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ORIGINAL ARTICLE

Simultaneous quantification of 37 synthetic cannabinoid metabolites in human urine by liquid chromatography-tandem mass spectrometry

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Abstract Despite efforts by legal authorities to control the abuse of synthetic cannabinoids, new derivatives have continually emerged on the market to circumvent regulations, and its abuse has become a threat to public health. Thus, development of analytical methods for confirming drug intake in biological fluids is essential to ensure effective drug control and to address further drug intoxication cases. Herein, a sensitive and reliable liquid chromatography–tandem mass spectrometry method was established and validated for the simultaneous determination of 37 synthetic cannabinoid metabolites, such as *N*-hydroxypentyl and carboxy metabolites, using 100 μl of urine. Urine specimens were treated by enzymatic hydrolysis and solid-phase extraction. Limits of detection for the evaluated drugs ranged from 0.1 to 1 ng/ml, and the linear range spanned from 0.25 or 1 to 100 ng/ml. Precision and accuracy bias were 1.4–12.1 % and −7.2–7.2 %, respectively. Matrix effects biases were in the range of 0.4 to 10.1 %, and extraction recoveries were 65–99 %. In addition, all analytes were stable under storage conditions of 4 °C and −20 °C for 14 days, and after three freeze–thaw cycles. The developed method was successfully applied to actual urine specimens obtained from synthetic cannabinoid users. The present method enabled simultaneous quantification of 37 synthetic cannabinoid metabolites, including their regioisomers, in urine in the field of clinical and forensic toxicology.

Keywords Synthetic cannabinoid · *N*-Hydroxypentyl metabolite · *N*-Pentanoic acid metabolite · LC–MS–MS · Urine · Regioisomer separation

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Introduction

Synthetic cannabinoids were originally synthesized to study the endocannabinoid system targeting CB_1 and CB_2 cannabinoid receptors for research purposes. However, herbal mixtures called "Spice" containing synthetic cannabinoids as synthetic additives have gained popularity globally since 2008 [1–4]. These mixtures are sold at head shops and gas stations and can be accessed via the Internet and even vending machines as incenses or air fresheners. Over time, these substances have been distributed and abused as legal alternatives to cannabis with little restriction. Since JWH-018 was first identified in herbal products [5], an enormous variety of compounds with cannabimimetic properties have continually been developed to circumvent drug control by legal authorities. Most synthetic cannabinoids possess higher binding affinity for cannabinoid receptors than Δ^9–tetrahydrocannabinol (THC) [6], and many of the synthetic cannabinoid metabolites themselves retain significant biological activities and may induce extensive pharmacological actions [7, 8]. Furthermore, the amount as well as the composition of synthetic cannabinoids has been found to vary for different herbal products (even those with the same brand name) [9]. These features may lead to unexpected, severe adverse symptoms including agitation, anxiety, paranoia, hallucination, psychosis, tachycardia, and seizures [10], and even a few fatal cases have been reported [11, 12].

The detection of synthetic cannabinoids and/or metabolites in biological matrices is of particular importance with respect to intoxication treatment as well as drug control. Immunoassay methods have been widely used as initial screening methods in the clinical and forensic fields. However, synthetic cannabinoids cannot be detected by most preliminary immunoassay screening procedures,

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which contribute to the prevalent use of these drugs. In spite of the recent development of immunoassays that target synthetic cannabinoids, they are still limited due to insufficient specificity and the continuous emergence of newly modified synthetic cannabinoids [13, 14]. Thus, some screening methods employing mass spectrometric techniques have been developed for selective and sensitive detection of synthetic cannabinoids and their metabolites in various biological matrices [15–19].

Urine is the specimen most commonly used to prove drug exposure. It is useful for drug screening due to its non-invasiveness, the longer retention time of drugs, and the applicability to routine drug testing. In general, after consumption of synthetic cannabinoids, the parent compounds are not detected in urine. Thus, determination of the intake of synthetic cannabinoids largely depends on metabolite detection. So far, several approaches for detection of synthetic cannabinoid metabolites in urine have been published, and recently presented nontargeted approaches enabled reduction of the time and effort required for detecting newly synthesized drugs [18, 20, 21]. High-resolution quadrupole time-of-flight mass spectrometry (QTOF–MS) screening methods have been developed as qualitative analytical techniques, facilitating easy update for new derivatives [22, 23]. More recently, Scheidweiler et al. [21] proposed liquid chromatography (LC)–QTOF–MS identifying method using SWATH™ (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra) acquisition for flexible inclusion of newly emerging compounds. Emergence of slightly modified derivatives of existing synthetic cannabinoids has become a recent trend, and drugs with structural similarities can produce common metabolites. Thus, quantification of detected metabolites as well as qualitative analysis of new analytes is critical for result interpretation. Previous studies have shown that fluorinated synthetic cannabinoids produce the same metabolites as those of their non-fluoro analogues through hydroxylation and carboxylation [24, 25]. Furthermore, our research group concluded that the concentration ratio of *N*-hydroxylated metabolites is a key factor for distinguishing the abuse of these drugs, especially because the availability of reference standards is limited [26, 27]. Because *N*-hydroxylated metabolites share the same ion transitions, careful column separation is required to quantify respective metabolites. However, baseline chromatographic separation of *N*-hydroxylated metabolites has been achieved in only a few studies [25–27].

Herein, we have developed and fully validated a comprehensive liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the determination of 37 synthetic cannabinoid metabolites from 17 parent compounds in urine, and demonstrate good chromatographic resolution of *N*-hydroxylated metabolites. In addition, actual urine samples submitted by the police or the prosecutor's office were analyzed to demonstrate the applicability of the developed method.

Materials and methods

Chemicals and materials

All chemicals were of analytical or HPLC grade. Sodium acetate trihydrate was purchased from Junsei Chemical (Tokyo, Japan); potassium dihydrogen phosphate from Samchun Pure Chemical (Pyeongtaek, Korea); β-glucuronidase (from *Helix pomatia*, type HP-2) from Sigma-Aldrich (St. Louis, MO, USA); ammonium formate and formic acid from Fluka Analytical (St. Louis, MO, USA); all metabolite standards and an internal standard (IS) from Cayman Chemical (Ann Arbor, MI, USA). Solid–phase extraction (SPE) was performed with a Clean Screen™ column (UCT, Bristol, PA, USA).

Instrumentation

The analysis was performed using an Agilent 1290 infinity UHPLC system (Agilent, Santa Clara, CA, USA) coupled to an AB Sciex 4,500 QTRAP tandem mass spectrometer from Applied Biosystems (Waltham, MA, USA). Data acquisition and quantification were performed using Analyst software version 1.6.2 (AB Sciex, Framingham, MA, USA).

Actual human urine specimens

Urine specimens were obtained from synthetic cannabinoid users apprehended by the police or the prosecutor's office from February 2013 to September 2014. The experimental procedures of this study were approved by the ethics committee at the National Forensic Service, Korea.

Sample preparation and analysis

One hundred microliters of urine was fortified with 20 µl of 100 ng/ml JWH-018 *N*-(5-hydroxypentyl) metabolite (*N*-5-OH M)-d_5 and 800 µl distilled water was added to it. After adding 60 µl of 2 M sodium acetate buffer (pH 4.5) and 40 µl of β-glucuronidase (approximately 10,000 units), the urine samples were incubated at 60 °C for 1 h for enzymatic hydrolysis. The samples were cooled to room temperature and centrifuged at 4,000 *g* for 5 min. The supernatant was subjected to SPE using an automatic equipment, Rapid Trace™ (Zymark, Hopkinton, MA, USA). The SPE columns were preconditioned by adding 1 ml of methanol, 1 ml of distilled water, and 1 ml of 0.1 M phosphate buffer (pH 6.0). The samples were loaded

onto the column, followed by washing with 1 ml of 0.1 N HCl and 1 ml of distilled water. Elution was sequentially performed with 1 ml of chloroform/acetone (1:1, v/v) and 1 ml of ethyl acetate/ammonia water (96:4, v/v). The eluates were evaporated to dryness under nitrogen at 45 °C and reconstituted in 150 µl of methanol/mobile phase A (1:1, v/v) prior to LC–MS–MS analysis.

Liquid chromatography

Chromatographic separation was carried out using a Zorbax Eclipse Plus C18 RRHD (2.1 × 100 mm, 1.8 µm) column from Agilent. Gradient elution was performed with 2 mM ammonium formate containing 0.2 % formic acid in water (A) and 2 mM ammonium formate containing 0.2 % formic acid in acetonitrile (B) at a flow rate of 0.3 ml/min. The initial condition was 10 % B, and the gradient was programmed as follows: 0–2 min to 50 % B, 2–8 min to 60 % B, 8–9 min to 95 % B and kept for 1 min. Finally, the initial condition was restored and held for 2 min to re-equilibrate the system. The total run time was 12 min. The column oven was maintained at 40 °C and the autosampler was set to 10 °C.

Mass spectrometry

The mass spectrometer was operated in the electrospray ionization (ESI) positive ion mode. The optimum source conditions were as follows: ion spray voltage, 5,500 V; curtain gas, 30 psi; collision gas, medium; source temperature, 600 °C; gas 1, 50 psi; gas 2, 55 psi. Detection of ions was performed in the multiple reaction monitoring (MRM) mode with two transitions for each analyte and one transition for JWH-018 *N*-5-OH M-d_5 (IS). The MRM transitions, retention times and parameters are listed in Table 1.

Method validation

A validation study was performed according to the method in the literature [28, 29]. Validation parameters included selectivity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy, matrix effects, extraction recovery, dilution integrity, carryover, hydrolysis efficiency, and stability.

Selectivity was assessed by analyzing blank urine specimens from 10 different individuals and by checking for potential endogenous interference. To evaluate potential interference from commonly used licit and illicit drugs (total 55 drugs) during quantification of the analytes, these drugs (500 ng/ml) were fortified into low quality control (QC) samples. The following is the list of drugs tested: 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, alprazolam, amphetamine, benzoylecgonine, bromazepam, buprenorphine, chlordiazepoxide, clobazam, clonazepam, cocaine, codeine, desalkylflurazepam, dextromethorphan, dextrorphan, diazepam, dihydrocodeine, diphenhydramine, doxylamine, ecgonine methyl ester, ephedrine, fentanyl, flunitrazepam, flurazepam, hydroxyalprazolam, hydroxymidazolam, hydroxytriazolam, ketamine, lorazepam, lormetazepam, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, methadone, methamphetamine, midazolam, 6-monoacetylcodeine, 6-monoacetylmorphine, morphine, nitrazepam, norcocaine, nordiazepam, norfentanyl, norketamine, norpethidine, oxazepam, oxycodone, pethidine, phentermine, pseudoephedrine, prazepam, temazepam, thebaine, triazolam, and zolpidem.

Linearity was determined by independent analysis of five sets of calibrators with at least seven concentrations across the linear range with a $1/x^2$ weighting factor. LODs and LOQs were evaluated by spiking drug-free urine with decreasing analyte concentrations. LOD was defined as the lowest concentration producing an acceptable peak shape and qualifier/quantifier ion ratio, and a signal-to-noise ratio of at least 3. LOQ was the lowest concentration satisfying LOD criteria with acceptable precision (<20 % coefficient of variation) and accuracy bias (within ±20 %). Intra- and interday precision and accuracy were determined from five replicates of urine samples at three QC concentrations (low, medium, and high) on five different days.

Matrix effect and recovery were assessed at two QC concentration levels (low and high) as proposed by Matuszewski et al. [29]. Briefly, three sets of samples spiked with analytes were prepared as follows: neat standard (set 1), urine extracts from five different sources fortified with analytes after extraction (set 2), and urine extracts from five different sources fortified with analytes before extraction (set 3). Matrix effects and extraction recovery were determined by comparing the analyte peak areas obtained from set 1, set 2, and set 3 as follows: matrix effect (%) = (peak area from set 2/peak area from set 1) × 100; extraction recovery (%) = (peak area from set 3/peak area from set 2) × 100.

Specimens with analyte concentrations exceeding the upper limit of linearity were diluted with blank urine. Thus, dilution integrity was evaluated by diluting a fortified urine sample containing 500 ng/ml of analytes with drug-free urine (1:10 dilution). Carryover was investigated by injecting extracted blank urine samples fortified at 500 ng/ml. To assess hydrolysis efficiency, blank urine was fortified with JWH-018 *N*-(5-hydroxypentyl) β-D-glucuronide (1,000 ng/ml) ($n = 3$), and the responses of the glucuronide with and without hydrolysis were compared.

To evaluate the stability of the analytes in urine samples under storage conditions, fortified urine samples ($n = 5$

Table 1 Multiple reaction monitoring transitions, retention times, and parameters for synthetic cannabinoid metabolites and internal standard

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Time (min)	DP (V)	EP (V)	CE (V)	CXP (V)
JWH-018 *N*-COOH M	372.1	155.2	5.59	106	10	29	4
		127.2		106	10	65	4
JWH-018 *N*-5-OH M	358.2	155.1	5.93	96	10	27	4
		127.1		96	10	57	4
JWH-018 *N*-4-OH M	358.1	155.2	6.08	96	10	29	4
		127.2		96	10	63	4
JWH-018 6-OH-indole M	358.3	155.2	8.56	111	10	33	4
		127.1		111	10	61	4
JWH-073 *N*-COOH M	358.0	155.2	5.15	101	10	31	4
		127.1		101	10	71	4
JWH-073 *N*-4-OH M	344.1	155.1	5.30	96	10	29	4
		127.1		96	10	65	4
JWH-073 *N*-3-OH M	344.1	155.1	5.82	101	10	29	4
		127.2		101	10	57	4
JWH-073 6-OH-indole M	344.3	155.0	7.13	111	10	29	4
		127.0		111	10	59	4
JWH-250 *N*-COOH M	366.1	121.1	4.69	91	10	27	4
		91.0		91	10	63	4
JWH-250 *N*-5-OH M	352.1	121.1	4.91	96	10	27	4
		91.1		96	10	59	4
JWH-250 *N*-4-OH M	352.1	121.1	5.01	91	10	27	4
		91.1		91	10	59	4
JWH-122 *N*-5-OH M	372.2	169.1	6.88	111	10	31	4
		141.2		111	10	59	4
JWH-122 *N*-4-OH M	372.1	169.1	7.06	121	10	29	4
		141.2		121	10	53	4
JWH-019 *N*-6-OH M	372.1	155.2	6.81	101	10	25	4
		127.1		101	10	63	4
JWH-210 *N*-5-OH M	386.2	183.1	8.14	121	10	29	4
		153.2		121	10	63	4
JWH-210 *N*-4-OH M	386.2	183.2	8.37	121	10	31	4
		153.2		121	10	61	4
JWH-081 *N*-5-OH M	388.1	185.2	6.44	111	10	29	4
		157.2		111	10	53	4
JWH-398 *N*-5-OH M	392.1	189.1	8.08	101	10	27	4
		126.1		101	10	93	4
JWH-398 *N*-4-OH M	392.1	189.0	8.30	126	10	27	4
		126.1		126	10	93	4
JWH-203 *N*-COOH M	370.2	125.1	5.29	80	10	38	4
		218.1		80	10	25	6
JWH-203 *N*-5-OH M	356.0	124.9	5.60	111	10	37	12
		186.0		111	10	23	6
JWH-203 *N*-4-OH M	356.1	124.9	5.71	101	10	37	12
		186.1		101	10	23	6
AM-2201 *N*-4-OH M	376.1	155.2	5.92	116	10	31	4
		127.2		116	10	59	4
AM-2201 6-OH-indole M	376.1	155.1	6.34	111	10	33	4
		127.1		111	10	65	4

Table 1 continued

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Time (min)	DP (V)	EP (V)	CE (V)	CXP (V)
MAM-2201 *N*-COOH M	385.9	169.1	6.41	121	10	29	4
		115.2		121	10	95	4
MAM-2201 *N*-4-OH M	390.1	169.2	6.85	126	10	33	4
		141.1		126	10	59	4
UR-144 *N*-COOH	342.2	125.2	7.06	101	10	25	4
		55.1		101	10	57	4
UR-144 *N*-5-OH M	328.2	125.2	7.54	111	10	27	4
		55.1		111	10	53	4
UR-144 *N*-4-OH M	328.1	125.2	7.73	96	10	25	4
		55.1		96	10	57	4
XLR-11 *N*-4-OH M	346.3	248.2	6.43	91	10	25	4
		144.1		91	10	45	4
AB-PINACA *N*-COOH M	360.9	315.9	3.01	86	10	21	10
		344.0		86	10	13	10
AB-PINACA *N*-4-OH M	347.0	302.1	3.04	81	10	21	8
		212.8		81	10	37	6
5F-AB-PINACA *N*-4-OH M	365.1	249.0	3.01	51	10	33	8
		320.0		51	10	21	10
AKB48 *N*-COOH M	396.0	134.9	8.12	91	10	27	12
		93.0		91	10	71	8
AKB48 *N*-5-OH M	382.0	135.0	8.80	111	10	27	12
		93.0		111	10	67	8
AKB48 *N*-4-OH M	382.1	135.1	8.96	106	10	29	12
		93.0		106	10	69	8
5F-AKB48 *N*-4-OH M	400.0	134.8	8.62	101	10	29	12
		92.9		101	10	75	10
JWH-018 *N*-5-OH M-d_5	363.07	155.1	5.89	91	10	25	4

The quantification transition is underlined

DP declustering potential, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential, *N-COOH M N*-pentanoic acid metabolite, *N-5-OH M N*-(5-hydroxypentyl) metabolite, *N-4-OH M N*-(4-hydroxypentyl) metabolite; for JWH-073: *N-COOH M N*-butanoic acid metabolite, *N-4-OH M N*-(4-hydroxybutyl) metabolite, *N-3-OH M N*-(3-hydroxybutyl) metabolite; for JWH-019: *N-6-OH M N*-(6-hydroxyhexyl) metabolite; *6-OH-indole M* 6-hydroxyindole metabolite

each) with low and high QC concentrations were stored at 4 °C and −20 °C for 14 days. Freeze–thaw stability was also tested by analyzing five replicates of QC urine samples at low and high concentrations before and after three freeze–thaw cycles. Autosampler stability was assessed by reinjecting low and high QC samples ($n = 5$) after 72 h of storage in the autosampler at 10 °C.

Results

Chromatographic separation

The chromatographic conditions were optimized to achieve good resolution and peak symmetry. Because *N*-hydroxylated metabolites share the same ion transitions and have similar retention times, complete separation on a column was first performed. A high resolution C_{18} column and the gradient condition described in the "Materials and methods" section were chosen through several trials and all of the evaluated regioisomers were fully separated on the chromatogram with acceptable accuracy and precision (Fig. 1).

Method validation

There was no endogenous interference originating from blank urine samples ($n = 10$) with the signals of the analytes or IS. The influence of other licit and illicit drugs on quantification of the analytes was also investigated, and the analytes at low QC concentrations could be quantified within 20 % deviation from the nominal concentration (data not shown).

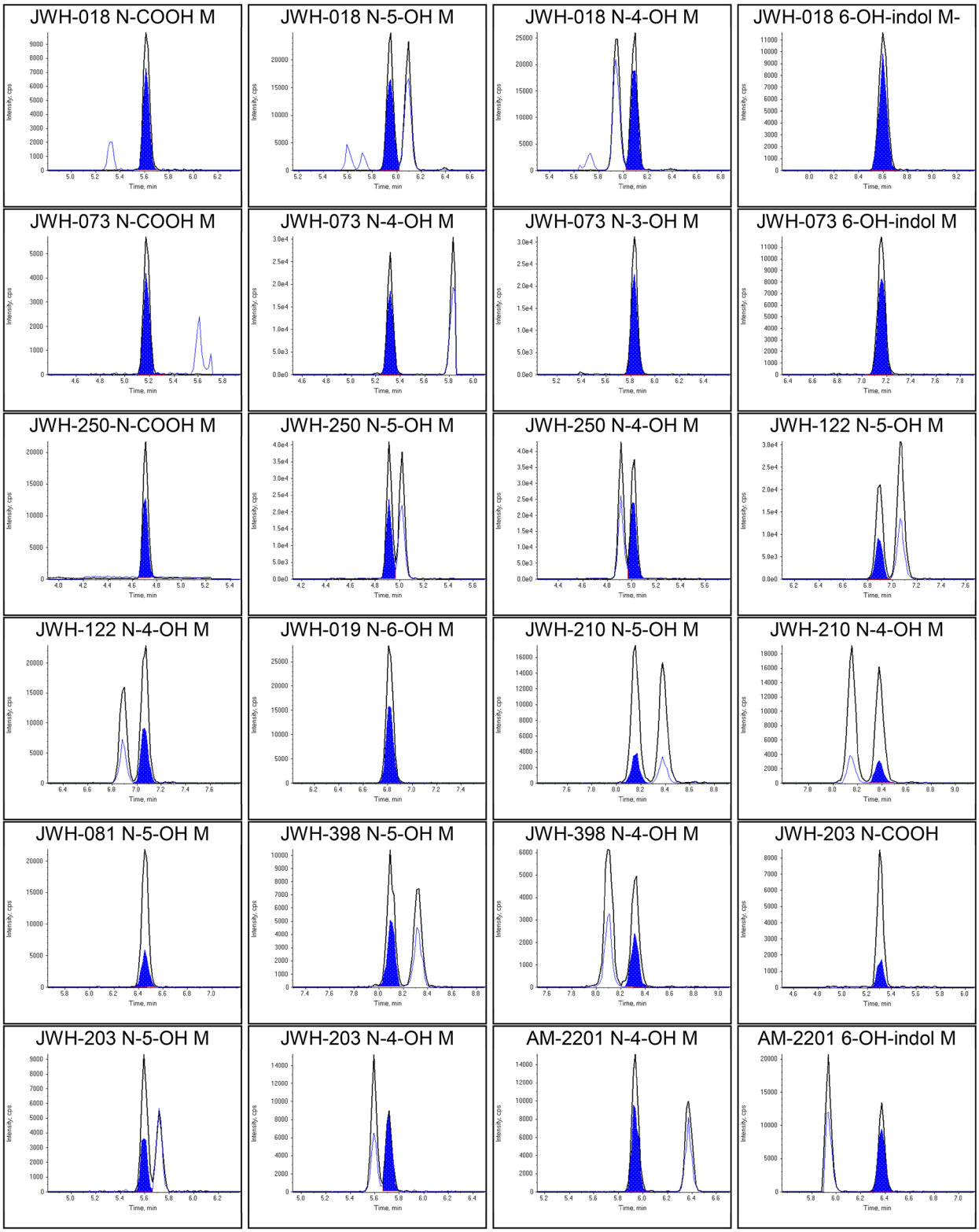

Fig. 1 Multiple reaction monitoring (MRM) chromatograms of synthetic cannabinoid metabolites in blank urine fortified at low quality control concentrations (0.5 or 2.5 ng/ml). Two MRM transitions were used for each analyte, choosing quantifier and qualifier transitions. The chromatographic peaks of analyte are indicated with colored shading of respective qualifier transition. Each pair of regioisomers can be differentiated each other by respective retention time on chromatogram due to sharing the same ion transitions, thus, corresponding regioisomer peaks are shown without shading

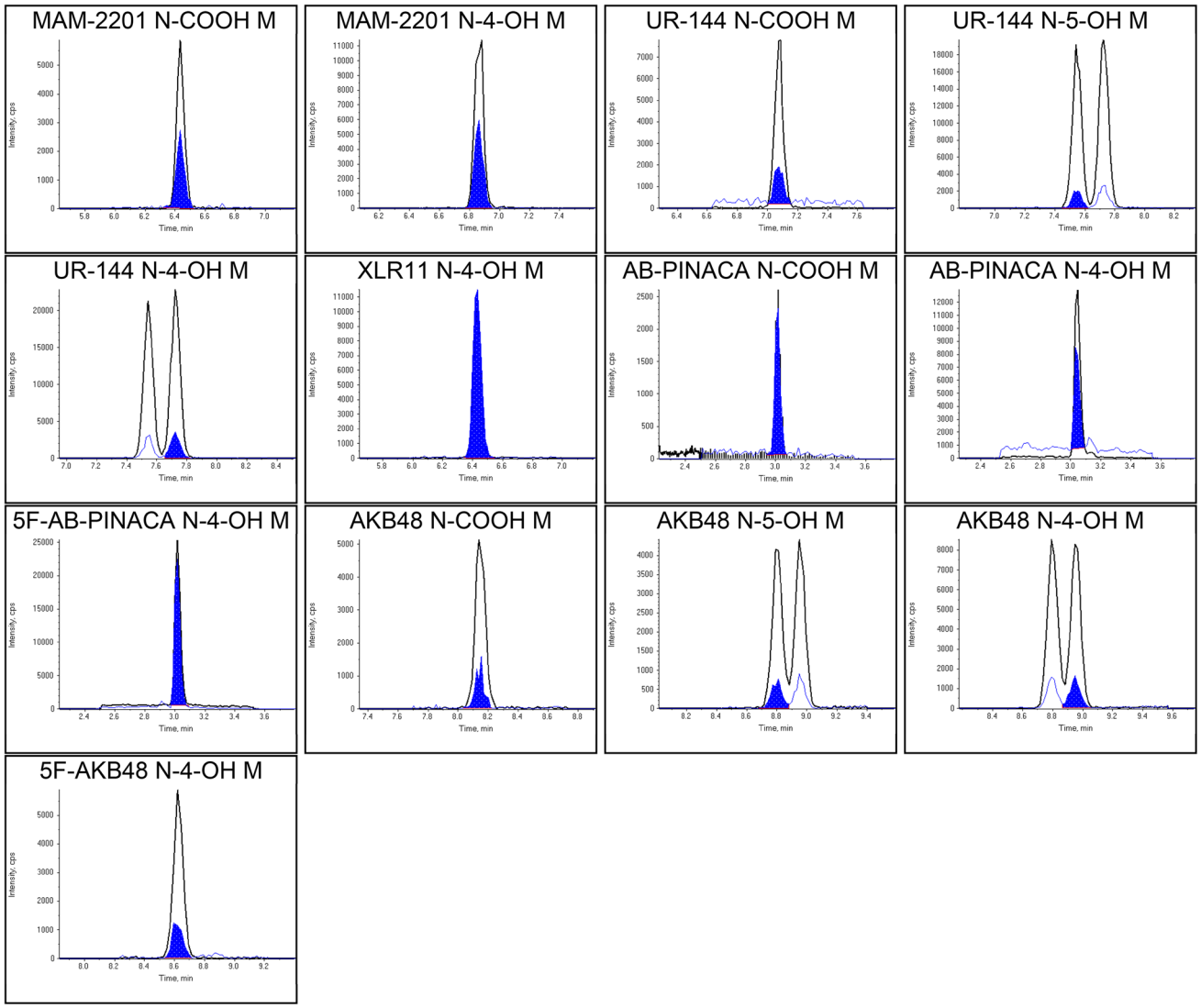

Fig. 1 continued

Linearity, LODs, and LOQs for all analytes are summarized in Table 2. Good linearity was achieved for all analytes within the range of 0.25 or 1–100 ng/ml. Average correlation coefficients (*r*) were greater than 0.99. LODs for 37 metabolites ranged from 0.1 to 1 ng/ml. LOQs were 0.25 ng/ml for all analytes, except for AB-PINACA *N*-pentanoic acid metabolite (*N*-COOH M), AB-PINACA *N*-(4-hydroxypentyl) metabolite (*N*-4-OH M), and 5F-AB-PINACA *N*-4-OH M where the LOQ was 1 ng/ml. Figure 1 shows the MRM chromatograms of blank urine sample fortified with analytes at low QC concentrations.

Precision and accuracy were expressed as coefficient of variation (CV) (%) and bias (%), respectively (Table 3). Intra- and interday precision and accuracy values satisfied the acceptance criteria at the evaluated QC concentrations for all analytes [28]. Intra- and interday precision were 1.4–12.1 % and 4.2–10.2 %, respectively. Intra- and interday accuracy bias ranged from −7.2 to 7.2 %.

Matrix effect and extraction recovery at low and high QC concentration levels are summarized in Table 4. Matrix effect derived from the presence of coeluting components from the matrix may affect ionization of the analytes. Mean values of matrix effect were 53 % (AB-PINACA *N*-4-OH M)–110 % (UR-144 *N*-COOH M). Variation of the relative matrix effect was found to be minimum among the samples obtained from different sources; i.e., CVs at 0.4–10.1 %, which were lower than the limit of 15 % proposed by Viswanathan et al. [30]. Extraction recoveries ranged from 65 % (MAM-2201 *N*-COOH M) to 99 % (5F-AB-PINACA *N*-4-OH M).

Table 2 Linearity, limit of detection (LOD), and limit of quantification (LOQ) for synthetic cannabinoid metabolites in human urine ($n = 5$)

Analyte	Calibration curve					LOD (ng/ml)	LOQ (ng/ml)
	Slope		Intercept		r		
	Mean	SD	Mean	SD			
JWH-018 *N*-COOH M	0.0273	0.0043	0.0023	0.0014	0.993	0.1	0.25
JWH-018 *N*-5-OH M	0.0644	0.0030	0.0036	0.0035	0.996	0.1	0.25
JWH-018 *N*-4-OH M	0.0758	0.0025	0.0059	0.0021	0.995	0.1	0.25
JWH-018 6-OH-indole M	0.0401	0.0075	0.0025	0.0021	0.997	0.1	0.25
JWH-073 *N*-COOH M	0.0167	0.0015	0.0012	0.0006	0.995	0.1	0.25
JWH-073 *N*-4-OH M	0.0651	0.0021	0.0062	0.0020	0.994	0.1	0.25
JWH-073 *N*-3-OH M	0.0822	0.0048	0.0062	0.0021	0.996	0.1	0.25
JWH-073 6-OH-indole M	0.0384	0.0065	0.0023	0.0017	0.996	0.1	0.25
JWH-250 *N*-COOH M	0.0484	0.0053	0.0038	0.0017	0.994	0.1	0.25
JWH-250 *N*-5-OH M	0.0919	0.0125	0.0070	0.0031	0.995	0.1	0.25
JWH-250 *N*-4-OH M	0.0946	0.0139	0.0056	0.0025	0.994	0.1	0.25
JWH-122 *N*-5-OH M	0.0590	0.0088	0.0050	0.0037	0.995	0.1	0.25
JWH-122 *N*-4-OH M	0.0628	0.0106	0.0050	0.0029	0.996	0.1	0.25
JWH-019 *N*-6-OH M	0.0743	0.0154	0.0064	0.0038	0.994	0.1	0.25
JWH-210 *N*-5-OH M	0.0596	0.0066	0.0053	0.0033	0.993	0.25	0.25
JWH-210 *N*-4-OH M	0.0508	0.0105	0.0033	0.0024	0.995	0.25	0.25
JWH-081 *N*-5-OH M	0.0580	0.0108	0.0034	0.0027	0.994	0.25	0.25
JWH-398 *N*-5-OH M	0.0201	0.0063	0.0020	0.0008	0.996	0.25	0.25
JWH-398 *N*-4-OH M	0.0177	0.0013	0.0014	0.0009	0.996	0.25	0.25
JWH-203 *N*-COOH M	0.0209	0.0042	0.0018	0.0010	0.991	0.25	0.25
JWH-203 *N*-5-OH M	0.0236	0.0018	0.0022	0.0011	0.995	0.1	0.25
JWH-203 *N*-4-OH M	0.0255	0.0026	0.0016	0.0008	0.996	0.1	0.25
AM-2201 *N*-4-OH M	0.0412	0.0069	0.0031	0.0013	0.995	0.1	0.25
AM-2201 6-OH-indole M	0.0357	0.0054	0.0027	0.0020	0.995	0.1	0.25
MAM-2201 *N*-COOH M	0.0157	0.0008	0.0019	0.0008	0.991	0.25	0.25
MAM-2201 *N*-4-OH M	0.0316	0.0045	0.0030	0.0013	0.994	0.1	0.25
UR-144 *N*-COOH	0.0276	0.0061	0.0012	0.0009	0.993	0.25	0.25
UR-144 *N*-5-OH M	0.0657	0.0133	0.0025	0.0022	0.996	0.25	0.25
UR-144 *N*-4-OH M	0.0710	0.0127	0.0048	0.0023	0.996	0.25	0.25
XLR-11 *N*-4-OH M	0.0278	0.0043	0.0025	0.0035	0.992	0.25	0.25
AB-PINACA *N*-COOH M	0.0010	0.0001	0.0005	0.0004	0.994	1.0	1.0
AB-PINACA *N*-4-OH M	0.0053	0.0010	0.0018	0.0007	0.996	1.0	1.0
5F-AB-PINACA *N*-4-OH M	0.0110	0.0016	0.0023	0.0009	0.998	1.0	1.0
AKB48 *N*-COOH M	0.0177	0.0023	0.0021	0.0008	0.993	0.25	0.25
AKB48 *N*-5-OH M	0.0146	0.0012	0.0015	0.0011	0.995	0.25	0.25
AKB48 *N*-4-OH M	0.0230	0.0038	0.0023	0.0013	0.998	0.25	0.25
5F-AKB48 *N*-4-OH M	0.0186	0.0024	0.0014	0.0004	0.995	0.25	0.25

SD standard deviation

Dilution integrity after tenfold dilution of the samples (500 ng/ml) with blank urine was acceptable (within 15 % of the nominal concentration). No carryover was observed after injection of the sample at 500 ng/ml. Hydrolysis efficiency was determined by comparing the changes in the peak areas of JWH-018 *N*-(5-hydroxypentyl) glucuronide and those of JWH-018 *N*-5-OH M with and without hydrolysis. More than 99 % cleavage of the glucuronide conjugate was achieved under the hydrolysis conditions employed in the present study.

Stability of the analytes in urine samples under various conditions was evaluated. As presented in Table 5, all analytes were stable under the given conditions, where the percentage recoveries ranged 86.3–101 % for 4 °C storage, 86.1–105 % for −20 °C storage, and 89.5–102 % after three freeze–thaw cycles.

Table 3 Intra- and interday precision and accuracy for synthetic cannabinoid metabolites in human urine

Analyte	Intraday ($n = 5$)						Interday ($n = 25$)					
	Precision (CV, %)			Accuracy (bias, %)			Precision (CV, %)			Accuracy (bias, %)		
	Low	Med	High	Low	Med	High	Low	Med	High	Low	Med	High
JWH-018 *N*-COOH M	7.6	6.2	5.7	−1.0	1.1	1.3	7.0	5.2	6.8	−3.4	3.1	−5.3
JWH-018 *N*-5-OH M	7.5	8.4	7.6	2.8	−0.6	1.1	6.1	6.5	7.7	3.2	2.6	−3.9
JWH-018 *N*-4-OH M	5.2	8.9	6.7	2.5	−0.5	2.4	7.0	5.5	6.5	0.6	1.4	−3.5
JWH-018 6-OH-indole M	7.4	9.8	6.1	−0.2	0.5	0.4	6.6	6.1	7.4	2.1	1.9	−2.1
JWH-073 *N*-COOH M	4.5	7.0	10.5	4.3	−2.1	1.7	7.5	5.5	8.6	−2.0	1.4	−4.2
JWH-073 *N*-4-OH M	2.5	9.2	5.5	−0.5	−0.4	−0.6	4.2	5.3	6.0	−0.2	1.5	−5.2
JWH-073 *N*-3-OH M	6.1	6.7	7.3	1.5	1.2	2.4	6.4	4.3	6.6	−0.5	2.7	−3.1
JWH-073 6-OH-indole M	8.6	9.7	5.2	−3.3	1.2	3.3	6.6	5.2	7.0	0.0	1.1	−3.5
JWH-250 *N*-COOH M	6.1	8.6	6.1	−3.8	1.8	−0.3	6.1	5.8	7.4	−4.3	2.2	−5.4
JWH-250 *N*-5-OH M	5.6	8.1	3.7	1.0	1.7	−1.9	6.0	4.9	7.0	−0.6	1.8	−5.5
JWH-250 *N*-4-OH M	3.7	8.6	5.1	5.0	1.5	0.4	5.5	5.1	7.3	0.9	1.5	−4.9
JWH-122 *N*-5-OH M	6.9	8.7	7.0	0.0	0.9	2.1	5.9	6.3	6.6	1.5	6.3	−3.1
JWH-122 *N*-4-OH M	4.8	8.9	6.2	0.2	0.4	2.0	4.7	5.2	6.4	1.9	5.3	−2.3
JWH-019 *N*-6-OH M	5.3	7.6	7.9	1.3	−2.1	−2.1	4.8	5.2	6.6	1.6	1.0	−3.3
JWH-210 *N*-5-OH M	6.6	8.7	6.3	−0.3	−2.6	−3.5	5.1	5.8	5.8	0.8	1.8	−3.0
JWH-210 *N*-4-OH M	4.6	9.9	5.3	0.0	−0.4	−0.7	5.5	6.7	5.5	−1.0	3.0	−1.0
JWH-081 *N*-5-OH M	10.9	8.1	5.8	−2.5	1.4	3.4	7.6	5.8	6.8	−1.2	1.0	−2.7
JWH-398 *N*-5-OH M	9.4	9.2	6.0	−1.7	−0.8	−2.0	7.1	6.2	5.9	1.8	2.9	−2.0
JWH-398 *N*-4-OH M	6.4	8.5	6.7	0.4	−1.5	0.2	6.4	6.8	5.8	−1.1	3.8	−0.7
JWH-203 *N*-COOH M	7.7	5.5	5.9	2.0	3.2	0.4	8.2	5.7	7.9	−2.4	5.5	−5.8
JWH-203 *N*-5-OH M	5.8	8.0	6.1	−0.8	3.1	4.7	6.6	5.8	7.0	−2.3	1.4	−3.1
JWH-203 *N*-4-OH M	6.0	6.3	4.2	1.0	−1.8	5.9	7.3	5.6	7.7	−0.8	1.8	−1.4
AM-2201 *N*-4-OH M	4.8	8.4	6.3	3.0	0.0	0.2	4.7	7.2	7.2	1.0	2.3	−3.6
AM-2201 6-OH-indole M	7.5	8.7	5.3	−5.3	−1.1	3.4	7.1	6.2	6.9	0.9	0.7	−1.0
MAM-2201 *N*-COOH M	7.4	5.0	6.4	−1.5	5.8	2.1	7.4	4.3	7.5	−3.9	4.9	−2.0
MAM-2201 *N*-4-OH M	4.2	8.5	5.9	2.0	−1.1	−0.1	5.0	5.5	5.6	0.2	2.5	−4.1
UR-144 *N*-COOH	5.3	9.0	4.6	2.1	2.7	0.4	6.9	5.4	8.5	−2.1	3.6	−5.5
UR-144 *N*-5-OH M	3.0	7.5	8.8	5.2	−2.0	−5.8	6.9	8.0	7.2	1.7	1.5	−1.5
UR-144 *N*-4-OH M	3.2	6.9	6.0	2.8	3.0	2.9	6.2	5.0	6.8	−1.4	3.7	−2.0
XLR-11 *N*-4-OH M	2.5	6.1	4.6	−1.4	−4.1	−5.7	10.2	6.8	6.8	−2.4	−2.1	−5.9
AB-PINACA *N*-COOH M	7.7	4.9	7.1	−1.0	−1.8	3.2	7.2	6.0	8.1	−0.2	5.4	−1.2
AB-PINACA *N*-4-OH M	2.8	6.6	5.8	7.2	4.1	5.4	5.1	5.8	6.3	5.3	5.6	0.4
5F-AB-PINACA *N*-4-OH M	1.4	6.4	8.7	4.6	0.4	−1.1	4.4	5.3	7.1	3.4	2.8	−3.3
AKB48 *N*-COOH M	12.1	6.3	7.6	−6.1	2.3	0.1	7.7	6.1	6.6	−7.2	5.0	−4.2
AKB48 *N*-5-OH M	6.4	10.3	7.4	2.2	0.5	0.0	5.4	6.4	5.3	0.5	3.2	−0.8
AKB48 *N*-4-OH M	2.8	6.6	5.8	6.4	4.1	5.4	6.2	4.8	6.8	1.4	4.3	0.4
5F-AKB48 *N*-4-OH M	7.3	9.1	7.4	5.5	−1.8	−0.4	6.3	6.4	5.3	0.3	1.7	−1.7

Low quality control concentrations were 0.5 ng/ml for all analytes except for AB-PINACA *N*-COOH M, AB-PINACA *N*-4-OH and 5F-AB-PINACA *N*-4-OH M (2.5 ng/ml). Median (Med) and high quality control concentrations were 25 and 70 ng/ml, respectively

CV coefficient of variation

Autosampler stability was assessed by placing the processed samples in an autosampler for 72 h; the percentage recoveries of all analytes were 92.4–103 % at low QC and 93.5–105 % at high QC (within ±15 % of the initial concentrations).

Application to actual specimens

The validated method was applied to urine specimens from suspected synthetic cannabinoid users arrested by the police or the prosecutor's office from February 2013 to September

Table 4 Matrix effect and extraction recovery for synthetic cannabinoid metabolites in human urine ($n = 5$)

Analyte	Matrix effect (%)				Extraction recovery (%)			
	Low		High		Low		High	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV
JWH-018 *N*-COOH M	93	10.1	87	4.6	73	4.9	70	2.4
JWH-018 *N*-5-OH M	94	6.1	90	1.5	87	5.8	80	1.0
JWH-018 *N*-4-OH M	92	6.0	92	2.4	89	3.3	84	4.0
JWH-018 6-OH-indole M	103	3.2	98	2.6	77	2.7	75	3.8
JWH-073 *N*-COOH M	83	9.2	84	4.3	80	6.6	73	3.6
JWH-073 *N*-4-OH M	91	7.6	86.	6.7	85	1.9	84	1.2
JWH-073 *N*-3-OH M	92	5.1	87	1.8	88	4.9	86	2.3
JWH-073 6-OH-indole M	101	4.5	97	1.7	79	2.8	78	3.2
JWH-250 *N*-COOH M	86	7.3	84	3.7	82	4.3	76	2.7
JWH-250 *N*-5-OH M	85	6.8	87	3.4	91	2.3	89	1.8
JWH-250 *N*-4-OH M	90	7.7	88	2.4	90	3.3	89	1.8
JWH-122 *N*-5-OH M	106	3.6	101	5.4	79	6.1	77	2.3
JWH-122 *N*-4-OH M	105	7.0	98	3.0	82	2.9	82	2.1
JWH-019 *N*-6-OH M	105	6.3	102	4.4	78	5.0	77	1.3
JWH-210 *N*-5-OH M	102	4.7	97	0.4	81	6.8	79	1.3
JWH-210 *N*-4-OH M	102	7.0	98	2.0	83	3.3	83	0.6
JWH-081 *N*-5-OH M	101	4.0	95	2.1	78	4.9	79	1.1
JWH-398 *N*-5-OH M	98	3.4	94	1.6	79	7.1	77	1.5
JWH-398 *N*-4-OH M	97	6.0	94	2.2	78	5.5	78	2.1
JWH-203 *N*-COOH M	96	3.7	90	2.4	73	7.8	72	2.7
JWH-203 *N*-5-OH M	90	7.2	87	4.7	90	5.7	82	3.5
JWH-203 *N*-4-OH M	93	7.3	90	1.8	85	5.7	86	1.8
AM-2201 *N*-4-OH M	94	7.1	88	2.8	83	2.4	84	2.6
AM-2201 6-OH-indole M	105	7.0	104	2.1	75	6.8	77	2.3
MAM-2201 *N*-COOH M	99	5.5	91	2.1	65	2.6	67	2.5
MAM-2201 *N*-4-OH M	103	3.3	102	5.9	83	4.0	77	0.5
UR-144 *N*-COOH	110	2.3	105	1.6	75	4.7	75	2.3
UR-144 *N*-5-OH M	96	4.4	94	2.3	90	2.0	85	2.0
UR-144 *N*-4-OH M	101	2.5	99	1.8	88	1.8	88	2.1
XLR-11 *N*-4-OH M	89	4.7	86	5.6	88	2.8	84	5.0
AB-PINACA *N*-COOH M	70	9.7	75	8.8	91	3.9	84	3.8
AB-PINACA *N*-4-OH M	53	4.7	58	8.6	94	4.7	89	3.7
5F-AB-PINACA *N*-4-OH M	67	6.8	75	6.3	99	6.5	94	4.0
AKB48 *N*-COOH M	99	5.2	91	1.6	73	4.6	71	0.9
AKB48 *N*-5-OH M	96	7.3	91	1.8	85	3.1	83	1.2
AKB48 *N*-4-OH M	101	3.5	98	1.2	86	4.0	84	2.5
5F-AKB48 *N*-4-OH M	99	2.3	91	2.1	83	3.9	86	1.5

Low quality control concentration were 0.5 ng/ml for all analytes except for AB-PINACA *N*-COOH M, AB-PINACA *N*-4-OH, and 5F-AB-PINACA *N*-4-OH M (2.5 ng/ml), and high quality control concentrations were 70 ng/ml

2014. Some positive cases and the results of identified drugs in the seized materials are presented in Table 6.

Discussion

In general, low dosages of synthetic cannabinoids are expected considering their high potency. Consequently, low concentrations (only a few ng/ml) of the metabolites in urine have been reported [25, 31], and the development of sensitive analytical methods is essential for comprehensive analysis of synthetic cannabinoid metabolites in urine. Yanes et al. [32] developed a LC–MS–MS method for the determination of the metabolites of JWH-018 and JWH-073 in 100 µl of urine with the linear range of 4–400 ng/ml. Scheidweiler and Huestis [33] developed a

Table 5 Stability data of synthetic cannabinoid metabolites in human urine ($n = 5$)

Analyte	14 Days at 4 °C (%)		14 Days at -20 °C (%)		3 Freeze–thaw cycles (%)	
	Low	High	Low	High	Low	High
JWH-018 *N*-COOH M	96.1	90.6	89.1	96.2	98.0	90.8
JWH-018 *N*-5-OH M	93.9	89.4	94.7	90.4	99.1	91.4
JWH-018 *N*-4-OH M	92.1	86.8	93.7	94.3	99.6	92.5
JWH-018 6-OH-indole M	91.3	88.4	91.3	91.5	97.7	94.0
JWH-073 *N*-COOH M	95.9	89.6	96.0	94.8	92.4	89.5
JWH-073 *N*-4-OH M	90.8	88.2	94.8	95.6	101	91.8
JWH-073 *N*-3-OH M	97.3	90.5	100	96.8	101	93.1
JWH-073 6-OH-indole M	93.4	90.0	93.2	99.3	97.7	93.5
JWH-250 *N*-COOH M	93.8	90.8	91.7	95.6	94.3	90.2
JWH-250 *N*-5-OH M	96.2	90.2	94.8	97.4	94.1	93.4
JWH-250 *N*-4-OH M	98.3	90.5	101	96.5	95.0	92.2
JWH-122 *N*-5-OH M	91.1	89.1	92.9	98.6	95.5	92.7
JWH-122 *N*-4-OH M	90.6	88.6	93.9	96.3	100	93.3
JWH-019 *N*-6-OH M	92.5	87.8	94.4	95.2	95.3	93.5
JWH-210 *N*-5-OH M	90.7	86.3	94.4	90.6	96.9	92.2
JWH-210 *N*-4-OH M	91.0	89.0	94.2	92.1	96.8	92.3
JWH-081 *N*-5-OH M	90.5	89.1	89.5	97.5	96.6	93.5
JWH-398 *N*-5-OH M	89.2	88.0	89.9	92.3	98.3	91.4
JWH-398 *N*-4-OH M	93.4	88.5	90.2	92.9	94.5	95.1
JWH-203 *N*-COOH M	98.0	92.4	96.8	96.4	97.4	92.9
JWH-203 *N*-5-OH M	94.0	96.9	92.3	101	95.8	92.8
JWH-203 *N*-4-OH M	94.5	92.5	96.7	100	94.9	90.9
AM-2201 *N*-4-OH M	97.8	90.1	93.7	90.3	98.9	93.1
AM-2201 6-OH-indole M	91.7	90.1	93.0	98.4	99.0	94.2
MAM-2201 *N*-COOH M	95.5	90.8	91.0	98.2	95.7	92.6
MAM-2201 *N*-4-OH M	95.2	91.3	97.0	93.8	102	93.6
UR-144 *N*-COOH	98.4	93.6	97.2	98.7	90.5	93.1
UR-144 *N*-5-OH M	99.6	92.4	105	97.1	96.3	92.9
UR-144 *N*-4-OH M	97.7	91.5	101	102	96.8	93.9
XLR-11 *N*-4-OH M	89.2	93.3	88.8	98.8	90.6	92.9
AB-PINACA *N*-COOH M	92.1	93.7	101	103	95.2	92.1
AB-PINACA *N*-4-OH M	96.3	95.2	97.3	104	99.4	94.7
5F-AB-PINACA *N*-4-OH M	101	94.9	102	99.9	92.7	92.2
AKB48 *N*-COOH M	91.4	90.0	86.1	94.4	98.0	94.8
AKB48 *N*-5-OH M	92.3	90.0	94.8	94.9	101	96.3
AKB48 *N*-4-OH M	96.3	95.2	97.3	104	100	95.2
5F-AKB48 *N*-4-OH M	94.7	88.9	93.8	95.1	97.3	100

Low quality control concentrations were 0.5 ng/ml for all analytes except for AB-PINACA *N*-COOH M, AB-PINACA *N*-4-OH, and 5F-AB-PINACA *N*-4-OH M (2.5 ng/ml), and high quality control concentrations were 70 ng/ml

comprehensive analytical method for 20 synthetic cannabinoids and 21 corresponding metabolites in 200 µl of urine with LODs of 0.1–0.5 ng/ml. However, it was not possible to distinguish isomeric *N*-hydroxylated metabolites and the metabolites were semi-quantified without assigning the hydroxyl group position. Hutter et al., [25] achieved chromatographic resolution of regioisomers of JWH-018 and JWH-073 metabolites and suggested a urinary marker to distinguish between JWH-018 and AM-2201 intake. The present method includes as many as 37 target metabolites, including recently emerging synthetic cannabinoids, and shows good sensitivities for all metabolites at low nanogram levels (LODs 0.1–1 ng/ml) using only 100 µl of urine. A total of nine pairs of positional isomers could be chromatographically separated with the present method (Fig. 1).

Among the actual specimens evaluated, specimens 1–3 were from the same case, in which both XLR-11 and 5F-AKB48 were identified in the seized materials. None of the specific metabolites for XLR-11 and 5F-AKB48

Table 6 Quantification of synthetic cannabinoid metabolites in actual human urine specimens

Specimen no.	Detected metabolites (ng/ml)	Seized material	
		Type	Detected drug
1	UR-144 *N*-COOH M (10.8), UR-144 *N*-5-OH (4.0), AKB48 *N*-5-OH M (2.0), AKB48 *N*-COOH M (0.8)	Smoking apparatus	XLR-11, 5F-AKB48
2	UR-144 *N*-COOH M (38), UR-144 *N*-5-OH (10.3), AKB48 *N*-5-OH M (0.7), AKB48 *N*-COOH M (0.4)		
3	UR-144 *N*-COOH M (8.6), UR-144 *N*-5-OH (3.0), AKB48 *N*-5-OH M (1.3), AKB48 *N*-COOH M (<LOQ)		
4	JWH-122 *N*-5-OH M (7.4), JWH-122 *N*-4-OH M (0.3), MAM-2201 *N*-COOH M (<LOQ), MAM-2201 *N*-4-OH M (<LOQ), UR-144 *N*-COOH M (18.9), UR-144 *N*-5-OH (3.3)	Dried leaves	MAM-2201, XLR-11, THC
5	AB-PINACA *N*-COOH M (420), AB-PINACA *N*-4-OH M (20.1), 5F-AB-PINACA *N*-4-OH M (9.2)	Cigarette butt	5F-AB-PINACA, nicotine

(i.e., XLR-11 *N*-4-OH M and 5F-AKB48 *N*-4-OH M) were detected in the specimens. Instead, certain metabolites of UR-144 and AKB48 (non-fluoro analogues of XLR-11 and 5F-AKB48, respectively) were identified. Carboxylated and *N*-5-hydroxylated metabolites were detected in all three specimens, whereas *N*-4-hydroxylated metabolites were not found. In our previous metabolism study of AM-2201 and MAM-2201, it was found that fluoro analogs produced mainly *N*-5-hydroxylated metabolites with generation of less *N*-4-hydroxylated metabolites through oxidative defluorination, whereas the converse was observed for non-fluoro analogs. In accordance with these findings, it can be inferred that the detected metabolites of UR-144 and AKB48 were produced after XLR-11 and 5F-AKB48 intake. Specimen No. 4 was obtained from a suspect possessing dried leaves containing MAM-2201 and XLR-11 with THC as a co-ingredient. JWH-122 *N*-5-OH M, the major metabolite of MAM-2201, was identified along with trace amounts of other metabolites. Similar to the case of specimens 1–3, the presence of UR-144 *N*-COOH M and UR-144 *N*-5-OH M with the absence of UR-144 *N*-4-OH M indicated XLR-11 intake. In the case of specimen No. 5, a cigarette butt containing 5F-AB-PINACA and nicotine was submitted by the police. The urine specimen contained AB-PINACA *N*-COOH M and AB-PINACA *N*-4-OH M along with 5F-AB-PINACA *N*-4-OH M, a characteristic metabolite of 5F-AB-PINACA. Similar to other fluorinated synthetic cannabinoids, it is expected that metabolism of 5F-AB-PINACA should produce a higher quantity of AB-PINACA *N*-5-OH M than AB-PINACA *N*-4-OH M. Because no reference standard of AB-PINACA *N*-5-OH M was commercially available at the time of method development, it was not possible to differentiate between AB-PINACA *N*-5-OH M and AB-PINACA *N*-4-OH M in specimen No. 5. However, there is a high probability that the specimen contained more AB-PINACA *N*-5-OH M, a presumed major metabolite of 5F-AB-PINACA, than AB-PINACA *N*-4-OH M.

Since late 2012, classical synthetic cannabinoids such as naphthoylindoles derivatives have been replaced by new compounds including adamantylindazoles and aminocarbonylindazoles. As shown in Table 6, 5F-AKB48 and 5F-AB-PINACA have recently appeared instead of classical synthetic cannabinoids such as JWH-018 or JWH-073. Another noticeable feature is the increase of fluorinated derivatives as presented in the aforementioned cases. These results are in accordance with a recent report on the trends of synthetic cannabinoids identified in Korea [3].

Conclusions

An LC–MS–MS method was developed and validated for simultaneous determination of 37 metabolites from 17 synthetic cannabinoids, including recently emerging drugs in urine. The present method showed satisfactory selectivity and sensitivity using a small volume of urine. In addition, all positional isomers were completely separated (Fig. 1), which enables discrimination of intake of structurally similar synthetic cannabinoids. The proposed method was successfully applied to actual urine specimens, and the results of metabolite analysis were consistent with the detected drugs in the seized materials. Synthetic cannabinoid abuse represents one of the major risks to the public health. The proposed method will be useful as a routine screening tool for the detection of the drugs in biological fluids in forensic and clinical cases.

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Conflict of interest There are no financial or other relation that could lead to a conflict of interest.

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