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ORIGINAL ARTICLE

Sensitive identification and quantitation of parent forms of six synthetic cannabinoids in urine samples of human cadavers by liquid chromatography–tandem mass spectrometry

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Abstract Human urine samples are easier to obtain than human blood samples due to noninvasiveness. The urine levels of synthetic cannabinoids (SCs) in unchanged forms, however, are usually much lower than their blood and tissue levels and cannot be detected in most cases. Therefore, in the present work a sensitive analytical method was devised for the determination of urine levels of six SCs in unchanged forms such as *N*-(1-amino-3-methy-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide (AB-PINACA), *N*-(1-amino-3-methy-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (AB-FUBINACA), *N*-[(1*S*)-1-(1-aminocarbonyl)-2-methyl-propyl]-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide (AB-CHMINACA), *N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide (MAB-CHMINACA), methyl-2-[1-(5-fluoropentyl)-1*H*-indazole-3-carboxamido]-3-methylbutanoate (5F-AMB) and methyl-2-[1-(5-fluoropentyl)-1*H*-indazole-3-carboxamido]-3,3-dimethylbutanoate (5F-ADB). These SCs were extracted from urine via liquid–liquid extraction. The identification and quantitation were performed by a relatively new type of an instrument for liquid chromatography–tandem mass spectrometry. The limits of detection were as low as 3–8 pg/mL, and the quantitation range was 10–1000 pg/mL using 400 µL of urine. The urine levels of AB-PINACA and AB-FUBINACA of victim 1 were 23 and 10 pg/mL, those of AB-CHMINACA and 5F-AMB of victim 2 were 239 and 19 pg/mL, and those of MAB-CHMINACA and 5F-ADB of victim 3 were 229 and 19 pg/mL, respectively. To our knowledge, this is the first report dealing with successful analysis of low levels of parent synthetic cannabinoids in authentic human urine specimens.

Keywords AB-PINACA · AB-FUBINACA · AB-CHMINACA · MAB-CHMINACA · Urine · Sensitive tandem mass spectrometry

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Introduction

A number of new compounds have been detected in the world since the first appearance of a psychotropic synthetic cannabinoid (SC) JWH-018 in the drug market in 2008 [1, 2]. Their potential toxicity and pharmacokinetic properties have not been well examined yet. Furthermore, the concentrations of these compounds varied from package to package. Different compounds are sold using the same brand names; several compounds are frequently mixed. Among them, *N*-(1-amino-3-methy-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide (AB-PINACA) and *N*-(1-amino-3-methy-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (AB-FUBINACA) were firstly identified and reported in 2013 [3]; methyl-2-[1-(5-fluoropentyl)-1*H*-indazole-3-carboxamido]-3-methylbutanoate (5F-AMB) and *N*-[(1*S*)-1-(1-aminocarbonyl-2-methyl-propyl)-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide (AB-CHMINACA) were reported in 2014 [4]; methyl-2-[1-(5-fluoropentyl)-1*H*-indazole-3-carboxamido]-3,3-dimethylbutanoate (5F-ADB) [5] and *N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1*H*-indazole-3-

carboxamide (MAB-CHMINACA) [6] were reported in 2015. The structures of these SCs are shown in Fig. 1.

The SCs distributed in drug markets are so divergent that the reports on forensic toxicology case studies for each of the six SCs were rather limited as follows. Shanks et al. [7] reported two fatalities where blood concentration of AB-PINACA was 32.8 ng/mL in one male and 12.2 ng/mL in the other male. Thornton et al. [8] reported unintentional exposure of a 10-month old female to AB-PINACA being contained in a cigarette [8], where her serum concentration of AB-PINACA was 42 ng/mL and AB-PINACA *N*-pentanoic acid metabolite 345 ng/mL, but fortunately she fully recovered later. Hess et al. [9] reported a fatality of a male via diabetic ketoacidosis following the consumption of ten SCs, where AB-FUBINACA was observed at 0.97 ng/mL in his blood. AB-FUBINACA and AB-CHMINACA together with other drugs were identified in the stomach contents of a male who intended a suicide, but their levels in body fluids were not reported there [10]. The tissue levels of AB-CHMINACA and 5F-AMB were firstly reported in 2015 [11], but their blood and urine levels were below their limit of quantitation. A first report on 5F-ADB was made for stomach contents and tissue samples, but the levels in blood and urine were below the limit of quantitation in the work [5]. The blood and tissue levels of MAB-CHIMINACA were firstly reported in 2015 [6] where the blood level was 10.6 ng/mL, but the urine level was below the limit of quantitation. The blood levels of MAB-CHMINACA obtained from four intoxicated individuals were reported to be 5.2, 1.3, 1.7 and 14.6 ng/mL in 2016 [12]. In all of the above studies [5–12]; however, the urine levels of the six SCs in unchanged forms were not reported. One of the reasons may be that these SCs are rapidly metabolized to water-soluble compounds to be excreted into urine, and; hence, the urine levels of the SCs in unchanged forms were much lower than their blood and tissue levels. This tendency was already noticed in 2010 when the monohydroxylated metabolites of JWH-018 were observed, but the parent compound was not detected in urine [13]. Therefore, the metabolites of SCs were used as indicators for SC intake for urine samples in several studies [14, 15]. However, the resulting metabolites produced from SCs with pentyl and 5-fluoropentyl side chains are known to be almost the same; the metabolites of XLR-11, 5F-AKB48, MAM-2201 and 5F-AB-PINACA were in common with those of UR-144, AKB48, JWH-122 and AB-PINACA, respectively [16]. Thus, it is most reliable to identify a parent SC in a urine sample to prove the compound that was taken, especially when the corresponding subject is alive.

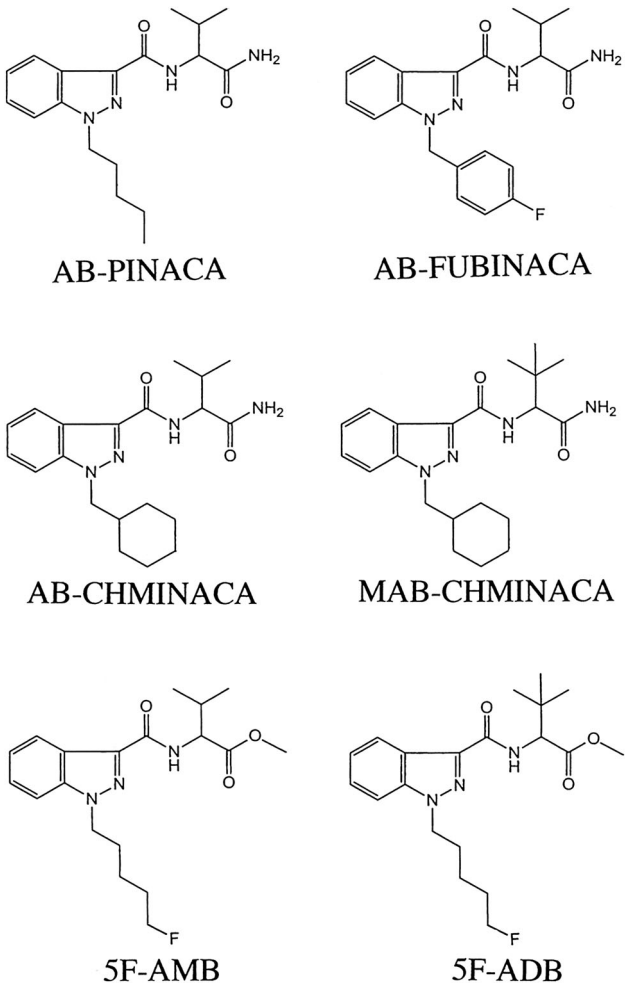

Fig. 1 Structures of AB-PINACA, AB-FUBINACA, AB-CHMINACA, MAB-CHMINACA, 5F-AMB and 5F-ADB

Therefore, in the present study, a sensitive method is presented for the determination of sub-nanogram urine levels of SCs in unchanged forms, and the method was applied to the determination of urine levels of six SCs obtained from three cadavers.

Case histories

Victim 1

A man aged in his twenties was found dead in a bathtub of his house, but the water level was lower than his shoulder. In his room, two open packages containing herbal blends named "ALADDIN PLATINUM" and "ALADDIN LIMITED" and a handmade aluminum foil pipe with herb ash were found. The autopsy was performed at our department. The postmortem interval of the deceased was about 2 days. The lung showed marked congestion and edema. No obvious changes were observed in other organs. Samples of urine, blood, solid tissues and stomach contents were collected at autopsy and kept frozen at −80 °C.

Victim 2

A man in his thirties was found dead in his car parked in a supermarket parking lot. He held a disposable lighter in his right hand. An opened package of herbal blend named "Herbal Incense, The Super Lemon" and another package of herbal blend without any label were found. The herb ash was found on the surface of a flattened coffee can in the car. The cadaver was stored in a refrigerated morgue at 3–4 °C for about 2 days until autopsy.

Victim 3

A man in his thirties was found dead in his room. Three silver-colored packages containing herbal blend mixtures were found. He was grasping a handmade aluminum foil pipe containing the herb ash in his right hand. The cadaver was stored in a refrigerated morgue at 3–4 °C for 1 day until autopsy.

Materials and methods

Materials

AB-PINACA, AB-FUBINACA, AB-CHMINACA, MAB-CHMINACA, 5F-AMB and 5F-ADB were purchased from Cayman Chemical (Ann Arbor, MI, USA); methanol and acetonitrile suitable for liquid chromatography (LC)–mass chromatography (MS), 1-chlorobutane (CB) suitable for amino acid analysis and other chemicals of analytical grade came from Wako Pure Chemical Industries, Osaka, Japan. Pure water with a specific resistance of 18 MΩ cm was used (Millipore, Bedford, MA, USA).

Urine samples from normal subjects, under their permission with informed consent, were used as blank samples, and those spiked with several amounts of SCs were used as quality control samples. The urine samples from three victims were obtained at the autopsies performed at our department, and stored at −80 °C until analyses. The drug testing of these samples had been asked officially by judicial authorities.

Standard solutions

Individual stock solutions of SCs were prepared separately by dissolving appropriate amounts of each drug in acetonitrile at 1 mg/mL and stored at −30 °C. Working calibration solutions and quality control solutions were prepared daily by diluting the stock solutions with blank urine at 10–1000 pg/mL. AB-CHMINACA at 1000 pg/mL in urine was used as the internal standard (IS) for the determination of AB-PINACA and AB-FUBINACA; AB-PINACA at 1000 pg/mL in urine was used as IS for the determination of AB-CHMINACA, MAB-CHMINACA, 5F-AMB and 5F-ADB.

Extraction procedure

For extraction, 400 μL of urine (under 10 °C) was mixed with 4 μL of the IS solution, 80 μL of 2 M K_2CO_3 solution and 0.8 mL of CB (under 10 °C). The mixture was vortexed for 60 s and centrifuged at 10,000 *g* for 2 min, and the supernatant was placed in a new tube. To the urine sample, 0.8 mL of CB was added again, vortexed for 60 s and centrifuged at 10,000 *g* for 2 min, and the supernatant was placed in another new tube. The two extracts from one urine sample were evaporated to nearly dryness at room temperature using a centrifugal dryer (miVac Duo LV; Genevac Ltd, Ipswich, England). The residues of the two extracts were combined and reconstituted with 100 μL of methanol, and centrifuged at 10,000 *g* for 20 s. The supernatant was used for the determination by LC–MS/MS.

Instrumental conditions

Electrospray ionization (ESI)-MS/MS was performed on a 4000 QTRAP MS/MS system (AB SCIEX, Framingham, MA, USA) in the positive ion mode. LC was performed using an Acquity instrument (Waters Co., Milford, MA, USA). A filter named SUMIPAX Filter PG-ODS (Sumika Chemical Analysis Service, Ltd., Osaka, Japan) was attached before LC separation. The LC column for the chromatographic separation was TSK-GEL ODS-100V (150 × 2.0 mm i.d., particle size 5 μm; Tosoh Co., Tokyo, Japan). The mobile phase consisting of 2% B (i.e., 98% A) was set at a flow-rate of 200 μL/min and then gradient elution was performed using 2% B to 70% B over 10 min, where solvent A was pure water containing 0.1% formic acid and 10 mM ammonium acetate, and solvent B, 100% methanol. The MS/MS conditions were: temperature, 700 °C; spray needle voltage, +5.5 kV; sheath gas pressure, 30 units for gas 1 and 60 units for gas 2; curtain gas flow, 20 units. The tandem MS collision energies and ion transitions were 53 V and *m/z* 369 → 109 for AB-FUMINACA, 35 V and *m/z* 331 → 215 for AB-PINACA, 35 V and *m/z* 357 → 241 for AB-CHMINACA, 35 V and *m/z* 371 → 241 for MAB-CHMINACA, 29 V and *m/z* 364 → 233 for 5F-AMB and 23 V and *m/z* 378 → 318 for 5F-ADB, respectively. A 5-μL aliquot of the final extract solution was injected into the LC–MS/MS instrument.

Examination of 24-h stability of the synthetic cannabinoids

The stabilities of SCs against heat and air oxidation were investigated as follows: to examine the stability of SCs in the air, 1.0 μL solution of an SC at 100 ng/mL (i.e., 100 pg) was placed in a glass vial (uncapped). These samples were maintained at 25, 10 and −30 °C, respectively. After 24 h, 100 μL of methanol was poured into the vial, and the levels

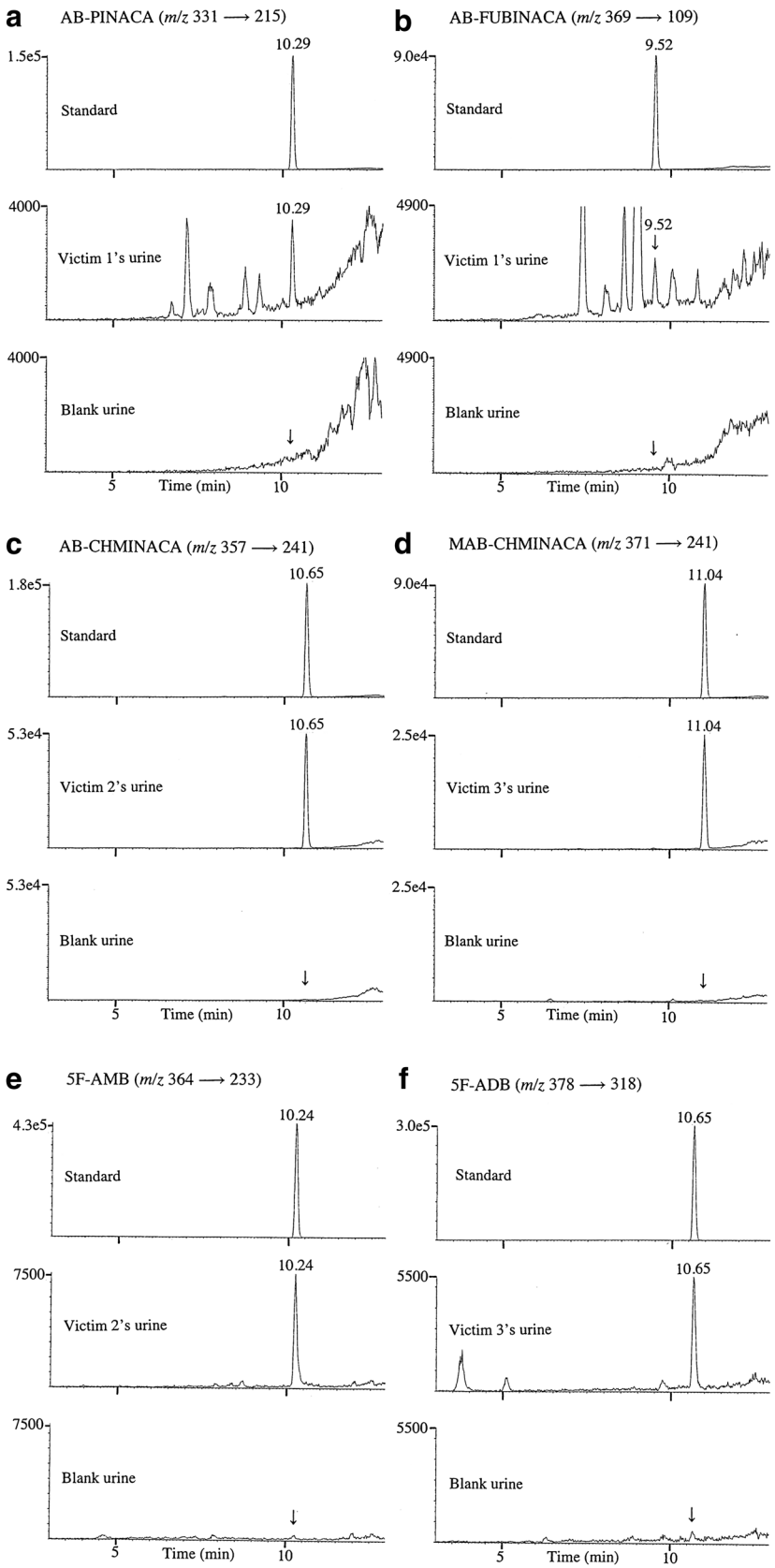

Fig. 2 Selected reaction monitoring chromatograms by liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the detection of AB-PINACA (**a**), where the reference standard is shown in the *top panel*, the urine extract from the victim shown in the *middle panel* and the urine extract from a normal subject shown in the *bottom panel*. The equivalent chromatograms are also shown for AB-FUBINACA (**b**), AB-CHMINACA (**c**), MAB-CHMINACA (**d**), 5F-AMB (**e**) and 5F-ADB (**f**)

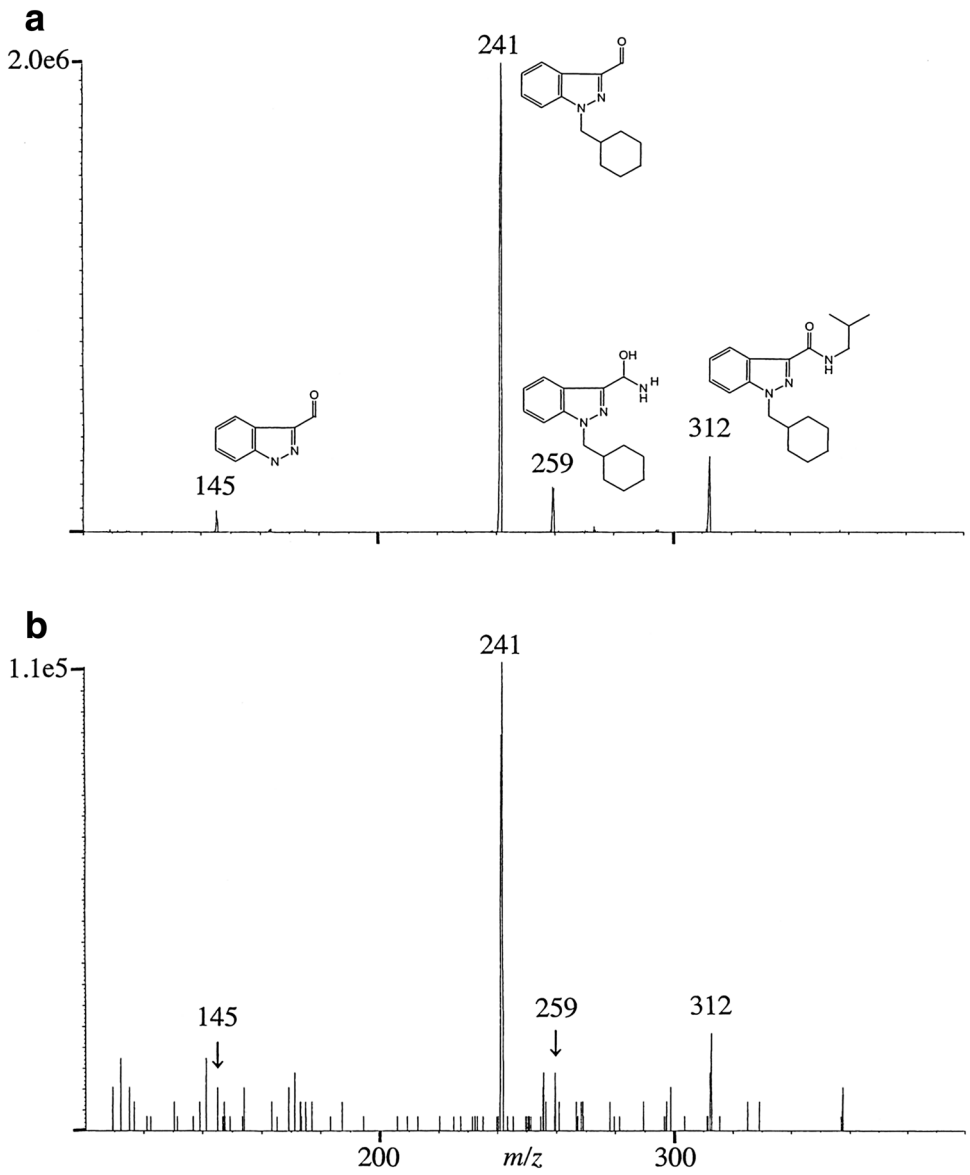

Fig. 3 Product ion spectrum of the reference standard AB-CHMINACA at 20 ng/mL (**a**) and that of the urine extract from victim 2 (**b**)

of SCs were measured by LC–MS/MS. To examine the stability of cannabinoids in methanol, 100 µL methanol solution of an SC at 1 ng/mL (i.e., 100 pg) was placed in a glass vial and capped (closed condition). These samples were maintained at 25, 10 and −30 °C for 24 h, respectively, and the levels of SCs were measured by LC–MS/MS.

Results and discussion

Selected reaction monitoring chromatograms and product ion spectra

The selected reaction monitoring (SRM) chromatograms by LC–MS/MS are shown for the detection of AB-PINACA (Fig. 2a), where the reference standard is shown at the top, the urine extract from the victim shown at the middle and the blank urine extract from the urine mixture of four normal subjects shown at the bottom; equivalent chromatograms are also shown for AB-FUBINACA, AB-CHMINACA, MAB-CHMINACA, 5F-AMB, and 5F-ADB (Fig. 2b–f), respectively. Each protonated molecular ion was used as precursor for acquisition of SRM chromatograms. The experiments were carried out very carefully not to contaminate the test sample measurements by the carry-over of the high level of reference standards. The carry-over could be completely avoided by washing the column with isopropanol, when it was necessary. Although various peaks appeared in the SRM chromatograms for detection AB-PINACA and AB-FUBINACA, unacceptable interference by blank urine was not detected at the retention times of the two SCs. For the other four SCs, the backgrounds were generally very low, and a

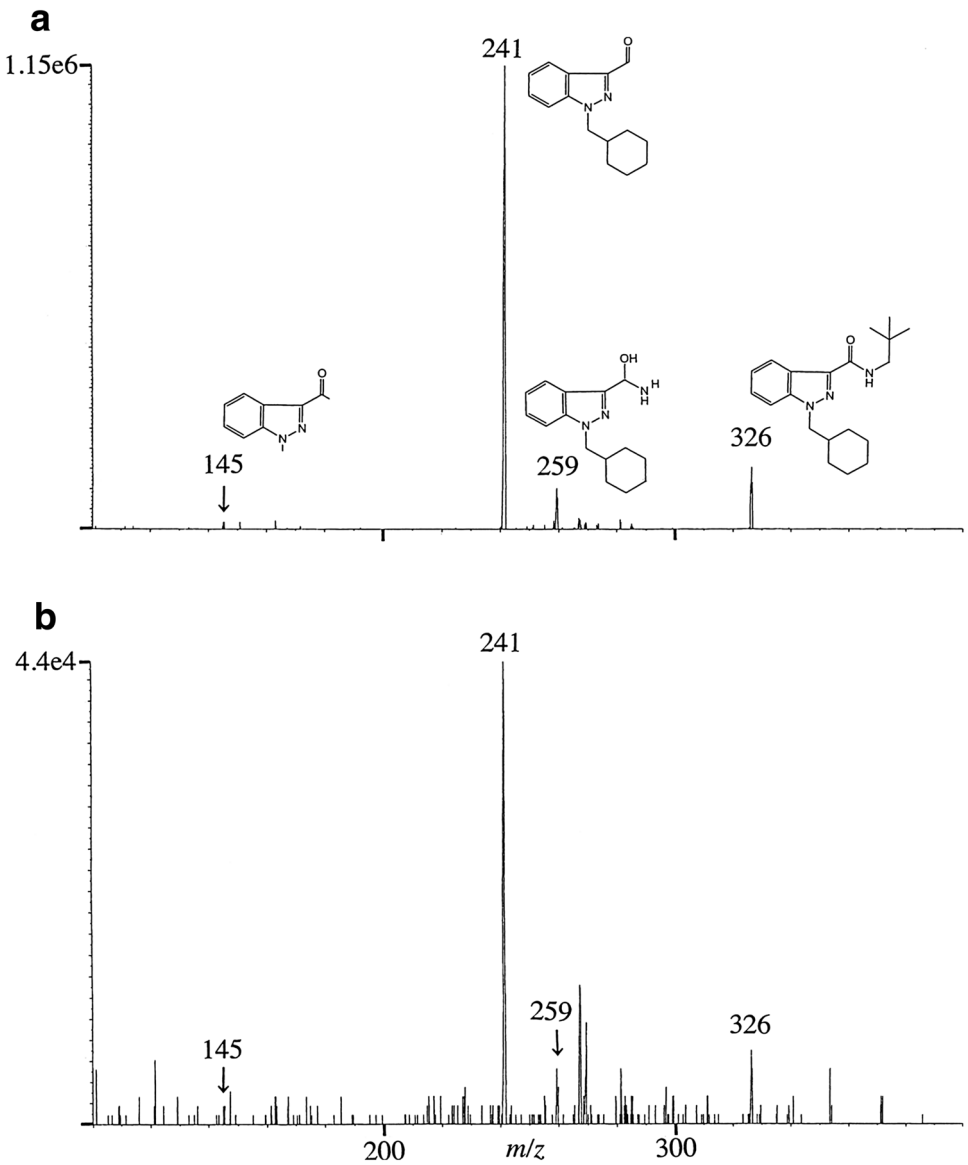

Fig. 4 Product ion spectrum of the reference standard MAB-CHMINACA at 20 ng/mL (**a**) and that of the urine extract from victim 3 (**b**)

few small peaks appeared apart from the test peaks (Fig. 2e, f).

Figure 3 shows the product ion spectra obtained from the reference standard of AB-CHMINACA and from the victim's urine for identification. Although many small peaks appeared at the base of the urine spectrum, the peaks at *m/z* 145, 241, 259 and 312 could be found in the spectrum, confirming that the SRM peak at *m/z* 241 (Fig. 2c) is certainly due to AB-CHMINACA. Figure 4 shows the equivalent comparison of product ion spectra between the reference standard MAB-CHMINACA and the victim's urine, resulting in the conclusion that the SRM peak at *m/z* 241 (Fig. 2d) is due to MAB-CHMINACA.

The product ion spectra of AB-PINACA, AB-FUBINACA, 5F-AMB and 5F-ADB in victims' urine could not be well demonstrated, because their concentrations in urine samples were too low. Therefore, peak height ratios of principal product ions at the signal-to-noise (S/N) ratio >3 derived from the respective standard protonated molecular ions and those at the S/N ratio >3 from victims' urine were compared by the SRM method for all SCs, and the results are listed in Table 1 by taking the highest product ions (quantifier ions) to be 100. The relative peak height ratios of the other principal product ions (qualifier ions) obtained from reference standards and those obtained from victims' urine samples almost agreed, confirming that the peaks from the victims' urine samples in Fig. 2 were due to the six SCs.

Reliability of the method

The extraction recovery was calculated by the ratio *A/B*. *A* was obtained from the relative peak height of an SC to IS in the following solution; an SC was spiked into 400 μL of

Table 1 Relative intensities of the product ions of six synthetic cannabinoids derived from the reference standard solutions and from victims' urine samples

Compound (collision voltage)	Protonated molecular ion (*m/z*) and sample	Percent product ion intensity (*m/z*)			
AB-PINACA	(331.2)	(215.1)	(233.1)	(286.1)	
(35 V)	RS (20 ng/mL)	100	9.8	4.2	
	Victim 1's urine	100	9.7	4.7	
AB-FUBINACA	(369.2)	(109.1)	(253.1)	(225.1)	
(53 V)	RS (20 ng/mL)	100	9.5	1.4	
	Victim 1's urine	100	9.0	Undetectable	
AB-CHMINACA	(357.2)	(241.1)	(312.1)	(259.1)	(145.1)
(35 V)	RS (20 ng/mL)	100	13.9	8.3	2.8
	Victim 2's urine	100	10.6	8.1	1.6
MAB-CHMINACA	(371.2)	(241.1)	(326.1)	(259.1)	(145.1)
(35 V)	RS (20 ng/mL)	100	13.2	8.0	1.4
	Victim 3's urine	100	11.1	7.7	2.0
5F-AMB	(364.2)	(233.1)	(304.1)	(251.1)	(213.1)
(29 V)	RS (20 ng/mL)	100	43.2	31.8	9.8
	Victim 2's urine	100	44.1	29.4	9.4
5F-ADB	(378.2)	(318.1)	(233.1)	(251.1)	(346.1)
(23 V)	RS (20 ng/mL)	100	23.2	9.8	9.1
	Victim 3's urine	100	22.5	8.9	7.8

RS reference standard

Table 2 Regression equations, correlation coefficients and limits of detection of six synthetic cannabinoids spiked into urine detected by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Compound	Equation	Correlation coefficient	Limit of detection (pg/mL)
AB-PINACA	$y = 0.000757\ x + 0.00569$	0.995	5
AB-FUBINACA	$y = 0.000317\ x + 0.000479$	0.996	5
AB-CHMINACA	$y = 0.00116\ x + 0.00359$	0.999	5
MAB-CHMINACA	$y = 0.000600\ x + 0.00250$	0.993	8
5F-AMB	$y = 0.00167\ x - 0.000301$	0.992	2
5F-ADB	$y = 0.00201\ x + 0.00394$	0.999	3

urine at either 10, 100 or 1000 pg/mL, and each extracted residue was dissolved in 100 μL of methanol solution containing IS at 4000 pg/mL. *B* was obtained from the relative peak height of an SC to IS in the following solution; to the extracted residue from 400 μL of blank urine, 100 μL of methanol solution containing IS at 4000 pg/mL and an SC at either 40, 400 or 4000 pg/mL was added. The recoveries at 10, 100 and 1000 pg/mL in urine were in the range of 72–99% (85.0% on average) for six SCs, which are acceptable for quantitative analysis.

The linearity of the present method was examined by spiking SCs at 0, 10, 100 or 1000 pg/mL to blank urine ($n = 6$ at each concentration) for calibration curves. The regression equations for the calibration curves are listed in Table 2 where the correlation coefficients were 0.992–0.999. The limits of detection (signal-to-noise ratio = 3) were calculated to be 2–8 pg/mL.

The precisions and the accuracies were assessed by analyzing urine samples spiked with SCs at 10, 100 or 1000 pg/mL three times a day as well as on three different days. The accuracy data were 74.4–129%, and the precision data were not greater than 23.1% for intraday and interday measurements as listed in Table 3; these data could be considered to be within the acceptable range for the quantitation.

The matrix effects were calculated by the ratio *C/D*. *C* was obtained from the peak height of an SC in the following solution; to the extracted residue from 400 μL of blank urine, 100 μL of methanol solution containing IS at 4000 pg/mL and an SC at either 40, 400 or 4000 pg/mL was added. *D* was obtained from the peak height of an SC in the 100 μL of methanol solution containing IS at 4000 pg/mL and an SC at either 40, 400 or 4000 pg/mL without the urine extract residue (neat

Table 3 Intraday ($n = 3$ each) and interday ($n = 3$ each) accuracy/precision data of synthetic cannabinoids spiked into blank urine

Concentration spiked (pg/mL)	Intraday		Interday	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
AB-PINACA				
1000	96.0	3.6	98.5	9.1
100	118	5.8	98.1	12.8
10	76.6	8.1	74.4	10.2
AB-FUBINACA				
1000	106	4.3	106	7.3
100	101	18.8	93.5	5.1
10	128	1.5	98.7	17.1
AB-CHMINACA				
1000	102	3.9	90.2	14.4
100	104	2.4	90.4	13.1
10	129	2.5	98.0	23.1
MAB-CHMINACA				
1000	109	2.0	94.0	9.0
100	107	4.6	97.5	9.4
10	74.8	3.9	107	22.7
5F-AMB				
1000	90.2	1.6	106	10.4
100	89.1	1.9	98.0	7.8
10	115	2.6	115	5.1
5F-ADB				
1000	99.4	2.9	102	4.9
100	117	9.8	105	6.4
10	82.9	10.3	89.1	21.6

sample). The matrix effects of SCs at 10, 100 and 1000 pg/mL in urine were in the range of 63–106% (83.0% on average) for six SCs. Although 400 µL of urine was finally concentrated to 100 µL of methanol solution, the matrix effects were found to be rather low. The extraction solvent CB, and the filter before the LC separation may have contributed suitably to the elimination of interfering substances.

The stability experiment showed that the remaining amounts of the six SCs in the air for 24 h were 77.4 ± 1.0, 86.2 ± 2.6 and 92.9 ± 1.4% at 25, 10 and −30 °C, respectively. The remaining amounts of SCs in methanol solution in capped vials for 24 h were 100 ± 3% at 25 or 10 °C by taking the amount of SCs stored at −30 °C to be 100%. These results suggest that the stability of SCs at lower temperature is higher than that at room temperature, and the stability of SCs in methanol solution under closed conditions is much higher than that in the air. Therefore, urine samples and CB were adjusted to be at around 10 °C before the extraction with CB, and the extracted residues from urine were dissolved in methanol solution at −30 °C and measured by LC–MS/MS within 24 h.

Concentrations of the parent forms of six synthetic cannabinoids in urine samples of abusers

As a result, the concentrations of AB-PINACA and AB-FUBINACA were 23 and 10 pg/mL in the urine of victim 1, those of AB-CHMINACA and 5F-AMB 232 and 19 pg/mL in the urine of victim 2, and those of MAB-CHMINACA and 5F-ADB 229 and 19 pg/mL in the urine of victim 3, respectively.

In this study, we succeeded in quantitation of 10–232 pg/mL of SCs in urine samples of abusers. The linear regression equations could be obtained in the range of 10–1000 pg/mL, and the limits of detection were 2–8 pg/mL (Table 2). Such high sensitivity is marvelous and is due to the capability of the relatively new type of LC-QTRAP MS/MS instrument.

Conclusions

The present LC–MS/MS method provides a simple and extremely sensitive method for the quantitation of synthetic cannabinoids even at 10 pg/mL using only 400 µL of

urine. The capability to detect picogram/mL levels of SCs in urine samples seems to be due to extremely high sensitivity of the LC-QTRAP MS/MS instrument used. This is the first report that quantitated AB-PINACA, AB-FUBINACA, AB-CHMINACA, 5F-AMB, MAB-CHMINACA and 5F-ADB in parent forms in real urine samples from three victims, although higher levels in blood and tissues for some SCs were reported previously. This simple and extremely sensitive method has the potential to be applied to the detection of low levels of unchanged drugs of abuse in the urine of living subjects at the scenes of emergency medicine and driving under the influence of drugs.

Compliance with ethical standards

Conflict of interest The authors declare that there are no financial or other relationships that could lead to a conflict of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the international and/or national committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all participants included in the study, who supplied about 40 mL each of urine for use as blank samples. The analysis of toxic substances from the cadavers was permitted by judicial authorities and supported by official documentation.

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