1.



Peaks	RT (min.)	Conc. (ng/mL)	MRM1	MRM2	MRM3
1. Suspected unknown metabolite	1.90	14.2	344.1/155.1	344.1/127.2	344.1/144.0
2. JWH-073 N-butanoic acid	2.13	13.7	358.1/155.1	358.1/127.3	358.1/144.1
3. JWH-018 N-pentanoic acid	2.59	9.9	372.1/155.2	372.1/127.1	372.1/144.1
4. JWH-018 5-hydroxypenty + unknown metabolite	2.55	29.5	358.1/155.2	358.1/127.1	358.1/230.3
5. JWH-018 6-hydroxyindole	3.99	<1	358.1/155.1	358.1/127.2	358.1/145.2

Column Ultra Biphenyl (cat.# 9109552)
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 5 µm
Pore Size: 100 Å
Temp.: 25 °C
Sample
Diluent: 50:50 mobile phase
Conc.: extracted authentic sample
Inj. Vol.: 10 µL
Mobile Phase
A: water + 0.05% acetic acid (pH approx. 3.4)
B: acetonitrile + 0.05% acetic acid

Time (min.)	Flow (mL/min.)	%A	%B
0	0.5	55	45
2.00	0.5	55	45
6.00	0.5	15	85
6.10	0.5	5	95
7.00	0.5	5	95
7.10	0.5	55	45
8.50	stop		

Detector API 4000
Model #: API 4000
Ion Source: TurboIonSpray®
Ion Mode: ESI+
Ion Spray Voltage: 3 kV
Curtain Gas: 40 psi (275.8 kPa)
Gas 1: 40 psi (275.8 kPa)
Gas 2: 40 psi (275.8 kPa)
Interface Temp.: 600 °C
Mode: MRM
Dwell Time: 30 ms
Instrument API LC MS-MS

Notes Since multiple transitions are shared between analytes, only 3 transitions are shown to simplify viewing. The transitions shown are: 344.1/155.1 (blue trace), 358.1/155.1 (green trace), 372.1/155.2 (gray trace). Internal standards are not shown.

CAD Gas was set to 4 psi.

Sample was prepared according to the following method:
1) Spike 1 mL blank urine sample with analytes and internal standards.

2) *Hydrolyze sample*:
- Add 1 mL solution of beta-glucuronidase from keyhole limpet (Sigma-Aldrich cat.# G8132). Solution is prepared at a concentration of 5,000 Fishman units/mL in 100 mM ammonium acetate buffer (pH = 5.0).
- Incubate at 60 °C for 3 hours.

3) *Extract sample on 6 mL, 500 mg C18 high-load endcapped Resprep® SPE cartridge (cat.# 24052)*:
- Add 1 mL 5 mM ammonium acetate + 0.1% acetic acid (pH = 4.2) to sample.
- Condition cartridge with 3x 1 mL acetonitrile.
- Condition cartridge with 3x 1 mL 5 mM ammonium acetate + 0.1% acetic acid.
- Apply sample and allow to pass through under gravity.
- Rinse with 3x 1 mL 5 mM ammonium acetate + 0.1% acetic acid.
- Dry cartridge with vacuum for 10 minutes.
- Elute with 3 mL acetonitrile followed by 3 mL butyl chloride.

4) *Concentrate sample:*
- Evaporate sample to dryness under nitrogen at 40 °C.
- Reconstitute in 0.5 mL water + 0.05% acetic acid:acetonitrile + 0.05% acetic acid (50:50).

Acknowledgement Special thanks to Cayman Chemical for reference standards

Table IV Quantitative results for authentic samples.

Compound	Sample 1 (ng/mL)	Sample 2 (ng/mL)	Sample 3 (ng/mL)	Sample 4 (ng/mL)	Sample 5 (ng/mL)	Sample 6 (ng/mL)
JWH-018 N-pentanoic acid	9.9	11.5	22.7	1.5	< 1	44.3
JWH-018 5-hydroxypentyl + unknown metabolite	29.5*	14.7*	84.2*	5.4*	1.4*	48.9
JWH-073 4-hydroxybutyl	ND	ND	ND	ND	ND	ND
Unknown metabolite	14.2	35.2	21.6	1.70	< 1	69.7
JWH-073 N-butanoic acid	13.7	1.2	9.3	1.3*	ND	1.4
JWH-018 4-hydroxyindole	ND	ND	ND	ND	ND	ND
JWH-018 5-hydroxyindole	ND	ND	< 1	ND	ND	ND
JWH-018 6-hydroxyindole	< 1	ND	1.1	ND	ND	ND
JWH-018 7-hydroxyindole	ND	ND	ND	ND	ND	ND
JWH-073 4-hydroxyindole	ND	ND	ND	ND	ND	ND
JWH-073 5-hydroxyindole	ND	ND	ND	ND	ND	ND
JWH-073 6-hydroxyindole	ND	ND	ND	ND	ND	ND
JWH-073 7-hydroxyindole	ND	ND	ND	ND	ND	ND

*Results did not meet ion ratio criteria (+/- 20%) for the second qualifier MRM transition.

Conclusions

Based on the results of this partial validation, the extraction and chromatographic methods developed here are suitable for the analysis of JWH-018 and JWH-073 metabolites in human urine. The mid-range pH SPE extraction allows both mono-hydroxylated and carboxylated metabolites to be recovered from a single extraction, providing a simpler alternative to separate high pH and low pH liquid-liquid extraction procedures.

The Ultra Biphenyl column used here provides enough retention for quantitative analysis of the hydrophilic carboxylated metabolites. The column also offers the selectivity needed to separate positional isomers of the mono-hydroxylated metabolites, which must be chromatographically resolved in order to report valid data. The methods established here can be applied in clinical and forensic laboratories to optimize screening procedures for synthetic cannabinoid metabolites in urine.

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