Characterizing the Novel Synthetic Cannabinoid, RCS-4's Metabolism – Human Hepatocytes Applicability in Forensic Drug Testing

For Research Use Only. Not for Use in Diagnostic Procedures

Adarsh S. Gandhi[1,#], Mingshe Zhu[2], Shaokun Pang[3], Ariane Wohlfarth[1], Karl B. Scheidweiler[1] and Marilyn A. Huestis[1]

[1] Chemistry and Drug Metabolism, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA. [#] Bioanalysis and Physiology, Lundbeck Research Inc. USA, Paramus, NJ, USA, [2] Department of Biotransformation, Bristol-Myers Squibb Research and Development, Princeton, NJ, USA, [3]AB Sciex, Redwood City, CA, USA

ABSTRACT

Introduction: Classification of first generation synthetic cannabinoids as Schedule 1 drugs led to the rapid emergence of novel synthetic drugs, such as RCS-4 (4-methoxyphenyl)-1-(pentyl-1H-indol-3-yl) methanone. Although RCS-4 phase I metabolism was studied preliminarily, phase II metabolism remains uncharacterized. Synthetic cannabinoid parent compounds are extensively transformed, with metabolites predominating in urine. Lack of phase I and II metabolite identification studies prevents effective RCS-4 monitoring in clinical and forensic urine testing. Once available, metabolite mass spectra generated using human hepatocytes and high-resolution mass spectrometry can be incorporated into reference libraries. Our goal was to generate a complete metabolite profile of RCS-4 using cryopreserved human hepatocytes and high-resolution accurate mass spectrometry.

Methods: Ten µM RCS-4 was incubated with human hepatocytes for up to 1 h at 37°C. Samples were collected at 0 and 1 h; protein was precipitated with acetonitrile. After centrifugation and dilution with mobile phase, samples were analyzed by HPLC coupled to a TripleTOF® 5600+ mass spectrometer (AB Sciex). Analysis consisted of a TOF survey scan and information-dependent acquisition (mass defect filtering and dynamic background subtraction) triggered product ion scans. Gradient elution was performed on a KinetexTM C18 column at 0.3 mL/min with 0.1% formic acid in water and acetonitrile, respectively. Data were analyzed with MetabolitePilot™ software (AB Sciex) employing peak finding algorithms and data mining tools such as product ion, neutral loss and mass defect filters.

Preliminary Data: We identified 18 phase I and II RCS-4 metabolites. Most metabolites were hydroxylated with or without demethylation, carboxylation, and dealkylation followed by glucuronidation. One sulfated metabolite was observed. O-demethylation was the most common biotransformation. More metabolites were observed after 3 compared to 1 h incubation.

Novel Aspect: Mass spectra of RCS-4 metabolites can be incorporated into spectral libraries for detecting RCS-4 consumption in forensic laboratories.

INTRODUCTION

1. Synthetic cannabinoids are labeled "not for human consumption" and consumed to avoid detection during drug testing.
2. They are primarily eliminated via oxidative metabolism followed by glucuronidation in humans (1-2). Adverse events from synthetic cannabinoid consumption are known; however, their metabolic and pharmacokinetic profiles remain uncharacterized.
3. RCS-4 [(4-methoxyphenyl)-1-(pentyl-1H-indol-3-yl) methanone] is a synthetic cannabinoid commonly consumed worldwide in 2010-2011. Use declined after banning in several European nations and the US.
4. Although previous report studied the phase I metabolism of RCS-4, we, identified phase I and phase II metabolites which are equally important (3).
5. Human hepatocytes better mimic the physiological liver environment than microsomes including bile canaliculi, uptake and efflux transporters and expression of phase II enzymes and essential cofactors; hepatocytes yield a more clinically relevant RCS-4 metabolic profile.

OBJECTIVE

To identify RCS-4 metabolites formed during hepatocyte incubation via Time of Flight (TOF) mass spectrometry coupled with high performance liquid chromatography (HPLC)

EXPERIMENTAL METHODS

Incubation of RCS-4 with human hepatocytes

1. Pooled cryopreserved human hepatocytes (BioreclamationIVT) (1.3x10^6 cells/mL) were incubated with 10.0 µM of RCS-4 at 37 ^{0}C. Diclofenac (CYP2C9 substrate) was used as a positive control.
2. Samples were collected after 0 and 1 h and reaction was terminated with an equal volume of acetonitrile.
3. Samples were centrifuged at 15,000 g for 5 min at 4 ^{0}C to remove cell debris and particulate matter. The supernatant was injected onto the LC column after 2X dilution with mobile phase.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

1. The samples were analyzed with a Shimadzu Prominence high performance liquid chromatograph coupled with an AB Sciex TripleTOF 5600+ mass spectrometer equipped with an electrospray ionization (ESI) source.
2. Samples were separated on a Kinetex C18 XB column (100 mm x 2.1 mm, 2.6 µm) with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as the mobile phase.
3. Gradient elution was performed at a flow rate of 0.3 mL/min from 10% B at 0 min to 95% B at 20.1 min and column was re-equilibrated at 10% B at 22 min for a total run time of 25 min.
4. MS analysis was performed in positive ESI mode by scanning the TOF masses from 100-950 Da. MS/MS analysis was performed with a combination of dynamic background subtraction (DBS) and mass defect filter (MDF) information dependent acquisitions (IDA). Four product ion scans (60-950 Da) were acquired with collision energy of 40±10 eV. The MDF list included masses for the parent, glucuronides, sulfates and dealkylated metabolites with a mass tolerance of 40 mDa.

Data analysis

1. Data acquired were analyzed by the data mining software MetabolitePilot™ (AB Sciex) that employs various peak-finding algorithms to generate metabolite candidates.
2. The processing parameters were optimized for detecting RCS-4 metabolites.

RESULTS

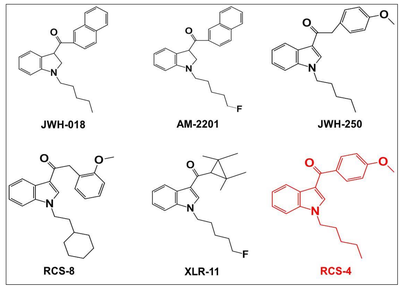

Fig.1. Chemical structures of related synthetic cannabinoids

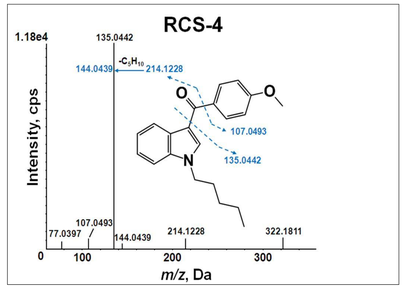

Fig.2. Structure and ESI MS/MS (m/z 322) spectra of RCS-4

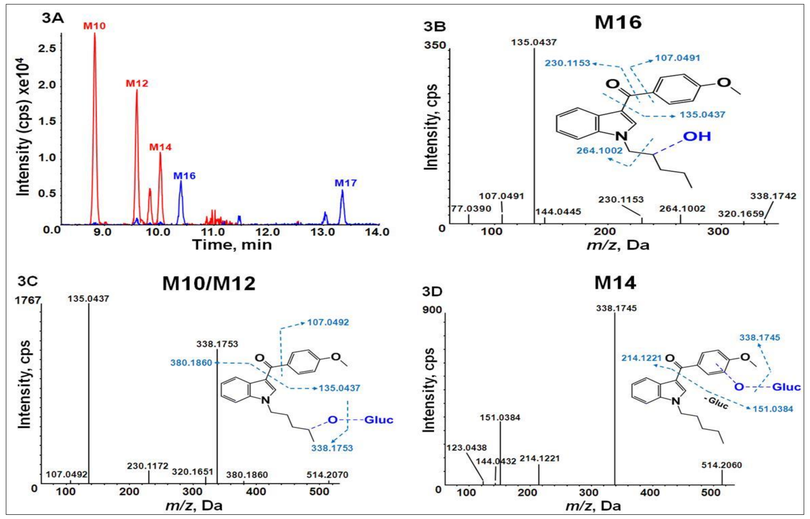

Fig.3. Extracted ion chromatograms (XICs) (A) and MS/MS spectra of monohydroxylated (B) and monohydroxylated/glucuronidated (C and D) RCS-4 metabolites

Peak ID	Biotransformation	Elemental composition	Precursor ion (m/z)	Diagnostic product ions (m/z)	Mass error (ppm)	RT (min)	MS Area
	Parent	$C_{21}H_{23}NO_2$	322.1811	107, 135, 144, 214	2.9	17.17	1.86E+06
M1	O-demethylation/Depentylation/ Glucuronidation	$C_{21}H_{19}NO_8$	414.1190	93, 121, 144, 238	1.5	4.11	1.79E+04
M2	O-demethylation/Dihydroxylation/ Glucuronidation	$C_{26}H_{29}NO_{10}$	516.1869	137, 186, 204, 340	0.8	5.27	4.95E+04
M3	O-demethylation/ Monohydroxylation/Glucuronidation	$C_{26}H_{29}NO_9$	500.1919	121, 130, 186, 204, 250, 32	0.8	5.51	8.82E+04
M4	Dihydroxylation/Glucuronidation	$C_{27}H_{31}NO_{10}$	530.2024	151, 186, 204, 230, 354	0.6	5.73	1.63E+04
M5	O-demethylation/ Monohydroxylation/Glucuronidation	$C_{26}H_{29}NO_9$	500.1920	121, 186, 204, 250, 324	1.1	5.81	2.26E+04
M6	O-demethylation/ Monohydroxylation/Glucuronidation	$C_{26}H_{29}NO_9$	500.1915	121, 130, 186, 204, 250, 324	0.0	6.15	1.13E+04
M7	O-demethylation/ Monohydroxylation/Sulfation	$C_{20}H_{21}NO_6S$	404.1170	121, 186, 200, 204, 250, 324	1.8	6.58	6.89E+04
M8	O-demethylation/Carboxylation	$C_{20}H_{19}NO_4$	338.1396	121, 144, 218, 244	2.7	7.60	3.25E+04
M9	O-demethylation/Monohydroxylation	$C_{20}H_{21}NO_3$	324.1602	121, 153, 186, 250	2.3	7.66	1.43E+04
M10	Monohydroxylation/Glucuronidation	$C_{27}H_{31}NO_9$	514.2070	135, 230, 338	-0.3	8.83	6.68E+04
M11	O-demethylation/ Monohydroxylation/Glucuronidation	$C_{26}H_{29}NO_9$	500.1915	137, 144, 188, 214, 324	0.0	9.49	4.67E+04
M12	Monohydroxylation/Glucuronidation	$C_{27}H_{31}NO_9$	514.2069	135, 230, 320, 338	-0.5	9.60	4.18E+04
M13	O-demethylation/Glucuronidation	$C_{26}H_{29}NO_8$	484.1961	121, 188, 214, 308	-1.0	9.92	5.85E+04
M14	Monohydroxylation /Glucuronidation	$C_{27}H_{31}NO_9$	514.2060	144, 151, 214, 338	-2.3	10.02	2.19E+04
M15	Carboxylation	$C_{21}H_{21}NO_4$	352.1547	107, 135, 144, 244	1.1	10.15	2.60E+04
M16	Monohydroxylation	$C_{21}H_{23}NO_3$	338.1754	107, 135, 144, 230, 264	0.9	10.40	1.80E+04
M17	Monohydroxylation	$C_{21}H_{23}NO_3$	338.1753	107, 135, 160, 230	0.6	13.34	1.36E+04
M18	O-demethylation	$C_{20}H_{21}NO_2$	308.1650	121, 132, 144, 188, 214	1.5	13.84	1.18E+05

Table 1: Accurate mass data, retention time (RT), mass error and diagnostic product ion data of RCS-4 and its postulated metabolites

RESULTS

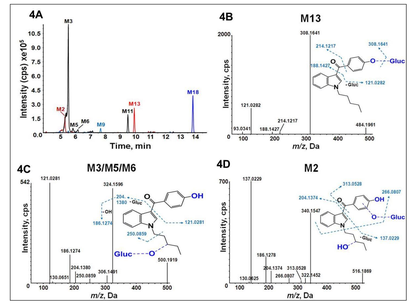

Fig.4. XICs (A) and MS/MS spectra of o-demethylated/ glucuronidated (B), o-demethylated/monohydroxylated/ glucuronidated (C), and o-demethylated/dihydroxylated/ glucuronidated (D) RCS-4 metabolites

Fig.5. (a) XICs (a) and MS/MS spectra of o-demethylated/depentylated/gucuronidated (b), o-demethylated/monohydroxylated/sulfated (c), and carboxylated (d) RCS-4 metabolites

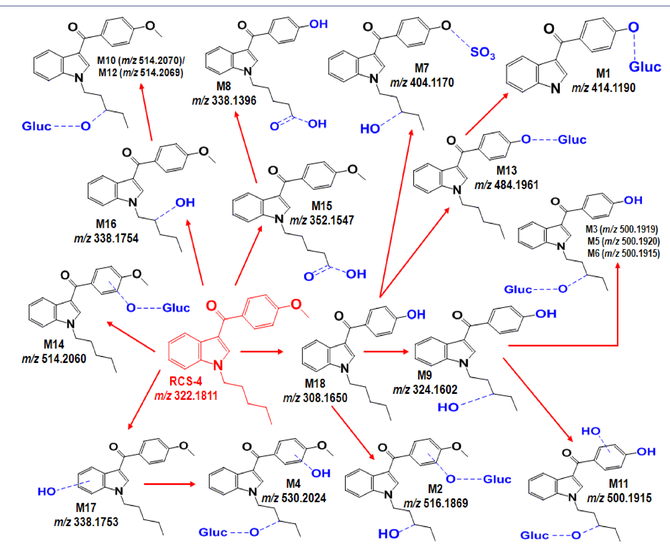

Fig.6. Proposed metabolic pathways of RCS-4 following human hepatocyte incubation

CONCLUSIONS

1. Eighteen Phase I and II RCS-4 metabolites, were identified by LC/TOFMS after incubation with human hepatocytes..
2. Our data lead us to propose monitoring of M3, M7, M10 and M18 in forensic analytical methods for detecting RCS-4 intake.
3. This is the first study to identify a sulfate conjugate of a synthetic cannabinoid.
4. These spectra could be into MS reference libraries for RCS-4 LC-MS/MS screening methods.
5. Human hepatocytes produce a more complete range of metabolites as compared to human liver microsomes and better predict *in vivo* metabolism.

REFERENCES

1. Elsohly MA, Gul W, Wanas AS, Radwan MM. Synthetic cannabinoids: analysis and metabolites. Life Sci 97:78-90 (2014).
2. Chimalakonda KC, Seely KA, Bratton SM, Brents LK, Moran CL, Endres GW, James LP, Hollenberg PF, Prather PL, Radominska-Pandya A, Moran JH. Cytochrome P450-mediated oxidative metabolism of abused synthetic cannabinoids found in K2/Spice: identification of novel cannabinoid receptor ligands. Drug Metab Dispos. 40:2174-84 (2012).
3. Kavanagh P, Grigoryev A, Melnik A, Simonov A. The identification of the urinary metabolites of 3-(4-methoxybenzoyl)-1-pentylindole (RCS-4), a novel cannabimimetic, by gas chromatography-mass spectrometry. J Anal Toxicol. 36:303-11 (2012).

ACKNOWLEDGEMENT

This work was supported by the Intramural Research Program NIDA/NIH