

Research Article

First Characterization of AKB-48 Metabolism, a Novel Synthetic Cannabinoid, Using Human Hepatocytes and High-Resolution Mass Spectrometry

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Abstract. Since the federal authorities scheduled the first synthetic cannabinoids, JWH-018 and JWH-073, new synthetic cannabinoids were robustly marketed. *N*-(1-Adamantyl)-1-pentylindazole-3-carboxamide (AKB-48), also known as APINACA, was recently observed in Japanese herbal smoking blends. The National Forensic Laboratory Information System registered 443 reports of AKB-48 cases in the USA from March 2010 to January 2013. In May 2013, the Drug Enforcement Administration listed AKB-48 as a Schedule I drug. Recently, AKB-48 was shown to have twice the CB₁ receptor binding affinity than CB₂. These pharmacological effects and the difficulty in detecting the parent compound in urine highlight the importance of metabolite identification for developing analytical methods for clinical and forensic investigations. Using human hepatocytes and TripleTOF mass spectrometry, we identified 17 novel phase I and II AKB-48 metabolites, products of monohydroxylation, dihydroxylation, or trihydroxylation on the aliphatic adamantane ring or *N*-pentyl side chain. Glucuronide conjugation of some mono- and dihydroxylated metabolites also occurred. Oxidation and dihydroxylation on the adamantane ring and *N*-pentyl side chain formed a ketone. More metabolites were identified after 3 h of incubation than at 1 h. For the first time, we present a AKB-48 metabolic scheme obtained from human hepatocytes and high-resolution mass spectrometry. These data are needed to develop analytical methods to identify AKB-48 consumption in clinical and forensic testing.

KEYWORDS: AKB-48; cannabinoids; human hepatocytes; metabolism; time-of-flight mass spectrometry.

INTRODUCTION

Although synthetic cannabinoids were synthesized in the 1990s as pharmacological tools for studying the endogenous cannabinoid system, they were first detected in material marketed as incense in 2004 (1). The authorities suspected that the plant material contained psychoactive substances and was being abused. Synthetic cannabinoids are sold in local head shops, on the Internet, and in gas stations under brand names

like K2 or Spice and labeled as “herbal incense” and “not for human consumption.” Similar to Δ^9 -tetrahydrocannabinol (THC), the active constituent of cannabis, synthetic cannabinoids act on cannabinoid CB₁ and CB₂ receptors in the brain and peripheral organs, mostly with a greater binding affinity than THC (2). Because synthetic cannabinoids are psychoactive, are not detected by routine cannabinoid urine screening methods, and when introduced are usually not scheduled illicit drugs, users consider these as legal cannabis alternatives that will not be detected by standard urine cannabinoid assays. More than 250 JWH compounds have been seized around the globe with major consumption in Europe (3,4), America (http://www.deadiversion.usdoj.gov/fed_regs/rules/2013/fr0412.htm), and Japan (5). Owing to their widespread abuse, regulatory authorities in Europe and the USA banned the manufacture, distribution, and possession of JWH-018, JWH-073, JWH-200, CP47,497, and HU-210, adding them to Schedule I illegal drugs. Synthetic cannabinoids also are associated with severe life-threatening toxicities, such as acute kidney damage or even death (6,7). However, mechanisms by which they act in the body to elicit toxicological responses remain unknown.

Although cannabimimetic effects are widely reported in online drug user forums, few clinical research studies of any synthetic cannabinoid compound exist, and their pharmacodynamic effects and pharmacokinetics remain incompletely characterized. The major barrier to conducting

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ABBREVIATIONS: AKB-48, *N*-(1-Adamantyl)-1-pentylindazole-3-carboxamide; CB, Cannabinoid; cps, Counts per second; CYP, Cytochrome P450; IDA, Information-dependent acquisition; LC-MS, Liquid chromatography mass spectrometry; MDF, Mass defect filter; MW, Molecular weight; NFLIS, National Forensic Laboratory Information System; THC, Delta 9-tetrahydrocannabinol; TOF, Time of flight; UDPGT, Uridine diphosphate glucuronosyltransferase; XICs, Extracted ion chromatograms.

controlled clinical studies with these compounds is the lack of toxicity data in preclinical species. A major problem with tying adverse events to causative agents is the lack of analytical methods to identify the specific synthetic cannabinoid producing the toxicity. When metabolism is unknown, determining targets for analysis is critical. Several liquid chromatography–tandem mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS) analytical methods are reported for detecting JWH-, AM-, WIN-, and RCS- series of synthetic cannabinoids in whole blood, urine, oral fluid, and hair (8–14). Many synthetic compounds share structural similarities such as a central indole ring attached by an amide or carbonyl linkage to the naphthyl/phenyl/adamantyl ring. Many include an *N*-pentyl side chain. Based on earlier studies (15), halogenation of the terminal carbon atom of the pentyl side chain was adopted by drug manufacturers to bypass legal issues and to increase receptor binding in the brain, e.g., AM-2201, a fluorinated analog of JWH-018. The chemical structures of some of these compounds are shown in Fig. 1.

Drug manufacturers continue to develop new unscheduled synthetic cannabinoids with numerous structural changes, but without loss of psychoactive potential. Clinical and forensic laboratories are constantly struggling to identify the latest new chemicals and to determine what the appropriate target analytes are and also are hampered by the lack of availability of commercial standard material, leading to poor identification of these drugs in biological fluids. Most synthetic cannabinoids are eliminated *via* CYP metabolism, with further glucuronidation by UDPGT (phase II enzymes). Oxidative metabolites and glucuronide conjugates predominate with negligible parent compound present in human urine (16–18). Thus, it is essential to characterize metabolic profiles of synthetic cannabinoids to identify target analytes that effectively document their consumption during clinical and forensic urine testing.

N-(1-Adamantyl)-1-pentylindazole-3-carboxamide (AKB-48), also known as APINACA, was identified in Japanese herbal blends in 2012 (19). The NFLIS registered 443 reports of AKB-48 usage from March 2010 to January 2013. The DEA announced that AKB-48 will be listed in the Schedule I category of illegal drugs from May 2013. AKB-48 has strong binding affinity,

although less than JWH-018, at the human CB₁ receptor compared to CB₂, based on data with recombinant human CB₁ and CB₂ membrane preparations (19). There are no *in vitro*, preclinical, or clinical studies characterizing AKB-48's pharmacological effects, potential toxicities, or pharmacokinetics. Based on the current knowledge of synthetic cannabinoids structurally similar to AKB-48, it is expected that AKB-48 metabolites will be important for identification of intake of this synthetic cannabinoid in urine.

AKB-48 is closely related to AB-001 (1-pentyl-3-(1-adamantoyl) indole), a synthetic cannabinoid found in herbal smoking blends in Ireland and Germany (20). Monohydroxylated metabolites with or without *N*-dealkylation were reported as major urinary metabolites of AB-001 in humans (20). To our knowledge, this is the first study to report the metabolic profile of AKB-48. Human hepatocytes were specifically selected over human liver microsomes to mimic the physiological liver environment, such as bile canalicular membrane, uptake and efflux transporters, and expression of phase II enzymes and essential cofactors, to aid in generating a realistic metabolic fingerprint of AKB-48.

Data were acquired by quadrupole–time-of-flight mass spectrometry coupled with high-performance liquid chromatography (QTOF-HPLC), and AKB-48 metabolites were identified with MetabolitePilot™ software. Tandem mass spectrometry with accurate mass measurement enabled structural elucidation of AKB-48 metabolites that can be targeted for documenting AKB-48 intake. These data are highly useful for clinical and forensic scientists to incorporate these spectra acquired from human hepatocyte metabolism studies into their spectral libraries to identify AKB-48 markers.

MATERIALS AND METHODS

Chemicals and Reagents

Cryopreserved human hepatocytes and thawing and incubation media were purchased from Celsis IVT (Baltimore, MD), and AKB-48 was obtained from Cerilliant (Round Rock, TX). Reagent grade acetonitrile and LC-MS grade acetonitrile

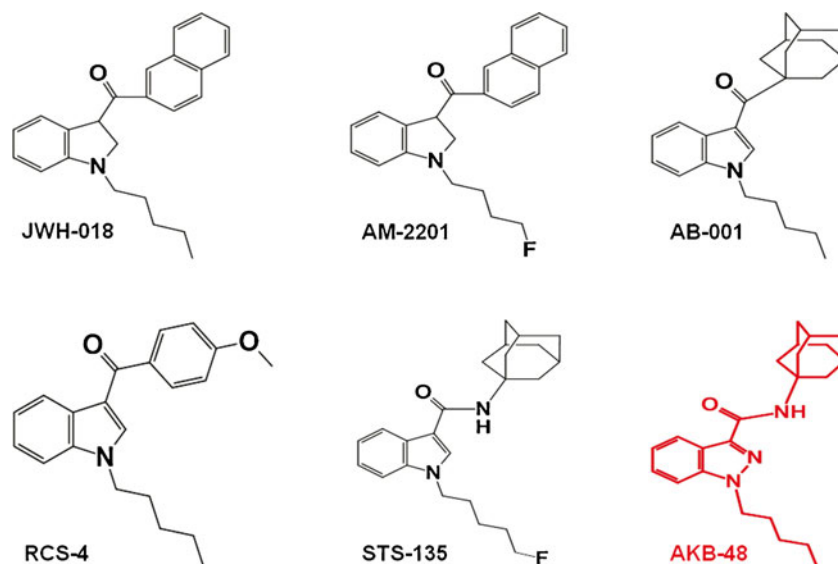


Fig. 1. Chemical structures of related synthetic cannabinoids

were supplied by Sigma-Aldrich (St. Louis, MO). Distilled water was prepared in house with an ELGA Purelab Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA).

Methods

Incubation of AKB-48 with Human Hepatocytes

Cryopreserved human hepatocytes pooled from three donors were thawed in a 37°C incubator. Hepatocytes were washed to remove dead cells and viability assessed with Trypan blue (0.4% v/v) exclusion method assuring greater than 80% viability. AKB-48 (10 µM) was incubated with human hepatocytes (1.3×10⁶ cells/mL) in 20-mL glass scintillation vials with constant shaking in an incubator set at 37°C. Diclofenac was incubated as a positive control to ensure metabolic capability under our experimental conditions. Samples were removed after 0, 1, and 3 h, and the reaction was stopped by protein precipitation with an equal volume of acetonitrile.

Sample Preparation

Samples were spun at 15,000×g for 5 min in a benchtop centrifuge (Eppendorf, Hamburg, Germany) to remove any cell debris or particulate matter. The supernatant was removed and injected onto the LC-MS/MS.

Instrumentation

Mass spectrometric analysis was performed on a 5600+ TripleTOF mass spectrometer (AB Sciex, Foster City, CA). Data were acquired with Analyst TF v. 1.6 and analyzed by MetabolitePilot™ v. 1.5 (AB Sciex, Foster City, CA). Chromatographic separation was achieved on a Shimadzu Prominence™ HPLC system consisting of two LC-20ADx pumps, a DGU-20A5R degasser, a SIL-20ACx autosampler, and a CTO-20 AC column oven (Shimadzu Corp., Columbia, MD).

LC-MS/MS Analysis

Chromatographic separation was performed on a Kinetex™ C18 column (100 mm×2.1 mm, 2.6 µm) fitted with a KrudKatcher Ultra HPLC In-Line Filter (0.5 µm×0.1 mm ID) (Phenomenex, Torrance, CA). The HPLC mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution was performed with 20% B for 1.5 min, increased to 50% B at 1.6 min, then ramped to 95% B over 10 min and held for 3 min before column re-equilibration at 20% for an additional 2.3 min for a total run time of 15.5 min. Column temperature was maintained at 40°C, and autosampler temperature was 4°C. A DuoSpray ion source was operated in positive electrospray ionization mode to acquire MS data by information-dependent acquisition (IDA) in combination with mass defect filter (MDF) and dynamic background subtraction (DBS). For IDA experiments, only spectra exceeding 100 cps were selected for the dependent scan, isotopes within 3 Da were excluded, and mass tolerance was 50 mDa. MDF-IDA criteria are shown in Table I; spectra were acquired scanning a mass range of 100–800 *m/z* followed by product ion scanning from 60 to 800 *m/z*. The declustering

potential was set at 80 V with a collision entrance potential of 10 V. Collision energy was set to 35 eV with a collision energy spread of ±15 eV. A standard calibration solution was run between every five samples using an automated calibrant delivery system to maintain instrument calibration.

Data Analysis

Data acquired by Analyst were analyzed by MetabolitePilot™ that employs peak-finding algorithms and data mining tools, such as common product ion and neutral loss, MDF, predicted biotransformation, and generic LC peak-finding to identify potential metabolites in the raw data. The processing parameters were adjusted to detect relevant metabolites that may play a role in drug disposition and toxicity. LC peak intensity threshold was set at 5,000 cps, MS at 3,500 cps, and MS/MS at 100 cps, respectively. Special attention was given to rule out any metabolites possibly generated as a result of in-source fragmentation or metabolite isotopes.

RESULTS AND DISCUSSION

Representative Extracted Ion Chromatogram and AKB-48 Mass Spectrum

Experimental conditions were successfully controlled by detection of diclofenac metabolites in hepatocyte samples. The AKB-48 mass spectrum is shown in Fig. 2a. The base peak at *m/z* 135 was identified as the adamantyl ion; collision energy optimization did not improve the intensity of other ions. Cleavage of the carbonyl-adamantane bond produced an *N*-pentylindazole acylium ion (*m/z* 215). Further loss of the pentyl side chain resulted in indazole acylium ion at *m/z* 145 (Fig. 2a).

Structural Characterization of AKB-48 Metabolites

The processing parameters in the MetabolitePilot™ software were set to collect relevant metabolites while filtering out background noise. Mass defect, neutral loss, and product ion filters also were selected, and mass tolerance was 50 ppm. A total of 17 metabolites were identified; representative extracted ion chromatograms (XICs) of parent AKB-48, monohydroxylation (M17), and dihydroxylations (M5, M7, M10, M11, M14, M15) are shown in Fig. 2b, and XICs of trihydroxylations (M1, M2, M4,

Table I. Mass Defect Filter (MDF)–Information-Dependent Acquisition (IDA) Criteria for Detection of Phase I and II AKB-48 Metabolites

Formula	MW (Da)	Width (Da)	Mass defect (mDa)
C ₂₃ H ₃₁ N ₃ O	365.2467	100	246.7129
C ₂₉ H ₃₉ N ₃ O ₇	541.2788	100	278.8011
C ₃₃ H ₄₈ N ₆ O ₇ S	672.3305	50	330.5202
C ₂₃ H ₃₁ N ₃ O ₄ S	445.2035	100	203.5286
C ₁₈ H ₂₁ N ₃ O	295.1685	100	168.4625
C ₁₃ H ₁₆ N ₂ O	216.1263	100	126.2633
C ₁₀ H ₁₇ N	151.1361	100	136.0997
C ₁₃ H ₁₇ N ₃ O	231.1372	100	137.1623
C ₁₀ H ₁₆	408.3756	100	375.6019

MW molecular weight

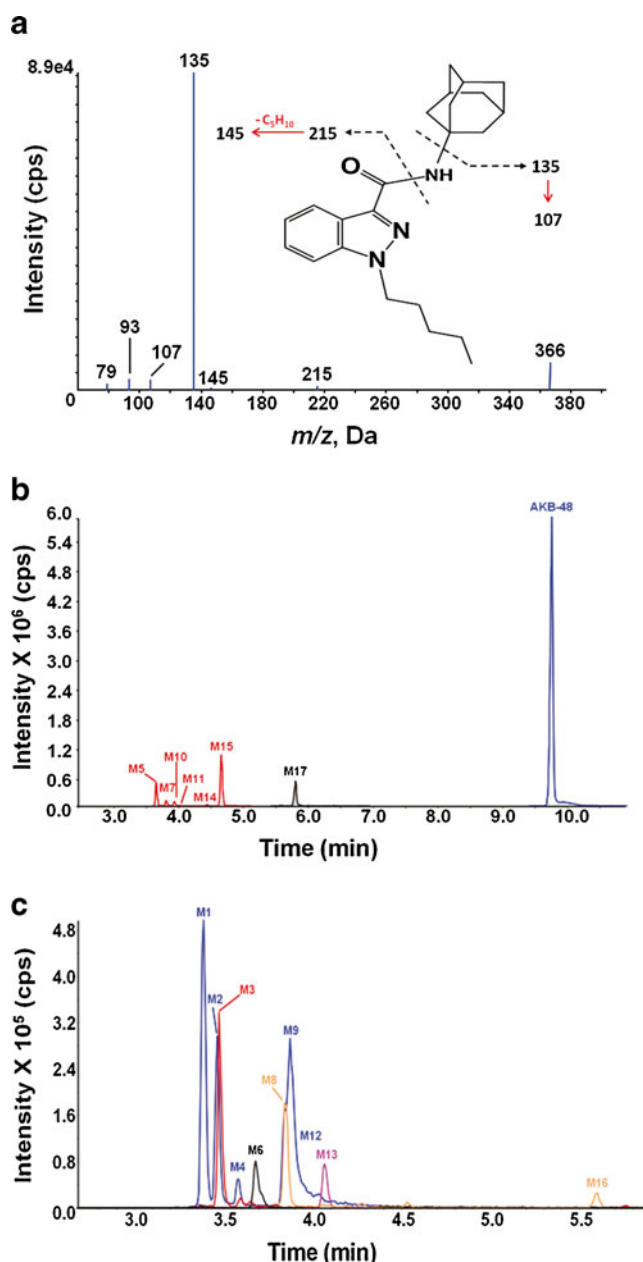


Fig. 2. **a** Product ion spectra of AKB-48, **b** extracted ion chromatograms (XIC) of AKB-48 and its mono- and dihydroxylation metabolites, and **c** XICs of trihydroxylation, mono- or dihydroxylation with glucuronidation, dioxygenation, and mono- or dihydroxylation with ketone formation AKB-48 metabolites. The chromatogram shows that major AKB-48 metabolites included monohydroxylation (M17), dihydroxylation (M5, M7, M10, M15), trihydroxylation (M1, M4, M9), mono- and dihydroxylation with glucuronide conjugation (M13 and M6), and dihydroxylation with ketone formation at the *N*-pentyl side chain (M3)

M9, M12), monohydroxylation with glucuronide conjugation (M13), dihydroxylation with glucuronide conjugation (M6), monohydroxylation with ketone formation (M16), dioxygenation (M8), and dihydroxylation with ketone formation (M3) are shown in Fig. 2c. Accurate mass data, elemental composition, diagnostic product ions, mass error, and retention times are listed in Table II. Parent AKB-48 eluted at 9.76 min, and the metabolites eluted between 3.39 and 5.80 min and were detected with a mass error of less than 2 ppm.

Monohydroxylation of AKB-48

M17 had a protonated molecular weight (MW) of 382, was hydroxylated on the aliphatic adamantane ring (m/z 151), had an unaltered *N*-pentylindazole moiety (m/z 145 and 215), and after water loss on the monohydroxylated adamantane ring produced m/z 133 (Fig. 3).

Dihydroxylation of AKB-48

We identified six dihydroxylated AKB-48 metabolites (M5, M7, M10, M11, M14, M15) with protonated MW of 398. M5 and M7 spectra contained m/z 133, 145, 151, 213, 230, 231, and 248, indicating one hydroxylation on the aliphatic adamantane ring and the other on the *N*-pentyl side chain (Fig. 4a). Water loss from m/z 151 and 231 produced m/z 133 and 213 fragments, respectively. M11 (similar to M5 and M7) spectrum contained m/z 133, 145, 151, 215, and 231 suggesting monohydroxylation on the aliphatic adamantane and the second hydroxylation on the *N*-pentyl side chain with unaltered indazole moiety. M10, M14, and M15 spectra contained m/z 133, 145, 149, 151, 167, 215, and 232 suggesting that both the hydroxyl groups were attached on the aliphatic adamantane ring with unaltered *N*-pentylindazole moiety, respectively (Fig. 4b). Water loss from m/z 151 and 167 produced M10 and M14, m/z 133 and 149 for M10 and M14, respectively. M15 spectrum contained m/z 131 and 149 due to loss of one or two water molecules from fragment ion m/z 167.

Trihydroxylation of AKB-48

Five trihydroxylated AKB-48 metabolites (M1, M2, M4, M9, M12) with a protonated MW of 414 were identified. M1, M2, and M4 spectra contained m/z 131, 145, 149, 167, 213, and 231 consistent with two hydroxyl groups attached on the aliphatic adamantane ring (m/z 167) and one on the *N*-pentyl side chain (m/z 231) and unchanged indazole moiety (m/z 145), respectively (Fig. 5a). Water loss from m/z 149, 167, and 231 produced m/z 131, 149, and 213, respectively. Metabolites M9 and M12 spectra contained m/z 145, 182, 215, and 232 suggesting that the three hydroxyl groups were attached to the aliphatic adamantane ring (m/z 182) with unaltered *N*-pentylindazole moiety (m/z 145) (Fig. 5b).

Mono- and Dihydroxylation of AKB-48 with Glucuronide Conjugation

M13, with a protonated MW of 558, contained m/z 133, 151, 309, and 327 indicating monohydroxylation (m/z 151) of the aliphatic adamantane ring followed by glucuronidation (m/z 327), and water loss produced m/z 133 and 309 fragments, respectively (Fig. 6a). The M6 spectrum, a dihydroxylated-glucuronide conjugate with protonated MW of 574, contained m/z 149, 167, 215, 343, and 398 suggesting dihydroxylation (m/z 167) of the aliphatic adamantane ring followed by glucuronidation (m/z 343) (Fig. 6b). Water loss from m/z 149 and 167 produced m/z 131 and 149, respectively. Glucuronide loss also was observed producing m/z 398 (Fig. 6b).

Table II. Accurate Mass Data, Elemental Composition, Diagnostic Product Ions, Mass Error, Retention Times (RT) and MS Peak Areas (1 and 3 h) of AKB-48 and Its Metabolites

Peak ID	Reaction	Elemental composition	Precursor ion (<i>m/z</i>)	Diagnostic product ions (<i>m/z</i>)	Mass error (ppm)	RT (min)	MS area, 1 h	MS area, 3 h
M1	Trihydroxylation	C ₂₃ H ₃₁ N ₃ O ₄	414.2391	145, 167, 213, 230, 248	0.9	3.39	3.83E+05	9.76E+05
M2	Trihydroxylation	C ₂₃ H ₃₁ N ₃ O ₄	414.2386	131, 145, 149, 167, 213, 231	-0.4	3.46	1.85E+05	5.88E+05
M3	Dihydroxylation and ketone formation	C ₂₃ H ₂₉ N ₃ O ₄	412.2232	145, 149, 167, 229	0.2	3.47	1.40E+05	5.70E+05
M4	Trihydroxylation	C ₂₃ H ₃₁ N ₃ O ₄	414.2386	145, 167, 215, 231, 248	-0.3	3.59	ND	1.31E+05
M5	Dihydroxylation	C ₂₃ H ₃₁ N ₃ O ₃	398.2441	133, 151, 213, 230, 248	0.7	3.65	7.23E+05	9.95E+05
M6	Dihydroxylation and glucuronide conjugation	C ₂₉ H ₃₉ N ₃ O ₉	574.2753	133, 149, 167, 215, 343, 398	-1.1	3.68	8.35E+04	2.15E+05
M7	Dihydroxylation	C ₂₃ H ₃₁ N ₃ O ₃	398.2437	145, 149, 167, 215, 232	-0.2	3.80	1.84E+05	2.98E+05
M8	Monohydroxylation and ketone formation	C ₂₃ H ₂₉ N ₃ O ₃	396.2279	133, 145, 151, 215, 229, 246	-0.8	3.84	2.17E+05	4.00E+05
M9	Trihydroxylation	C ₂₃ H ₃₁ N ₃ O ₄	414.2387	145, 164, 182, 215, 232	-0.1	3.87	6.01E+05	1.29E+06
M10	Dihydroxylation	C ₂₃ H ₃₁ N ₃ O ₃	398.2440	133, 145, 151, 167, 215, 232	0.4	3.93	1.04E+05	2.00E+05
M11	Dihydroxylation	C ₂₃ H ₃₁ N ₃ O ₃	398.2437	133, 145, 151, 215, 231	-0.4	4.01	ND	3.65E+04
M12	Trihydroxylation	C ₂₃ H ₃₁ N ₃ O ₄	414.2387	145, 182, 215, 232	0.0	4.04	ND	7.25E+04
M13	Monohydroxylation and glucuronide conjugation	C ₂₉ H ₃₉ N ₃ O ₈	558.2799	133, 151, 309, 327	-2.0	4.07	8.68E+04	1.86E+05
M14	Dihydroxylation	C ₂₃ H ₃₁ N ₃ O ₃	398.2437	145, 149, 167, 215, 232	-0.3	4.44	ND	7.38E+04
M15	Dihydroxylation	C ₂₃ H ₃₁ N ₃ O ₃	398.2443	145, 149, 167, 215, 232	1.2	4.66	1.89E+06	2.46E+06
M16	Dioxidation	C ₂₃ H ₂₉ N ₃ O ₃	396.2284	145, 147, 165, 215, 232	0.6	5.60	6.64E+04	6.72E+04
M17	Monohydroxylation	C ₂₃ H ₃₁ N ₃ O ₂	382.2487	133, 145, 151, 215	-0.6	5.80	1.35E+06	1.32E+06
Parent		C ₂₃ H ₃₁ N ₃ O	366.2547	77, 109, 135, 145, 215	1.9	9.76	1.62E+07	1.87E+07

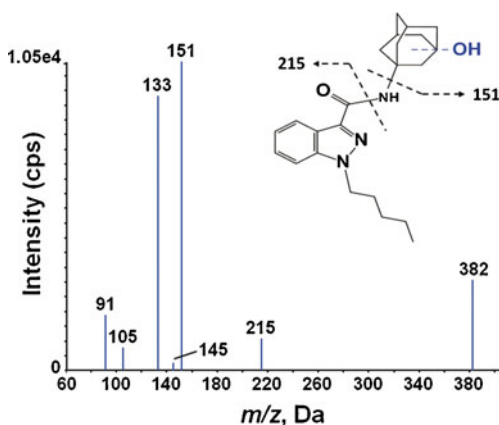
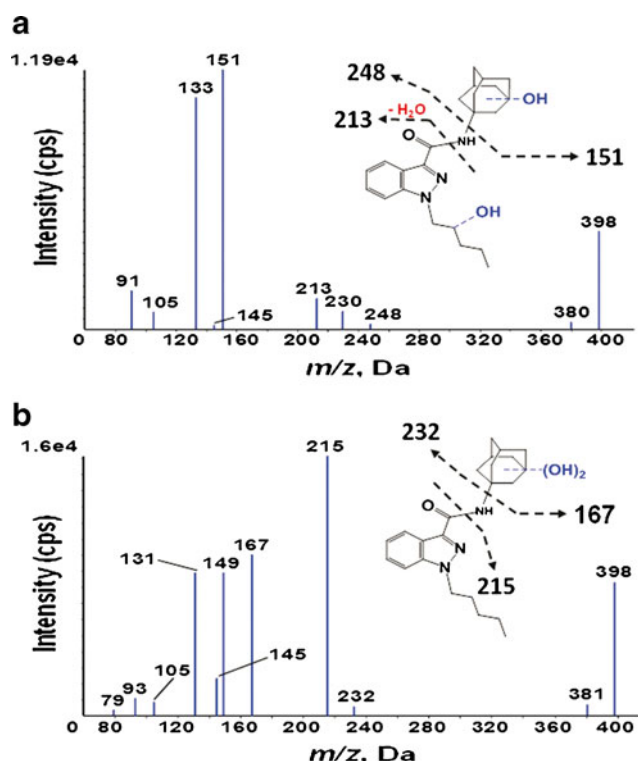
ND not detected

Mono- or Dihydroxylation and Ketone Formation of AKB-48

M8, protonated MW of 396, was consistent with monohydroxylation on the adamantane ring with ketone formation on the *N*-pentyl side chain. The spectrum contains *m/z* 133, 145, 151, 215, and 229 supporting monohydroxylation on the aliphatic adamantane ring (*m/z* 151) with oxidation of the *N*-pentyl side chain forming a ketone (*m/z* 229) and unaltered indazole moiety (*m/z* 145), respectively (Fig. 7a). Water loss from fragment *m/z* 151 produced fragment *m/z* 133.

We also identified M16, a dioxidation product of AKB-48 with protonated MW of 396 (Fig. 7b). The M16 spectrum contained *m/z* 145, 147, 165, 215, and 232 suggesting oxidation of

the aliphatic adamantane ring (*m/z* 147) and monohydroxylation (*m/z* 165) on the aliphatic adamantane ring with unaltered *N*-

**Fig. 3.** Product ion spectra of monohydroxylated (M17) AKB-48 metabolite**Fig. 4.** a Product ion spectra of dihydroxylated (M5 and M7) and b (M10, M14, and M15) AKB-48 metabolites

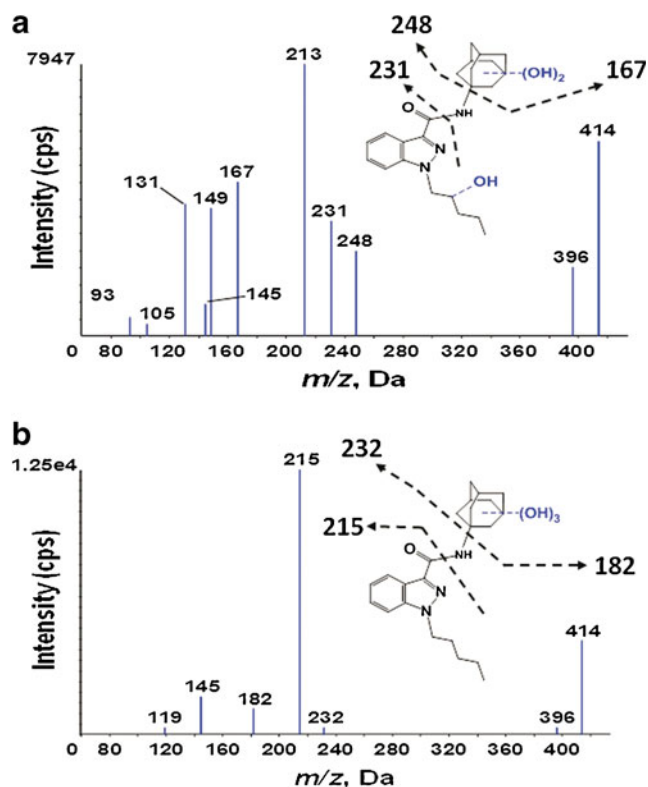


Fig. 5. **a** Product ion spectra of trihydroxylated (M1, M2, and M4) and **b** (M9 and M12) AKB-48 metabolites

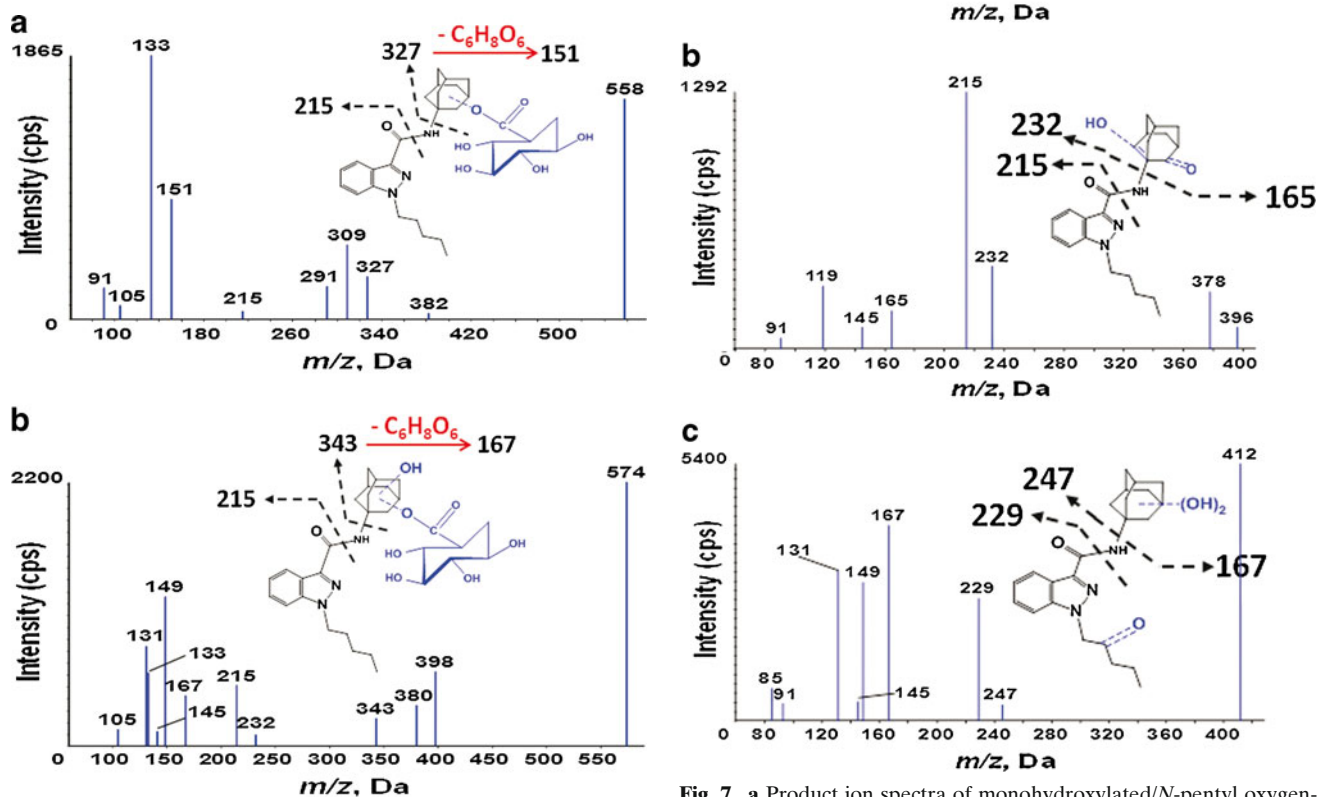


Fig. 6. **a** Product ion spectra of monohydroxylated/glucuronidated (M13) and **b** dihydroxylated/glucuronidated (M6) AKB-48 metabolites

pentylindazole moiety (m/z 145) (Fig. 7b). Water loss from m/z 396 produced m/z 378 (Fig. 7b).

Trace levels of AKB-48 metabolite M3 with protonated MW of 412 was identified, consistent with dihydroxylation and oxidation of the *N*-pentyl side chain subsequently forming a ketone. The M3 spectrum contained m/z 145, 167, and 229 suggesting dihydroxylation on the aliphatic adamantane ring (m/z 167), oxidation on the *N*-pentyl side chain (m/z 229) forming a ketone, and unaltered indazole moiety (m/z 145), respectively (Fig. 7c). Water loss from m/z 149 and 167 fragments formed m/z 131 and 149 fragments.

Correlation of Metabolites Formed Between 1- and 3-h Samples

A correlation profile of AKB-48 metabolites after 1- and 3-h hepatocyte incubation was generated with MetabolitePilotTM software. Fewer metabolites were found in the 1- than 3-h sample (Table II). Only 4 di- and 3-trihydroxylated metabolites

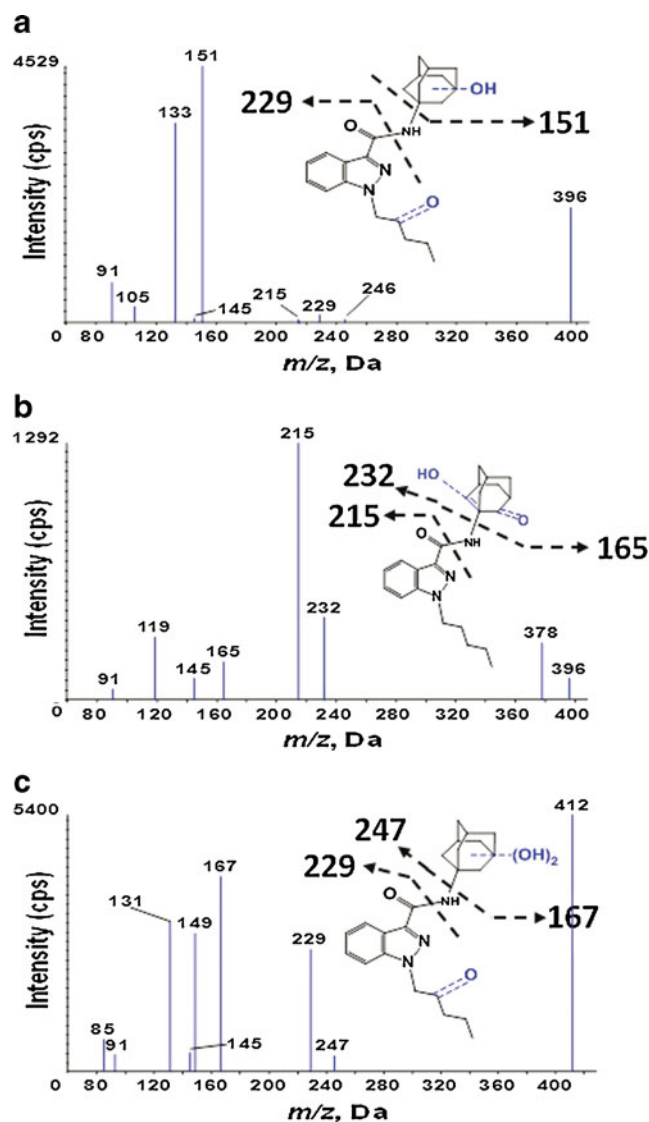


Fig. 7. **a** Product ion spectra of monohydroxylated/*N*-pentyl oxygenated (M8), **b** dihydroxylated (M16), and **c** dihydroxylated/*N*-pentyl oxygenated (M3) AKB-48 metabolites

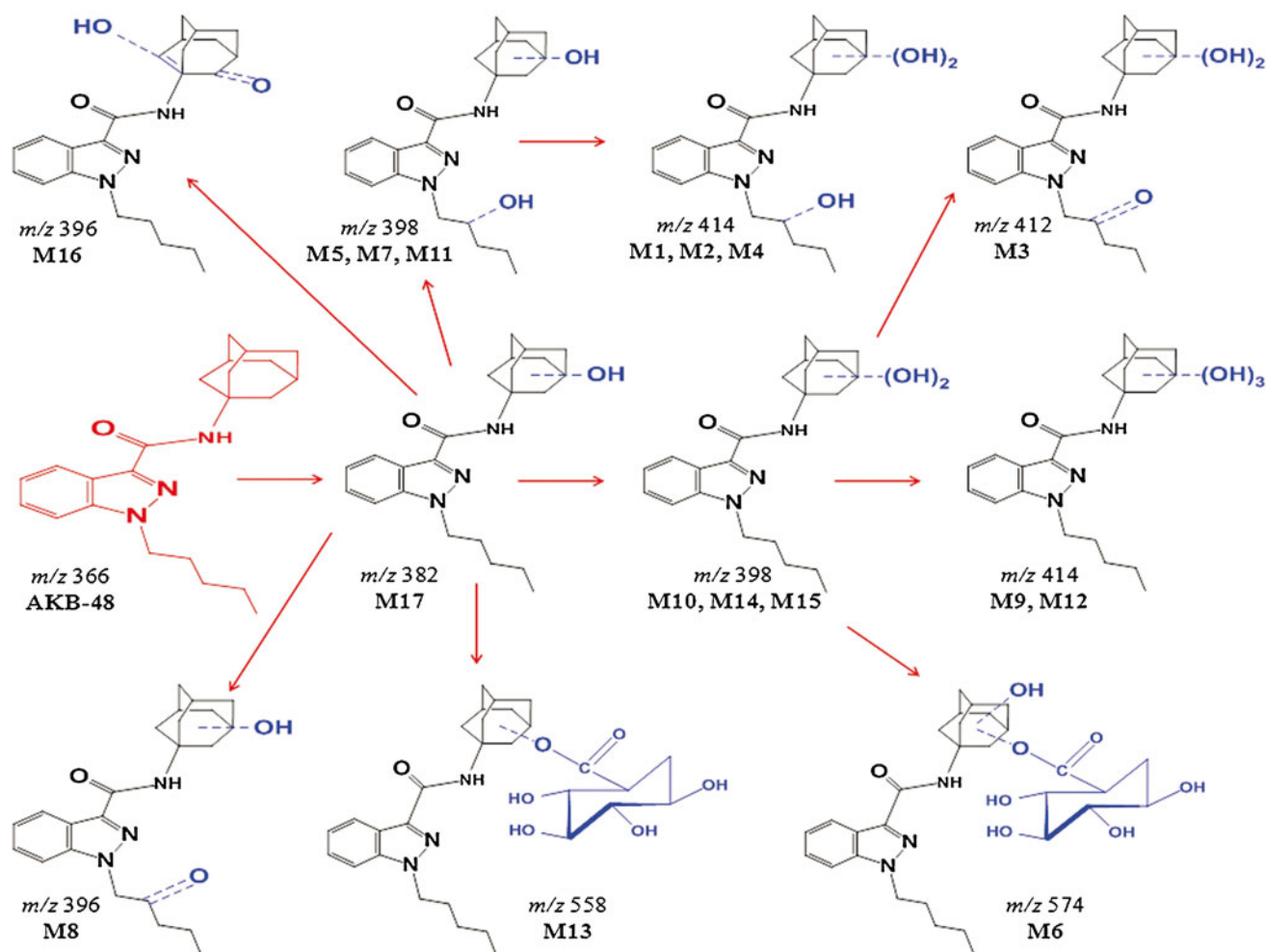


Fig. 8. Proposed metabolic pathways of AKB-48 in humans (m/z values correspond to protonated molecules)

were identified compared to the more complex profile at 3 h. Additionally, we observed increased peak areas for phase I and II metabolites after 3 h compared to 1 h, highlighting efficient metabolic capacity even after 3 h of incubation. However, we did not observe a significant difference in peak areas of parent AKB-48 between 1 and 3 h. This could be due to a high AKB-48 concentration (10 μ M) that may have saturated enzyme capacity; however, these high concentrations would never be achieved *in vivo*. Furthermore, based on data on other synthetic cannabinoids, it is likely that AKB-48 metabolites would predominate in urine. Metabolite profiling data obtained from our human hepatocyte study identify metabolites useful for identifying AKB-48 intake. Also, human clinical studies cannot be conducted until adequate *in vitro* and animal drug safety data are obtained to support an Investigational New Drug Application from the Food and Drug Administration. For forensic purposes, we propose that targeting metabolites identified during our hepatocyte studies along with parent AKB-48 in urine provides the best monitoring approach for AKB-48 intake.

Human AKB-48 Hepatic Metabolic Pathway

Human hepatocytes offer many advantages over human liver microsomes. Hepatocytes are a more relevant physiological

system to generate metabolite data for drugs of abuse due to the presence of phase I and II drug metabolizing enzymes, necessary cofactors, uptake and efflux drug transporters, and drug-binding proteins. Although hepatocyte studies are expensive to conduct, the high-quality metabolite profiling data generated justify their use over human liver microsomes. In addition to providing useful data to guide urine assay development for monitoring intake of AKB-48, metabolite samples from human hepatocytes can serve as references for structural confirmation of metabolites detected in human urine, although their definitive structures (*i.e.*, location of hydroxyl groups) remain undetermined.

The metabolic pathway of AKB-48 in humans is unknown. Based on the metabolite profile observed following human hepatocyte incubation, we propose a scheme for AKB-48 phase I and II metabolism in humans (Fig. 8). Our results showed that the major pathways are hydroxylation on the aliphatic adamantane ring followed by glucuronidation. Oxidation of the *N*-pentyl side chain forming a ketone appears to be a minor metabolic pathway. Based on the published studies for other synthetic cannabinoids, AKB-48 shares some common metabolic pathways, *e.g.*, mono- or dihydroxylation of the *N*-pentyl side chain or the indole moiety similar to JWH-018, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, or RCS-4 (17,21) or hydroxylation of the aliphatic adamantane ring similar to AB-001 (20), followed by glucuronidation (22,23). These data

improve detection of AKB-48 consumption in forensic and clinical toxicological testing and document the need for synthesis of AKB-48 metabolites as reference standards for developing quantitative methods. Additional analytical procedures such as nuclear magnetic resonance spectroscopy (for structural identification and confirmation of metabolites), isolation and purification of individual metabolite fractions by HPLC-UV analysis, or stereochemical analysis by GC-MS/MS will enable development of reference AKB-48 metabolites.

CONCLUSION

Eleven major AKB-48 metabolites were identified based upon a predefined metabolite filtering criterion of peak area $>1 \times 10^5$. Major metabolites included monohydroxylated (M17), dihydroxylated (M5, M7, M10, M15), trihydroxylated (M1, M4, M9), and mono- and dihydroxylated glucuronide conjugates (M13 and M6) and dihydroxylated with ketone formation at the *N*-pentyl side chain (M3). No glutathione or sulfate conjugates were found. An AKB-48 metabolic scheme was determined by analyzing *in vitro* human hepatocyte samples with high-resolution mass spectrometry employing MDF- and DBS-IDA. Human hepatocytes produce a complete range of metabolites as compared to human liver microsomes and better predict *in vivo* metabolism. Synthetic cannabinoid metabolites predominate in urine, and parent compounds are rarely present, making metabolite identification critical for documenting new synthetic cannabinoid intake. For the first time, markers of the new synthetic cannabinoid AKB-48 are available for synthesis of reference standards and development of analytical methods for clinical and forensic investigations.

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Conflict of Interest None

REFERENCES

- Dresen S, Ferreira N, Putz M, Westphal F, Zimmermann R, Auwarter V. Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds. *J Mass Spectrom.* 2010;45(10):1186–94.
- Wilson RI, Nicoll RA. Endocannabinoid signaling in the brain. *Science.* 2002;296(5568):678–82.
- Auwarter V, Dresen S, Weinmann W, Muller M, Putz M, Ferreira N. 'Spice' and other herbal blends: harmless incense or cannabinoid designer drugs? *J Mass Spectrom.* 2009;44(5):832–7.
- Lindigkeit R, Boehme A, Eiserloh I, Luebbecke M, Wiggermann M, Ernst L, *et al.* Spice: a never ending story? *Forensic Sci Int.* 2009;191(1–3):58–63.
- Uchiyama N, Kikura-Hanajiri R, Ogata J, Goda Y. Chemical analysis of synthetic cannabinoids as designer drugs in herbal products. *Forensic Sci Int.* 2010;198(1–3):31–8.
- Wikstrom M, Thelander G, Dahlgren M, Kronstrand R. An accidental fatal intoxication with methoxetamine. *J Anal Toxicol.* 2013;37(1):43–6.
- Thornton SL, Wood C, Friesen MW, Gerona RR. Synthetic cannabinoid use associated with acute kidney injury*. *Clin Toxicol (Phila).* 2013;51(3):189–90.
- Kneisel S, Auwarter V, Kempf J. Analysis of 30 synthetic cannabinoids in oral fluid using liquid chromatography-electrospray ionization tandem mass spectrometry. *Drug Test Anal.* 2013 (in press).
- Hutter M, Kneisel S, Auwarter V, Neukamm MA. Determination of 22 synthetic cannabinoids in human hair by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2012;903:95–101.
- 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.
- Shanks KG, Dahn T, Terrell AR. Detection of JWH-018 and JWH-073 by UPLC-MS-MS in postmortem whole blood case-work. *J Anal Toxicol.* 2012;36(3):145–52.
- Kneisel S, Speck M, Moosmann B, Corneillie TM, Butlin NG, Auwarter V. LC/ESI-MS/MS method for quantification of 28 synthetic cannabinoids in neat oral fluid and its application to preliminary studies on their detection windows. *Anal Bioanal Chem.* 2013;405:4691–706.
- Wohlfarth A, Scheidweiler KB, Chen X, Liu HF, Huestis MA. Qualitative confirmation of 9 synthetic cannabinoids and 20 metabolites in human urine using LC-MS/MS and library search. *Anal Chem.* 2013;85(7):3730–8.
- de Jager AD, Warner JV, Henman M, Ferguson W, Hall A. 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.
- Usami N, Okuda T, Yoshida H, Kimura T, Watanabe K, Yoshimura H, *et al.* Synthesis and pharmacological evaluation in mice of halogenated cannabidiol derivatives. *Chem Pharm Bull (Tokyo).* 1999;47(11):1641–5.
- Moller I, Wintermeyer A, Bender K, Jubner M, Thomas A, Krug O, *et al.* Screening for the synthetic cannabinoid JWH-018 and its major metabolites in human doping controls. *Drug Test Anal.* 2011;3(9):609–20.
- Hutter M, Broecker S, Kneisel S, Auwarter V. 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(1):54–65.
- Sobolevsky T, Prasolov I, Rodchenkov G. Detection of urinary metabolites of AM-2201 and UR-144, two novel synthetic cannabinoids. *Drug Test Anal.* 2013 (in press).
- Uchiyama N, Kawamura M, Kikura-Hanajiri R, Goda Y. URB-754: a new class of designer drug and 12 synthetic cannabinoids detected in illegal products. *Forensic Sci Int.* 2013;227(1–3):21–32.
- Grigoryev A, Kavanagh P, Melnik A. The detection of the urinary metabolites of 3-[(adamantan-1-yl)carbonyl]-1-pentylindole (AB-001), a novel cannabimimetic, by gas chromatography-mass spectrometry. *Drug Test Anal.* 2012;4(6):519–24.
- Wintermeyer A, Moller I, Thevis M, Jubner M, Beike J, Rothschild MA, *et al.* In vitro phase I metabolism of the synthetic cannabimimetic JWH-018. *Anal Bioanal Chem.* 2010;398(5):2141–53.
- Seely KA, Brents LK, Radominska-Pandya A, Endres GW, Keyes GS, Moran JH, *et al.* A major glucuronidated metabolite of JWH-018 is a neutral antagonist at CB1 receptors. *Chem Res Toxicol.* 2012;25(4):825–7.
- Chimalakonda KC, Bratton SM, Le VH, Yiew KH, Dineva A, Moran CL, *et al.* Conjugation of synthetic cannabinoids JWH-018 and JWH-073, metabolites by human UDP-glucuronosyltransferases. *Drug Metab Dispos.* 2011;39(10):1967–76.