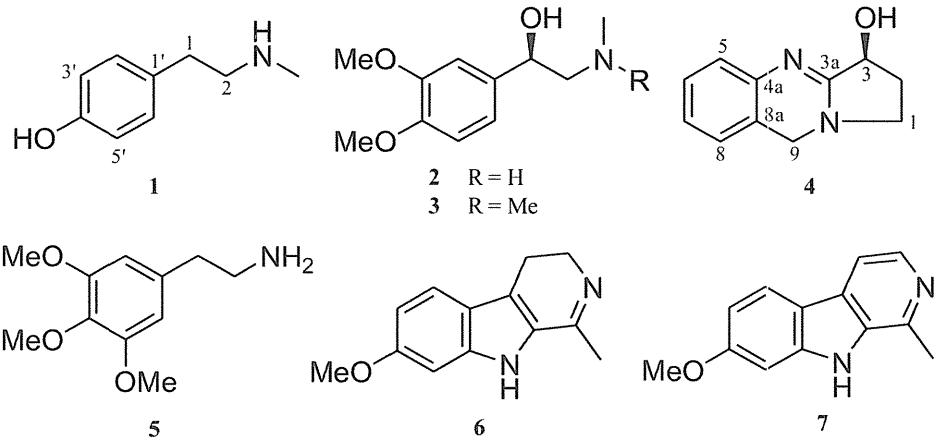

Fig. 1 Chemical structures of compounds detected in the herbal product. **1**, *N*-methyltyramine; **2**, (*R*)-normacromerine; **3**, (*R*)-macromerine; **4**, (*S*)-vasicine; **5**, mescaline; **6**, harmaline; **7**, harmine

N-Me), 3.07 (1H, br d, J = 12.3 Hz, H-2a), 3.30 (1H, br t, J = 12.3 Hz, H-2b), 3.88 (3H, s, OMe-4′), 3.90 (3H, s, OMe-3′), 5.12 (1H, br d, J = 9.6 Hz, H-1), 6.85 (1H, d, J = 8.3 Hz, H-5′), 6.89 (1H, br d, J = 8.3 Hz, H-6′), 6.99 (1H, br s, H-2′); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 43.1 (*N*-Me), 45.4 (*N*-Me), 55.9 (OMe-3′, 4′), 65.3 (C-2), 68.2 (C-1), 108.8 (C-2′), 111.1 (C-5′), 117.9 (C-6′), 132.2 (C-1′), 149.0 (C-4′), 149.3 (C-3′); DART-TOFMS *m/z* 226.1444 $[M+H]^+$ (calculated for $C_{12}H_{20}NO_3$, 226.1443).

Compound **4** [(*S*)-vasicine]: $[\alpha]_D^{20}$ −38° (*c* 0.5, $CHCl_3$); ^{1}H NMR (600 MHz, CD_3OD) δ_H 2.11 (1H, m, H-2a), 2.65 (1H, m, H-2b), 3.67 (1H, m, H-1a), 3.75 (1H, m, H-1b), 4.80 (1H, d, J = 15.4 Hz, H-8a), 4.88 (1H, d, J = 15.4 Hz, H-8b), 5.11 (1H, t, J = 8.1 Hz, H-3), 7.13 (1H, d, J = 7.7 Hz, H-5), 7.20 (1H, d, J = 7.7 Hz, H-8), 7.26 (1H, br t, J = 7.7 Hz, H-7), 7.34 (1H, t, J = 7.7 Hz, H-6); ^{13}C NMR (125 MHz, CD_3OD) δ_C 30.8 (C-2), 47.5 (C-9), 51.9 (C-1), 72.5 (C-3), 118.2 (C-8a), 118.3 (C-4a), 128.3 (C-8), 128.4 (C-7), 130.5 (C-6), 132.2 (C-4a), 164.8 (C-3a); DART-TOFMS *m/z* 189.1024 $[M+H]^+$ (calculated for $C_{11}H_{13}N_2O$ 189.1028).

A small amount of the plant product was extracted with methanol in the same way, and directly subjected to LC-MS analysis for compounds, the authentic standards of which were available at hand. The methanol extract of the product showed seven peaks at 2.1, 3.7, 4.1, 4.3, 8.4, 17.1, and 17.7 min on a total ion mass chromatogram obtained by LC-MS analysis (Fig. 2). Among them, the three peaks detected at 8.4, 17.1, and 17.7 min were identified as mescaline (**5**), harmaline (**6**), and harmine (**7**), respectively, by comparing the ultraviolet spectra, mass spectra, and retention times with those of the authentic standards available. The peaks **1**–**4** appearing in the LC-MS chromatogram (Fig. 2) were confirmed to be due to compounds **1**–**4** already identified as above.

The sample was further analyzed by GC-MS to obtain a chromatographic profile, under the conditions previously reported [11]. One major peak was detected at 22.5 min in a total ion chromatogram (Fig. 3A). The mass spectrum exhibited four ion peaks at *m/z* (% relative intensity) 225 (0.9), 207 (8.1), 192 (7.8), and 58 (100) (Fig. 3B). The mass spectrum and retention time of this peak corresponded to those of (*R*)-macromerine (**3**).

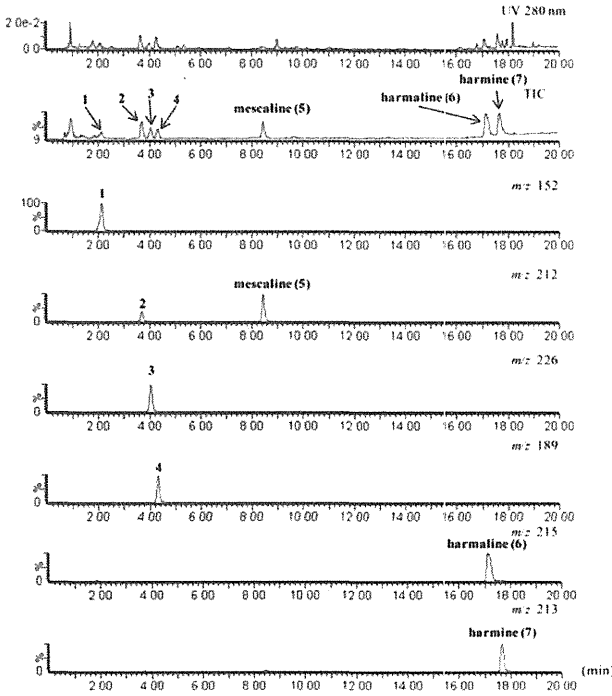

Fig. 2 Ultraviolet ultra-performance liquid chromatography (*UPLC*) chromatogram, total ion chromatogram (*TIC*), and mass chromatograms of the methanol extract from the plant sample. The peaks at *m/z* 212, 215, and 213 were identified as the $[M+H]^+$ ions of mescaline (**5**), harmaline (**6**), and harmine (**7**), respectively, by comparison with each authentic standard. The peaks at *m/z* 152, 212, 226, and 189 were also identified as compounds **1**–**4**, respectively, which were identified by various methods without authentic standards

N-Methyltyramine (**1**) has been isolated from a variety of plant species such as *Coryphantha missouriensis* (Cactaceae), *Pilosocereus maxonii* (Rose), and *Acacia schweinfurthii* (Leguminosae) [16–18]. It has been reported that compounds **2** and **3** caused hallucinogenic effects in animals [14,19], and were extracted from the genus *Coryphantha* (Cactaceae) [20–22]. (*S*)-Vasicine (**4**), harmaline (**6**), and harmine (**7**) were found in the seeds of *Peganum harmala*. Among them, the β-carboline derivatives **6** and **7** are known as potent inhibitors of monoamine oxidase (MAO) [23,24]. MAO is an enzyme that converts various monoamines, such as serotonin and dopamine, into psychologically inactive aldehydes. Therefore, a combination of monoamines, such as **2** and **3**, with β-carboline derivatives (**6** and **7**) might cause prolongation of the hallucinogenic effects. Mescaline (**5**) is known to be a psychoactive phenethylamine present in peyote (*Lophophora williamsii*) and San Pedro (*Trichocereus pachanoi*). This compound is strictly controlled by the Narcotics and Psychotropic Control Law in Japan, although the source plants are not controlled.

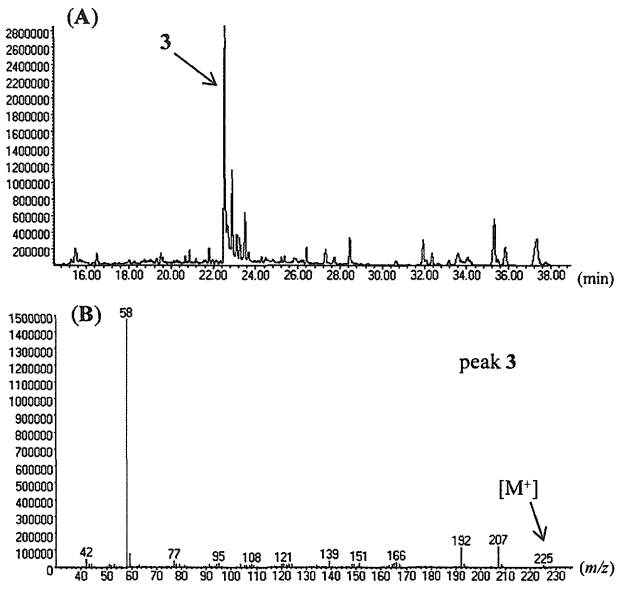

Fig. 3A, B Gas chromatography-mass spectrometry analysis of the methanol extract from the plant sample. **A** TIC of 10 mg/ml of the sample solution. **B** Mass spectrum of the peak detected at 22.5 min

Finally, the identified psychotropic compounds **2**, **3**, **5**, **6**, and **7** in the product were quantitated by LC-MS. The calibration curves of the individual compounds showed good linearity in the indicated ranges (Table 1). The contents of these compounds in the plant sample were 5.3, 1.8, 0.8, 5.8, and 2.1 mg/g product, respectively (Table 1).

Identification of plant species by DNA sequence analysis

To clarify the origins of the plant species in the product, we performed genetic analyses of *trnL-F* and ITS regions. Noncoding regions of DNA, such as the nucleotide sequences of *trnL-F* and ITS regions, provide useful phylogenetic information, and are useful as molecular markers for species identification. Therefore, many noncoding regions, including introns and intergenic spacers, have recently been sequenced to assess intrageneric relationships [25,26]. The nucleotide sequence alignment based on the BLAST search revealed two high-homology sequences corresponding to *P. harmala* and *Turnera diffusa* (data not shown). Because compounds **2** and **3** are often extracted from the genus *Coryphantha*, the product was assumed to contain plant material of the genus *Coryphantha*. Therefore, we surveyed the ribosomal protein L16 (*rpl16*) intron regions to compare the sequence data of three kinds of *Coryphantha* cacti: *Coryphantha elephantidens* (accession no. AY545231), *Coryphantha pallida* (AY545232), and *Coryphantha durangensis* (AY545230). The *rpl*16 sequences of these three cacti have been previously reported [27]. However, the sequence derived from the herbal product did not agree with the sequence data of those *Coryphantha* species (each identity; 85%) (Fig. 4). Thus, we extracted the DNA from *Coryphantha macromeris* purchased via the Internet, and the *rpl16* intron sequence was compared with that of the herbal product. The *rpl16* intron sequence of the herbal product showed the highest simi-

Table 1 Parameters of the calibration curves and contents for the five targeted compounds in the product

Compound	Range (μg/ml)	Equation	Correlation coefficient (r^2)	LOD (μg/ml)	LOQ (μg/ml)	Content (mg/product)
(*R*)-Normacromerine (**2**)	0.05–10	$y = 0.0256x - 0.0017$	0.998	0.5	1.0	5.3
(*R*)-Macromerine (**3**)	0.05–10	$y = 0.0724x + 0.0037$	0.991	0.5	1.0	1.8
Mescaline (**5**)	0.05–10	$y = 0.2847x - 0.0017$	0.995	0.1	0.1	0.8
Harmaline (**6**)	0.05–10	$y = 0.5838x + 0.0258$	0.997	0.05	0.1	5.8
Harmine (**7**)	0.05–5.0	$y = 1.2613x + 0.0234$	0.998	0.05	0.05	2.1

LOD, Limit of detection (signal-to-noise ratio = 3); LOQ, limit of quantitation (signal-to-noise ratio = 10)

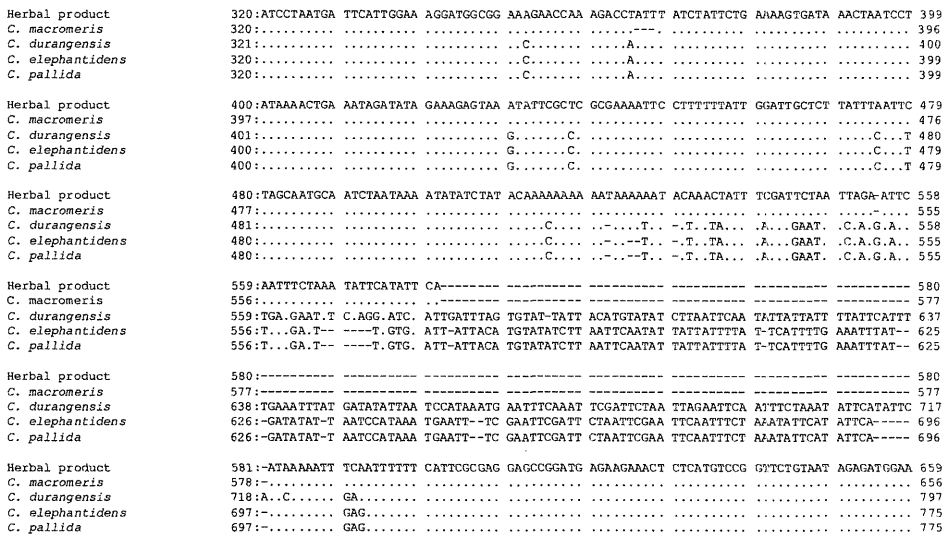

Fig. 4 Multiple alignments of *rpl16* intron sequences from the herbal product and plants of genus *Coryphantha*. Identical sequences are indicated by *dots*; *dashes* are used to show the maximum similarity

larity to that of *C. macromeris* (identity; 99%). Therefore, it is strongly suggested that the herbal product contained *C. macromeris*.

Coryphantha macromeris, the so-called Dona Ana cactus, is known to be a psychoactive cactus with about 20% of the potency of peyote, which is a cactus containing the hallucinogenic compound mescaline. The plant is also known as nipple beehive cactus, and is distributed in New Mexico and Texas [22]. Previous chemical investigations of this plant led to the isolation of various methylated phenethylamine derivatives. Of these phenethylamines, (*R*)-normacromerine is by far the most abundant.

Relationship between chemical constituents and plant species for the product

On the basis of the DNA sequence analysis of the herbal product, we estimated that it contained at least three kinds of plants, such as *C. macromeris*, *T. diffusa*, and *P. harmala*. Considering the chemical constituents and plant species detected in the product, it was suggested that compounds **2** and **3** originated from *C. macromeris*, and compounds **4**, **6**, and **7** were from *P. harmala*. Compound **5** was detected at low yield in the product, but we could not identify the sequences from peyote or San Pedro cactus in the DNA analysis. Therefore, we speculated that the extracts of such cacti were added to the product. *Turnera diffusa* (Damiana) is sometimes detected in herbal products sold as incense with narcotic pharmacologic effects in Japan, but its active compound(s) are still not known. Further studies are needed to clarify this problem.

Essentially, there seems to be no contradiction between chemical constituents and the plant species identified by DNA analyses, except for compound **5**. The cactus *C. macromeris*, containing psychoactive compounds, was first detected in herbal products distributed in the illegal drug market. When we consider the contents of psychotropic compounds probably derived from natural sources, the narcotic-like effects of the product seem to be weak, even if the entire 3-g package is smoked [14,19]. However, it seems likely that the distribution of products containing uncontrolled psychotropic plants, such as *C. macromeris*, will increase in the illegal drug markets. Therefore, we will continuously monitor such products.

Acknowledgments Part of this work was supported by a Health and Labour Sciences Research Grant from the Ministry of Health, Labour, and Welfare, Japan.

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Forensic Science International 198 (2010) 62–69

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Determination of a new designer drug, *N*-hydroxy-3,4-methylenedioxymethamphetamine and its metabolites in rats using ultra-performance liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form 29 January 2010
Accepted 9 February 2010
Available online 17 March 2010

Keywords:
N-OH MDMA
MDMA
MDA
Rats
Biological samples
UPLC–MS/MS

ABSTRACT

An *N*-hydroxy analogue of 3,4-methylendioxymethamphetamine (MDMA), *N*-hydroxy MDMA (*N*-OH MDMA), has recently been distributed as a new designer drug in some drug markets. Very little data is available to the metabolic and pharmacological properties of *N*-OH MDMA, although it has been reported that the *N*-demethyl analogue, *N*-hydroxy-3,4-methylenedioxyamphetamine (*N*-OH MDA), is mainly metabolized to MDA in rats. In this study, an analytical method for the determination of *N*-OH MDMA and its metabolites in biological samples was developed, and the metabolic properties of *N*-OH MDMA in rats were investigated.

After the *i.p.* administration of *N*-OH MDMA to pigmented hairy rats (5 mg/kg/day, 10 days), *N*-OH MDMA and its *N*-dehydroxy and *N*-demethyl metabolites (MDMA, *N*-OH MDA and MDA) in rat plasma, urine and hair samples were determined by ultra-performance LC (UPLC)–MS/MS. The hair sample was extracted by 1-h sonication and overnight soaking in 5 M hydrochloric acid–methanol (1:20). The plasma, urine, and hair extract samples were purified using a solid-phase extraction procedure. *N*-OH MDMA in the samples could be precisely analyzed by avoiding an alkaline environment. The parent compound very rapidly disappeared from the rat plasma (<15 min) and urine (<10 h), and most of the *N*-OH MDMA was excreted in the rat urine as MDMA and MDA in 72 h. In the rat hair samples collected 4 weeks after the first administration, *N*-OH MDMA (0.03 ng/mg) and *N*-OH MDA (0.13 ng/mg) were clearly detected as well as MDMA (149 ng/mg) and MDA (52 ng/mg). This analytical method will be useful for the analysis of *N*-OH MDMA and its metabolites in biological samples.

1. Introduction

Various designer drugs of 3,4-methylenedioxymethamphetamine (MDMA) have appeared as street drugs in recent years. Besides *N*-alkyl derivatives of MDMA such as 3,4-methylenedioxyethylamphetamine (MDEA), the use of beta-keto compounds such as methylone, 1-(3,4-methylenedioxyphenyl-2-(pyrrolidin-1-yl)-1-pentanone (MDPV), 2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one (bk-MBDB), and 2-ethylamino-1-(3,4-methylenedioxyphenyl)propan-1-one) (bk-MDEA) has become widely spread throughout the world [1–7]. Moreover, an *N*-hydroxyl analogue of MDMA, *N*-hydroxy MDMA (*N*-OH MDMA, FLEA), has also been distributed as a new designer drug in some drug markets [5,8].

The *N*-hydroxy group has been found to have unique analytical properties in similar compounds such as *N*-hydroxy-3,4-methylenedioxyamphetamine (*N*-OH MDA; *N*-demethyl analogue of *N*-OH MDMA). *N*-OH MDA is unstable at high temperatures (e.g. GC–MS analysis) and decomposes to MDA and the oxime of 3,4-methylenedioxyphenyl-2-propanone [9]. Moreover, *N*-OH MDA (p*K*a value = 6.22) is considerably less basic than MDA (p*K*a value = 10.04), and thus its capacity factors show greater variation with mobile phase pHs in the 2.5–6.0 range for HPLC analysis, while retention of the primary amine, MDA, and *N*-alkyl MDAs remains relatively constant over this range [10]. The aqueous solution stability of *N*-OH MDA has been found to decrease with increases in the aqueous solution pH, and the degradation half-life decreases to a value of 2.57 h at pH 10 [10]. In an alkaline environment, *N*-OH MDA is mainly transformed to its corresponding oxime. This pH-dependent mechanism is different from that in the pyrolysis of *N*-OH MDA, as observed in the GC–MS analysis. On the other hand, Ravis et al. [11] have reported that *N*-OH MDA is rapidly metabolized to MDA in rats and that no other major metabolites could be detected in a rat liver slice, plasma, or urine sample. As compared with *N*-OH MDA, very little data is available as to the analytical, metabolic, and pharmacological properties of

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doi:10.1016/j.forsciint.2010.02.013

N-OH MDMA, although it has been reported that direct GC–MS analysis of *N*-OH MDMA gives no indication of the parent compound and that only MDMA (*N*-dehydroxy compound) and MDA (*N*-dehydroxy and *N*-demethyl compound) are detected as the major components of the sample [8].

In this study, the stability of *N*-OH MDMA in sample solutions under various conditions (including wide pH ranges) was studied to establish suitable conditions for animal studies. Furthermore, a rapid and sensitive analytical method for the simultaneous determination of *N*-OH MDMA and its metabolites in rat plasma, urine, and hair samples was developed using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS), and the metabolic properties of *N*-OH MDMA in rats were investigated.

2. Materials and methods

2.1. Chemicals and reagents

MDMA hydrochloride [12], MDA hydrochloride [12], *N*-OH MDA hydrochloride [13] and 2-methylamino-1-phenylpropane-2,3,3,3-d4 hydrochloride (methamphetamine(MA)-d4, used as an internal standard) [14] were prepared, as previously reported. *N*-OH MDMA oxalate was synthesized from 3,4-methylenedioxyphenetylacetone and *N*-methylhydroxylamine according to the procedure reported by Noggle et al. [9]. Its structure and purity were confirmed by the measurements of accurate mass, the infrared spectrum [8], GC–MS (acetylated derivatives) [8], LC–MS [8], and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) [5]. The accurate mass of $[M+H]^+$ was *m/z* 210.11309 in the positive scan mode by AccuTOF JMS-T100 (JEOL, Tokyo, Japan). The error between the observed mass and the theoretical mass of $[M+H]^+$ ($C_{11}H_{16}NO_3$) was +0.07 mmu. The structures of these drugs are shown in Fig. 1. A solid-phase extraction column (Bond Elut Plexa, 30 mg/1 mL) was obtained from Varian (Harbor City, CA, USA), and the membrane filter (Ultrafree-MC, 0.45 μm) was from Millipore Corporation (Bedford, MA, USA). All other chemicals and solvents were of an analytical reagent grade or HPLC grade (Wako Chemicals, Osaka, Japan).

2.2. Instrumentation

The UPLC analysis was performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA). The separations were achieved using an Acquity HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm) from Waters (Milford, MA, USA). The column temperature was maintained at 40 °C, and the following gradient system was used with a mobile phase A (1% formic acid) and mobile phase B (1% formic acid/acetonitrile) delivered at 0.3 mL/min: 90% A/10% B (0 min)–70% A/30% B (8 min). The mobile phase was used as a wash solvent to avoid any carry-over from previous injections. The auto-sampler was maintained at 4 °C and the injection volume was 2 μL. The total run time for each sample analysis was 8.0 min. Quantitation was achieved by MS/MS detection in a positive ion mode using a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface. Quantification was performed using multiple reaction-monitoring (MRM) of the transitions of *m/z* 210.2 → 163.2 for *N*-OH MDMA (4.5 min), *m/z* 196.2 → 163.2 for *N*-OH MDA (3.9 min), *m/z* 194.3 → 163.2 for MDMA (3.3 min), *m/z* 180.2 → 163.2 for MDA (2.9 min), and *m/z* 154.2 → 92.1 for IS (3.1 min), with a scan time of 0.05 s per transition. The cone voltage and collision energy were set at 20 and 15 for *N*-OH MDMA, MDMA, and IS, and at 20 and 10 for *N*-OH MDA and MDA, respectively. The optimal MS parameters obtained were as follows: capillary 3.0 kV, source temperature 120 °C, and desolvation temperature 400 °C. Nitrogen was used as the desolvation and cone gas, with a flow rate of 800 and 50 L/h, respectively. Argon was used as the collision gas, with a flow rate of 0.25 mL/min. All data collected in the centroid mode were processed using MassLynx™ NT4.1 software with a QuanLynx™ program (Waters, Milford, MA, USA).

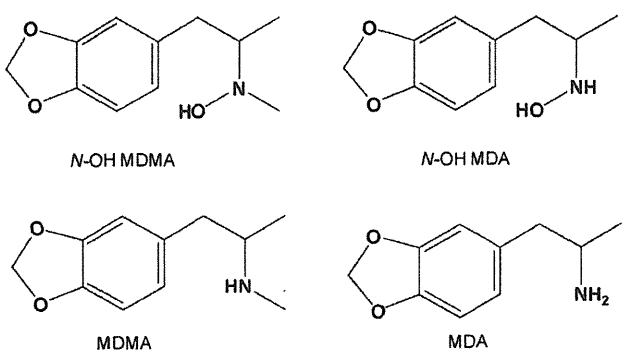

Fig. 1. Structures of *N*-OH MDMA and its metabolites.

2.3. Animal experiments

The animal experimental model was designed as shown in our previous reports [15,16]. All experiments were carried out with the approval of the Committee for Animal Care and Use of National Institute of Health Sciences, Japan. *N*-OH MDMA oxalate was administered to male dark agouti (DA) pigmented rats, which were 5 weeks old and around 90 g mean weight (Japan SLC, Shizuoka, Japan). The drugs were given once daily at 5 mg/kg by intraperitoneal injection for 10 successive days. Blood samples were collected 5, 15, 30, 60 120, and 360 min after the first administration from the orbital vein plexus. Plasma samples were prepared by centrifugation at 10,000 × *g* for 3 min and stored at −20 °C until analysis. The area under the plasma concentration time curve (AUC) was calculated by the conventional method [15]. Urine samples were collected 0–10, 10–24, 24–34, 34–48, and 48–72 h after the last administration and stored at −20 °C. To prevent the degradation of *N*-hydroxy compounds in the urine samples, 1 mL of 1 M phosphate buffer (pH 3) was added in advance to the collection vials and then cooled in an ice-bath (4 °C), and the pH of the collected urine was kept under acidic conditions. Each animal had been shaved on the back just before the first drug administration. The new growing hair samples were collected 28 days after the first administration.

2.4. Sample preparation

2.4.1. Stock solution

An individual standard solution of 1.0 mg/mL of each drug, *N*-OH MDMA, *N*-OH MDA, MDMA, and MDA, was prepared in methanol and stored at 4 °C. The IS solutions of 2 μg/mL of MA-d4 in methanol for the analysis of hair samples and those of 2 μg/mL of MA-d4 in distilled water for plasma and urine samples were also prepared.

2.4.2. Stability of *N*-OH MDMA

To investigate the stability of *N*-OH MDMA under various pH conditions, 0.5 mL of sample solutions containing 0.1 μg/mL of *N*-OH MDMA and MA-d4 (IS) were prepared with 0.1 M phosphate buffers at various pHs. The pHs of the buffer solutions, containing either mono- or di-basic potassium phosphate, were adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 by adding 1 M phosphoric acid or 1 M potassium hydroxide, respectively. Fifty microliters of each sample solution was pipetted into the corresponding test tube, into which was previously added 0.45 mL of the mixed solution of methanol and acetonitrile (1:1) at 0, 1, 2, 4, 5, and 24 h after sample preparation, and analyzed using the UPLC–MS/MS system. The results were calculated using the peak-area ratios of the ions monitored for the target compounds versus IS, and indicated as a percentage of the remaining *N*-OH MDMA.

To establish suitable conditions for animal studies, *N*-OH MDMA and MA-d4 (IS) were dissolved in the rat drug-free urine (pH 8–9) at concentrations of 1 μg/mL. The stability of *N*-OH MDMA in the urine was studied under six different conditions described as follows: (1) kept at room temperature, (2) added 1 mL of 1 M phosphate buffer (pH 3.0) and kept at room temperature, (3) kept on ice, (4) added 1 mL of 1 M phosphate buffer (pH 3.0) and kept on ice, (5) the control urine was heated at 70 °C for 15 min before the addition of the drugs and was kept at room temperature, and (6) the urine was heated at 70 °C for 15 min and 1 mL of 1 M phosphate buffer (pH 3.0) was added before addition of the drugs. One hundred microliters of the sample under each condition was pipetted into the corresponding test tube, into which was previously added 1 mL of 0.1 M of phosphate buffer (pH 3.0) at 0, 1, 2, 4, 5, and 24 h after the sample preparation, and immediately the solution was treated with Bond Elut Plexa and analyzed as below. For the rat plasma samples, *N*-OH MDMA and MA-d4 (IS) were dissolved in the rat drug-free plasma (pH 7) at concentrations of 1 μg/mL. After being maintained at room temperature or on ice for 1, 2, and 4 h, 100 μL of each sample was pipetted and analyzed using the same method as with the urine samples.

2.4.3. Extraction of *N*-OH MDMA and its metabolites from plasma and urine samples

To a 50-μL plasma sample or a 100-μL urine sample were added 50 μL of the IS aqueous solution and 1 mL of 0.1 M phosphate buffer (pH 3.0), respectively. Before the quantitative analysis of MDMA and MDA, due to their high concentrations, the urine (0–10, 10–24, and 24–48 h) and plasma samples were diluted with the control specimens 100 times and 5 times as concentrations, respectively. After a Bond Elut Plexa was pre-activated with methanol and distilled water, the sample solution was applied to the Bond Elut Plexa. After the column was washed with 0.5 mL of distilled water, 0.5 mL of the solution of 2% formic acid/methanol was passed through the column to elute the target drugs. Following evaporation of the solvent under a nitrogen stream, the residue was dissolved in 0.5 mL of the mixed solution of methanol and acetonitrile (1:1). Two microliters of the solution was automatically injected into the UPLC–MS/MS.

2.4.4. Extraction of N-OH MDMA and its metabolites from hair samples

Hair samples were washed three times with distilled water under ultrasonication. After the sample was dried under a nitrogen stream at room temperature, approximately 10 mg of finely cut hair was precisely weighed and extracted with 1.5 mL of methanol/5 M hydrochloric acid mixed solution (20:1) containing 50 μL of each IS methanol solution for 1 h under ultrasonication. For the quantitative analysis of MDMA and MDA, 2-mg hair samples were used separately. Following overnight storage at room temperature, the hair was filtered off, the filtrate was evaporated with a nitrogen stream, and the residue was dissolved in 1 mL of 0.1 M phosphate buffer (pH 3.0). The solution was treated with Bond Elut Plexa and analyzed as above.

2.4.5. Linearity, precision, and recovery of the analytical method

The drug concentrations in the samples were calculated using the peak-area ratios of the ions monitored for the target compounds versus IS. The calibration curves for the determination were constructed by analyzing extracted drug-free control samples spiked with the standard solution, as described above. The calibration samples containing 0, 0.5, 1, 5, 10, 50, 100, and 500 ng/mL of the target drugs for the rat plasma and urine samples were prepared just before analysis. The samples containing 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 0.75 ng/mg of *N*-OH MDMA and *N*-OH MDA, and 0, 1, 2.5, 5, 10, 25, 50, and 75 ng/mg of MDMA and MDA for the hair samples were also prepared. For the urine analysis, 1 M phosphate buffer (pH 3.0) was added to the drug-free control urine samples before the addition of the standard solution of target drugs, to prevent the degradation of *N*-OH compounds. The limit of quantitation of each drug was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance.

The precision of the method was evaluated by five consecutive analyses of the plasma and urine samples that were spiked with the standard solutions containing 0.5, 50, and 500 ng/mL of the target drugs, respectively. For the hair analyses, the control samples, spiked with the standard solutions each containing 0.01, 0.05, and 0.5 ng/mg of *N*-OH MDMA and *N*-OH MDA and 1, 5, and 50 ng/mg of MDMA and MDA, were evaluated. The limit of detection (LOD) was defined as concentrations in a sample matrix resulting in peak areas with signal-to-noise (S/N) ratios of 3. The extraction recoveries of the four analytes, using the solid-phase extraction column, were determined using 0.1 M phosphate buffer (pH 3.0) spiked with the analytes at a concentration of 100 ng/mL, respectively. To determine the recoveries, the responses of the analytes spiked in the solutions before and after extraction were compared.

3. Results and discussion

3.1. Stability of N-OH MDMA

N-OH MDA, an *N*-demethyl analogue of *N*-OH MDMA, was unstable at high temperatures or in alkaline environments. It mainly decomposed to the oxime and/or its *N*-dehydroxy compound [9,10]. To evaluate the stability of *N*-OH MDMA oxalate in the stock solution, the methanol or aqueous solution of *N*-OH MDMA at a concentration of 1 mg/mL was kept at 4 °C for 2 weeks and the ratio of the remaining drug was measured. In both solutions, more than 90% of *N*-OH MDMA remained and thus they can be used as stock solutions for at least 2 weeks. In the same way, the stability of *N*-OH MDMA in the solution for the UPLC–MS/MS measurement was also studied. The ratios of the remaining drug of the solutions of 0.1% formic acid, acetonitrile, methanol, methanol/acetonitrile (1:1), 0.1% formic acid/10% acetonitrile (the initial composition of the mobile phase for the UPLC–MS/MS analysis) and 2% formic acid/methanol (the solution for eluting the drugs from the solid-phase extraction column) at a concentration of 0.1 μg/mL were analyzed over 24 h. As a result, the mixed solution of methanol/acetonitrile (1:1) was found to be the most suitable for the measurement of the UPLC–MS/MS system in this study.

To investigate the stability of *N*-OH MDMA under various pH conditions, the ratios of the remaining drugs in the buffer solutions under various pHs were analyzed over 24 h. *N*-OH MDMA was relatively stable in acidic conditions below pH 5, although it rapidly decomposed under basic conditions. Almost no parent compound was detected in the buffer solutions above pH 8 (Fig. 2). Beckett and Al-Sarraj [17] have reported that *N*-hydroxyamphetamine is readily decomposed into both the corresponding *syn*- and *anti*-oximes in alkaline solutions. The rate of the decomposition of *N*-hydroxyamphetamine is increased by dissolved oxygen in the solution, and a free radical mechanism has been proposed [17]. In contrast, Valaer et al. [10] have reported that *N*-OH MDA is chemically decomposed to its oxime and that this mechanism is pH-dependent. In our additional study, we detected the oxime- and nitroso-compounds of MDA as the decomposed products of *N*-OH MDMA in alkaline environments by NMR analyses [18]. Under these conditions, as compared with *N*-OH MDA, some other mechanism would be responsible for the chemical transformation of *N*-OH MDMA to its corresponding *N*-demethyl oxime- and nitroso-compounds.

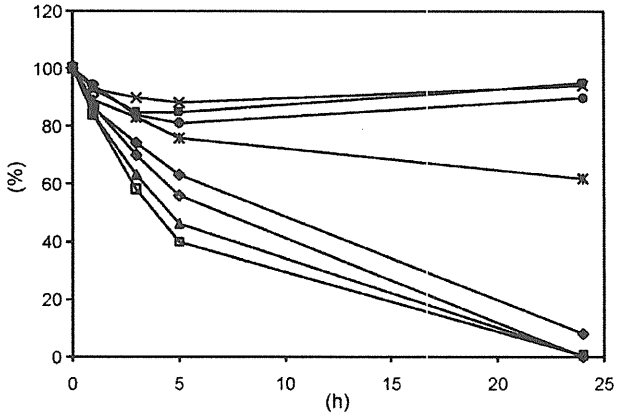

Fig. 2. Stabilities of *N*-OH MDMA oxalate in phosphate buffers at different pHs. ■, pH 3.0; ×, pH 4.0; ●, pH 5.0; *, pH 6.0; ◆, pH 7.0; ▲, pH 8.0; □, pH 9.0; ◇, pH 10.0.

Because of the instability of *N*-OH MDMA in an alkaline environment, it appears likely that this drug would decompose in rat urine samples (pH 8–9). To set suitable conditions for animal studies, the ratios of the remaining drug in the rat control urine (pH 8–9) with added *N*-OH MDMA were analyzed under six different conditions over 24 h (Fig. 3). The ratios of the remaining *N*-OH MDMA of the urine samples kept on ice were approximately 80% after 4 h and 50% after 24 h, while no *N*-OH MDMA was detected in the urine kept at room temperature after 24 h. When the urine samples were heated before adding the drugs to remove the influences of bacteria and endogenous enzymes, the ratios of the remaining drug were almost the same as those of the non-treated urine samples and no *N*-OH MDMA was detected after 24 h at room temperature. Furthermore, when the pH of the urine samples was adjusted to pH 4–5 before adding the drugs, the ratios of the

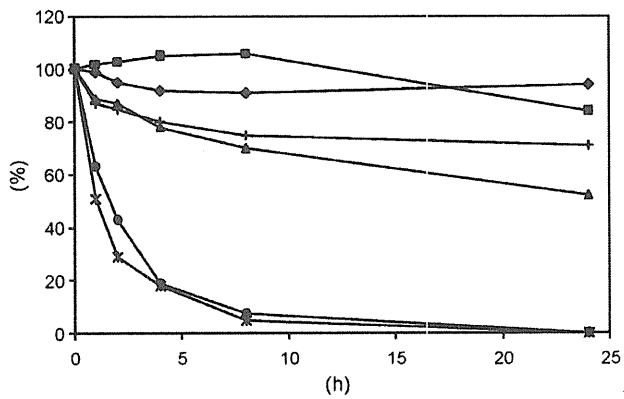

Fig. 3. Stabilities of *N*-OH MDMA oxalate in urine samples kept under different conditions. ●, kept at room temperature; ■, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept at room temperature; ▲, kept on ice; ◆, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept on ice; *, the control urine was heated at 70 °C for 15 min before addition of the drugs and was kept at room temperature, +, the urine was heated at 70 °C for 15 min and 1 mL of 1 M phosphate buffer (pH 3.0) was added before addition of the drugs.

Table 1
Linear ranges, calibration curves, and RSDs of analyses of *N*-OH MDMA and its metabolites in rat plasma, urine, and hair samples.

Compounds	Linear ranges	Calibration curves[a]	Conc. added	Conc. measured	RSDs (%)[b]
Plasma (ng/mL)					
N-OH MDMA	0.5–500		0.5	0.7	17
		$y = 0.5797x + 0.1111$	50.0	69.8	2.4
		$r^2 = 0.9988$	500.0	534.5	4.3
N-OH MDA	0.5–500		0.5	0.7	26
		$y = 0.4413x + 0.0647$	50.0	64.7	3.7
		$r^2 = 0.9983$	500.0	493.3	4.6
MDMA	0.5–500		0.5	0.3	7.1
		$y = 1.2243x + 0.2837$	50.0	58.5	2.3
		$r^2 = 0.9984$	500.0	513.2	2.3
MDA	0.5–500		0.5	0.4	17
		$y = 1.2047x + 0.2660$	50.0	61.8	1.8
		$r^2 = 0.9994$	500.0	530.9	1.8
Urine (ng/mL)					
N-OH MDMA	0.5–500		0.5	0.3	5.5
		$y = 0.8636x + 0.2218$	50.0	44.2	2.2
		$r^2 = 0.9981$	500.0	540.0	2.2
N-OH MDA	0.5–500		0.5	0.3	6.8
		$y = 0.3836x - 0.0111$	50.0	46.2	2.7
		$r^2 = 0.9983$	500.0	449.5	1.1
MDMA	0.5–500		0.5	0.3	6.9
		$y = 0.4741x + 0.2510$	50.0	53.0	2.5
		$r^2 = 0.9920$	500.0	522.7	16
MDA	0.5–500		0.5	0.3	27
		$y = 0.5269x + 0.2294$	50.0	45.2	4.9
		$r^2 = 0.9812$	500.0	465.3	2.1
Hair (ng/mg)					
N-OH MDMA	0.01–0.75		0.01	0.01	4.6
		$y = 4.8339 - 0.0019$	0.05	0.03	7.1
		$r^2 = 0.9900$	0.50	0.42	2.3
N-OH MDA	0.01–0.75		0.01	0.01	4.3
		$y = 2.3578 - 0.0056$	0.05	0.04	5.7
		$r^2 = 0.9892$	0.50	0.56	2.6
MDMA	1–50		1.0	1.2	2.5
		$y = 3.2766 + 0.8837$	5.0	5.2	2.4
		$r^2 = 0.9953$	50.0	53.1	1.5
MDA	1–50		1.0	1.1	2.5
		$y = 2.4426 + 0.2389$	5.0	5.5	1.9
		$r^2 = 0.9980$	50.0	55.3	1.2

[a] Weighting: $1/x$.
[b] RSD: relative standard deviation ($n = 5$).

Table 2
Time courses of drug concentrations in rat urine after the last administration of *N*-OH MDMA (5 mg/kg, *i.p.*).

Rat	Compounds	Concentrations in urine (μg/mL)				
		0–10 h	10–24 h	24–34 h	34–48 h	48–72 h
Rat 1	*N*-OH MDMA	0.05	TR	TR	TR	TR
	N-OH MDA	0.34	0.04	TR	TR	TR
	MDMA	75.3	12.4	1.97	0.68	0.17
	MDA	53.6	13.0	2.09	0.53	0.18
	Volume (mL)	2.5	5.3	2.8	3.6	6.2
Rat 2	*N*-OH MDMA	TR	TR	ND	ND	ND
	N-OH MDA	0.16	0.01	TR	TR	TR
	MDMA	63.4	7.35	1.79	0.73	0.36
	MDA	42.8	7.69	1.64	0.72	0.39
	Volume (mL)	2.7	4.3	3.0	3.6	6
Rat 3	*N*-OH MDMA	TR	TR	TR	ND	ND
	N-OH MDA	0.16	0.03	TR	TR	TR
	MDMA	69.9	12.5	3.43	0.64	0.66
	MDA	31.3	13.4	2.51	0.51	0.51
	Volume (mL)	3.5	3.8	3.0	2.8	3.7

TR: trace level, <10 ng/mL. ND: not detected.

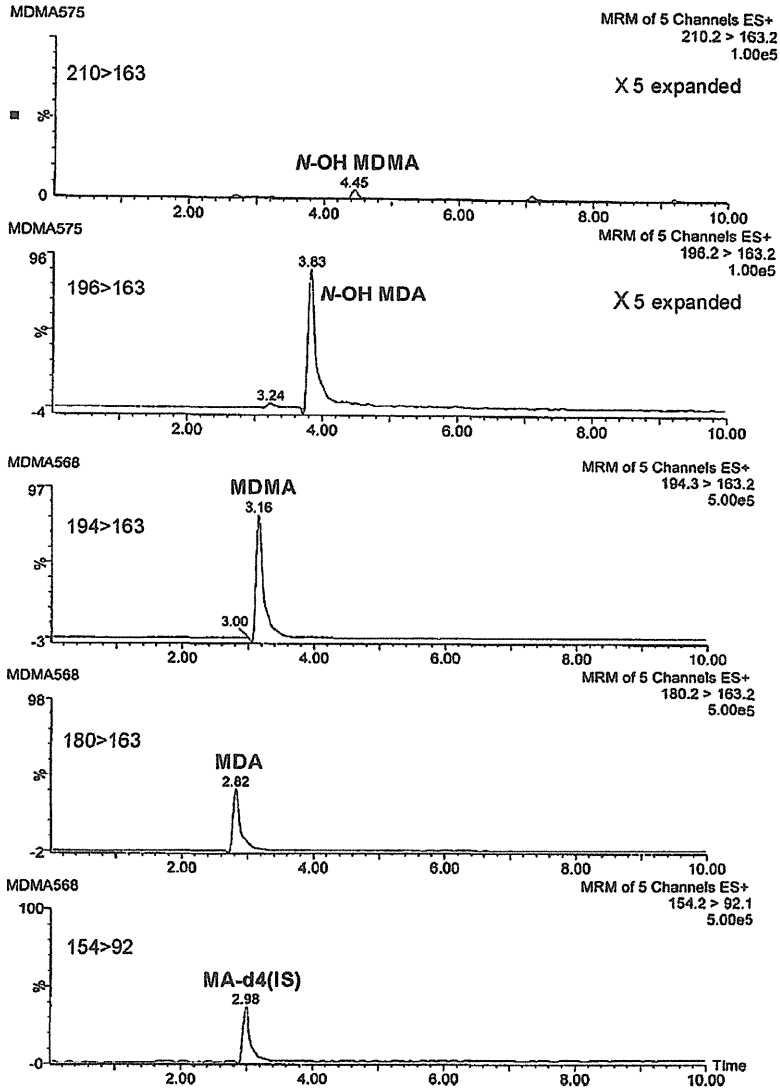

Fig. 4. UPLC–MS/MS MRM chromatograms of the extract from the rat urine 0–10 h after the last administration of *N*-OH MDMA (5 mg/kg, *i.p.*, rat 1).

remaining drugs after 24 h were approximately 95% on ice and 85% at room temperature (Fig. 3). On the basis of these results, adequate volumes of the phosphate buffer (pH 3.0) were added in advance to the collection vials in an ice-bath (4 °C), and the collected urine was kept under the acidic conditions in this study. On the other hand, no serious decomposition of *N*-OH MDMA in the plasma control samples kept on ice or at room temperature was observed for at least 2 h.

3.2. Pre-treatment steps

N-OH MDMA was unstable under the basic conditions, as described above. Moreover, it has been reported that *N*-OH MDA is considerably less basic than MDA and that its capacity factors show greater variation with mobile phase pH values for HPLC analysis using a C18 column [10]. Therefore, for the simultaneous extraction of target drugs from biological samples, a polymer-based solid-phase extraction column (Bond Elut Plexa™) was used without any basic solvents. According to the method described in Section 2, the recoveries of *N*-OH MDMA and its metabolites, *N*-OH MDA, MDMA, and MDA, from the samples added with their standard solutions (100 ng/mL), were 93.0, 85.5, 101.5 and 94.8%, respectively.

3.3. Linearity and precision of the analytical method for the rat urine, plasma, and hair samples

Under the chromatographic conditions used, there was no interference with any of the drugs or the internal standard by any extractable endogenous materials in the control rat plasma, urine, and hair extracts. The calibration curves were linear over the concentration range 0.5–500 ng/mL for rat plasma and urine, and 0.01–0.75 ng/mg (*N*-OH MDMA and *N*-OH MDA) and 1–50 ng/mg (MDMA and MDA) for rat hair with good correlation coefficients of $r^2 \geq 0.981$, respectively. The LODs of each drug were 0.1 ng/mL for the urine and plasma samples and 5 pg/mg for the hair samples, respectively. The precision data from the analytical procedure ($n = 5$) for the rat urine, plasma and hair samples, spiked with standard solution of *N*-OH MDMA, *N*-OH MDA, MDMA, and MDA, are presented in Table 1.

3.4. Time course of excretion of N-OH MDMA and its metabolites into rat urine

After intraperitoneal administration of *N*-OH MDMA oxalate to 3 rats at 5 mg/kg, the concentrations of *N*-OH MDMA and its metabolites in the rat urine were monitored using UPLC–MS/MS.

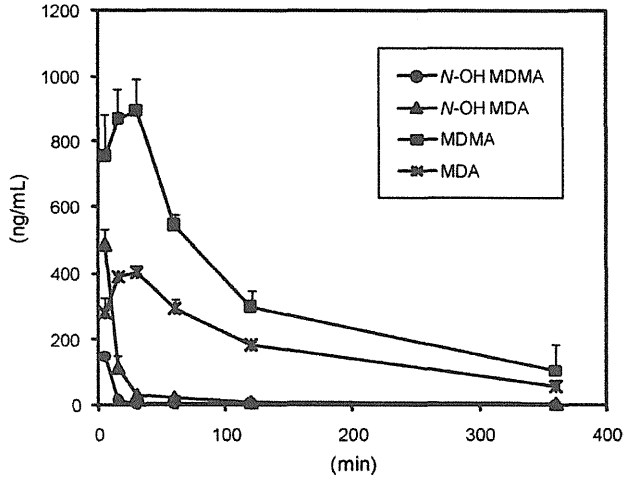

Fig. 5. Time courses of rat plasma drug concentrations after the first administration of *N*-OH MDMA (5 mg/kg, *i.p.*, $n = 3$). The error bar indicates each standard deviation.

The time courses of excretion of *N*-OH MDMA, *N*-OH MDA, MDMA, and MDA in the urine over 72 h are shown in Table 2. Fig. 4 shows LC–MS/MS MRM chromatograms of the extract from the rat urine 0–10 h after the last administration of *N*-OH MDMA (rat 1). The major metabolite excreted in the rat urine was MDMA (the *N*-dehydroxy metabolite), and 63–76 μg/mL of MDMA was detected from 0 to 10 h after administration. MDA (the *N*-dehydroxy and *N*-demethyl metabolite) was also detected at high concentrations of 31–54 μg/mL in the 0–10 h urine. In contrast, *N*-OH MDMA and *N*-OH MDA (the *N*-demethyl metabolite) were slightly detected only in 0–10 h urine and 0–24 h urine, and accounted for approximately 0.01 and 0.16% of the dose, respectively. More than 90% of the dose was excreted as MDMA and MDA in the rat urine in 72 h (Table 3), although other minor metabolites were not examined in this study. *N*-OH MDA has also been reported to be rapidly metabolized to MDA, and no other major metabolites have been detected in rats [11]. The rapid *N*-dehydroxylation of *N*-OH MDMA/*N*-OH MDA would make it difficult to discriminate *N*-OH MDMA/*N*-OH MDA use from MDMA/MDA use by urine analysis.

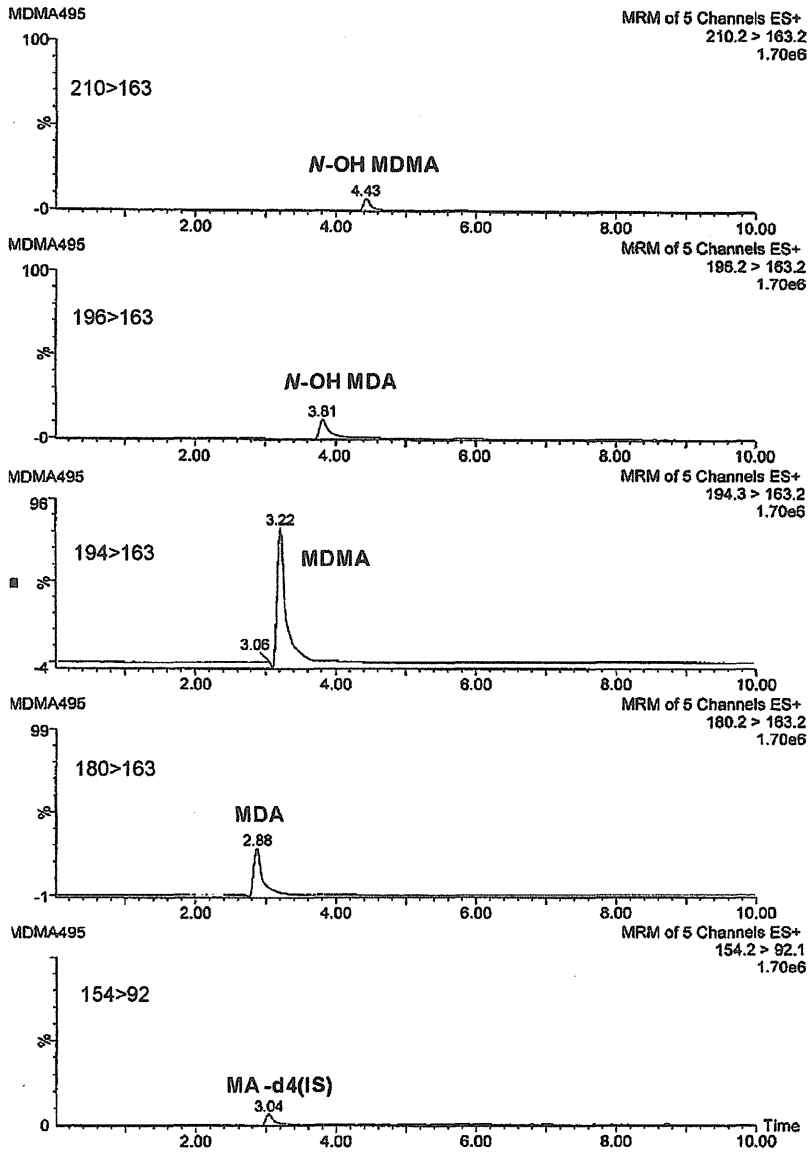

Fig. 6. UPLC–MS/MS MRM chromatograms of the extract from the rat plasma 5 min after the first administration of *N*-OH MDMA (5 mg/kg, *i.p.*, rat 1).

Table 3
The amounts of *N*-OH MDMA and its metabolites in urine, plasma, and hair.

Rat	Compounds	Total excretion into urine (μg, 0–72 h)	Plasma AUC (μg min/mL)	Concentrations in hair (ng/mg)
Rat 1	*N*-OH MDMA	0.1	1.1	0.03 ± 0.00
	N-OH MDA	1.1	7.2	0.07 ± 0.00
	MDMA	263.0	149.6	163.3 ± 1 7.3
	MDA	211.8	74.0	48.7 ± 0.7
Rat 2	*N*-OH MDMA	0.0	1.4	0.02 ± 0.00
	N-OH MDA	0.5	7.5	0.08 ± 0.00
	MDMA	212.9	130.9	117.6 ± 17.6
	MDA	158.8	77.6	41.4 ± 1.7
Rat 3	*N*-OH MDMA	0.0	1.4	0.03 ± 0.006
	N-OH MDA	0.7	10.2	0.23 ± 0.02
	MDMA	306.7	143.2	164.5 ± 11.1
	MDA	171.5	89.0	66.3 ± 1.4

3.5. Concentrations of N-OH MDMA and its metabolites in rat plasma

Fig. 5 shows the time courses of the rat plasma concentrations of *N*-OH MDMA and its metabolites over 360 min after the first administration of *N*-OH MDMA oxalate at 5 mg/kg. LC–MS/MS MRM chromatograms of the extract from the rat plasma 5 min after the administration of *N*-OH MDMA (Rat 1) are shown in Fig. 6. The concentrations of *N*-OH MDMA and *N*-OH MDA were

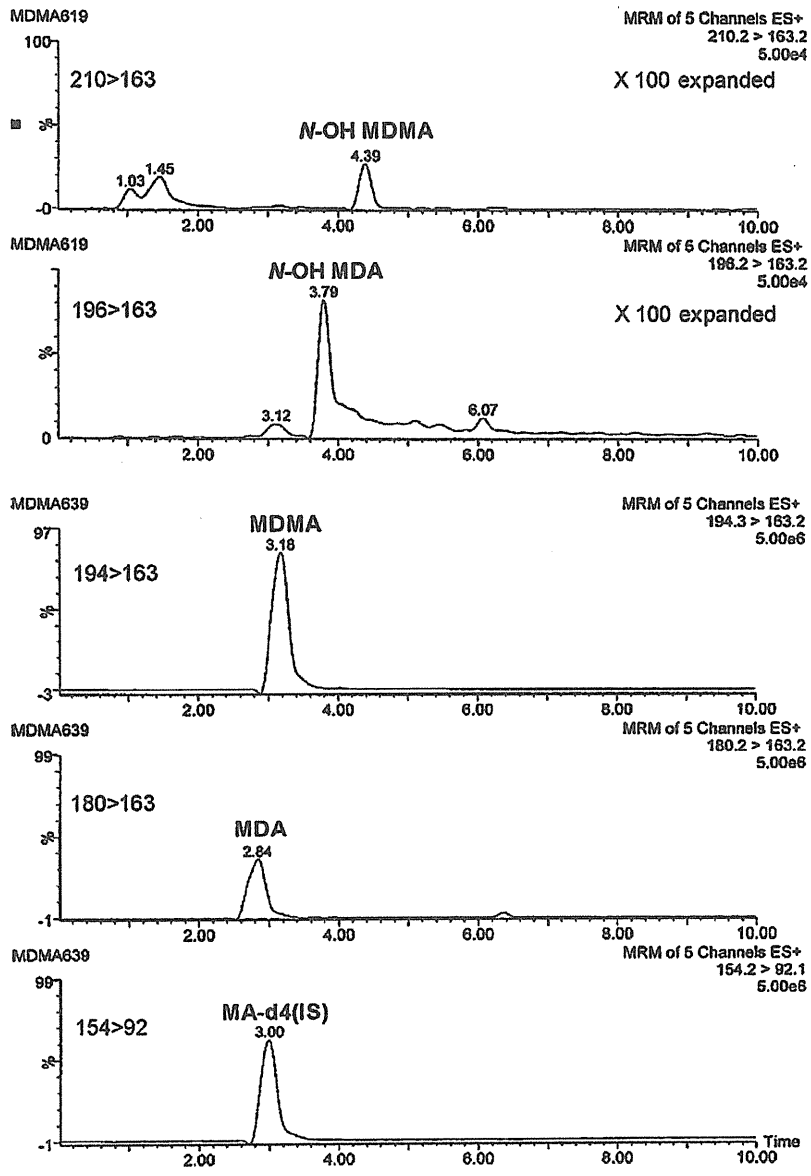

Fig. 7. UPLC–MS/MS MRM chromatograms of the extract from the rat hair collected 4 weeks after the first administration of *N*-OH MDMA (5 mg/kg × 10 days, *i.p.*, rat 1).

extremely low and their average peak concentrations ($n = 3$) were 130 ng/mg at 5 min and 490 ng/mL at 5 min, respectively. It was difficult to detect *N*-OH MDMA in the plasma at 120 min after administration. The concentrations of the major metabolites, MDMA and MDA in the plasma showed peaks (970 and 410 ng/mL) within 30 min. The AUC values of *N*-OH MDMA, *N*-OH MDA, MDMA, and MDA in the rat plasma were 1.1–1.4, 7.2–10.2, 130.9–149.6, and 74.0–89.0 μg min/mL, respectively, as shown in Table 3. The AUC values of MDMA and MDA were approximately 110 and 65 times larger than those of *N*-OH MDMA, respectively.

3.6. Drug concentrations in rat hair

Various procedures for the extraction of drugs from hair samples have been reported, including digestion with alkali, acid extraction, and enzymatic treatment [19,20]. Because *N*-OH MDMA is unstable under alkaline conditions, the procedures using alkali digestion (above pH 10) and enzymatic treatment (above pH 7) may not be acceptable for the extraction of *N*-OH MDMA. We have reported that the mixed solution of methanol and 5 M hydrochloric acid (20:1) is suitable for the extraction of phenethylamine-type compounds from hair samples [20,16]. Therefore, in this study, the acidic organic solvent was used for the extraction of *N*-OH MDMA and its metabolites from the rat hair. To investigate the stability of these drugs during the extraction procedure described in Section 2, the rat control hair samples, with added *N*-OH MDMA and its metabolites (10 ng/mg each), were analyzed. As a result of the analysis, almost no degradation of the *N*-OH compounds was observed.

Fig. 7 shows UPLC–MS/MS MRM chromatograms of the extract from the rat hair collected 4 weeks after the first administration of *N*-OH MDMA (5 mg/kg × 10 days, *i.p.*, rat 1). In the rat hair samples, although MDMA (149 ng/mg) and MDA (52 ng/mg) were mainly detected in large quantities, *N*-OH MDMA (0.03 ng/mg) and *N*-OH MDA (0.13 ng/mg) were also clearly detected. The detection of *N*-OH compounds from the hair samples might provide useful information for distinguishing *N*-OH MDMA use from MDMA use over a long period. However, it has been reported that *N*-OH MDMA and *N*-OH MDA are also detectable as *N*-hydroxylated metabolites in the urine of horses, orally administered with MDMA [21]. It may therefore be difficult to conclude whether *N*-OH MDMA detected in biological samples is the parent compound or the *N*-hydroxylated metabolite of MDMA. In further studies, the ratios of the parent compound to the metabolites in the samples obtained from *N*-OH MDMA users and MDMA users should be examined to deduce the source of the compound detected.

4. Conclusions

In this study, we have established a detailed procedure for the analysis of *N*-OH MDMA, *N*-OH MDA, MDMA, and MDA in rat urine, plasma, and hair samples using UPLC–MS/MS. Moreover, the established method was applied to investigate the metabolic properties of *N*-OH MDMA in rats. *N*-OH MDMA in biological samples could be precisely analyzed by avoiding alkaline environments. *N*-OH MDMA very rapidly disappeared from rat plasma and urine, and most of the *N*-OH MDMA was excreted in rat urine as MDMA and MDA in 72 h. The rapid *N*-dehydroxylation of *N*-OH MDMA would make it difficult to discriminate *N*-OH MDMA use from MDMA use by urine analysis. In the rat hair samples collected 4 weeks after the first administration, *N*-OH MDMA and *N*-OH MDA were clearly detected as well as MDMA and MDA, which were found to be the major metabolites in hair. The proposed analytical method will be useful for the analysis of *N*-OH MDMA and its metabolites in biological samples.

Acknowledgement

Part of this work was supported by a Health and Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare in Japan.

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Forensic Science International 198 (2010) 31–38

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Chemical analysis of synthetic cannabinoids as designer drugs in herbal products

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ARTICLE INFO

Article history:
Received 19 August 2009
Received in revised form 5 January 2010
Accepted 7 January 2010

Keywords:
Cannabicyclohexanol
JWH-018
Oleamide
CP-47,497
JWH-073
Synthetic cannabinoid

ABSTRACT

Several synthetic cannabinoids were found in 44 of 46 different kinds of herbal products that are currently distributed on the illegal drug market in Japan due to their expected narcotic effects. Gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS) analyses indicated that most of the products contained two major synthetic cannabinoids: (1*RS*,3*SR*)-3-[2-hydroxy-4-(2-methylnonan-2-yl)phenyl]cyclohexan-1-ol, renamed cannabicyclohexanol with the agreement of Pfizer Inc., and/or 1-naphthalenyl(1-pentyl-1*H*-indol-3-yl)methanone, named JWH-018. Oleamide (*cis*-9,10-octadecenoamide), which is an endogenous cannabinoid, was also detected in 7 products. Additionally, two synthetic cannabinoids were identified as minor components in some products. One was (1*RS*,3*SR*)-3-[2-hydroxy-4-(2-methyloctan-2-yl)phenyl]cyclohexan-1-ol, which is named CP-47,497 and is a homolog of cannabicyclohexanol. The other was 1-naphthalenyl(1-butyl-1*H*-indol-3-yl)methanone, which is named JWH-073 and is a homolog of JWH-018. These compounds were reported as synthetic cannabinoids possessing pharmacological cannabimimetic activity. The concentrations of cannabicyclohexanol, JWH-018 and oleamide in the products ranged from 1.1 to 16.9 mg/g, 2.0 to 35.9 mg/g and 7.6 to 210.9 mg/g, respectively, and showed considerable variation. In this study, details of the analysis and identification of these synthetic cannabinoids in herbal products being sold on the Japanese drug market are described.

1. Introduction

Cannabis sativa L. (cannabis, marijuana) is widely abused throughout the world because it contains psychoactive cannabinoids such as Δ^9-tetrahydrocannabinol. In Japan, the abuse of cannabis has recently increased, along with the abuse of a number of herbal products that are also distributed on the drug market for their cannabis-like effects when smoked. Although the active components of these herbal products were not identified, we recently found two synthetic cannabinoids as adulterants in herbal products that had been sold commercially as incense. One was (1*RS*,3*SR*)-3-[2-hydroxy-4-(2-methylnonan-2-yl)phenyl]cyclohexan-1-ol (cannabicyclohexanol, **1**), which was detected together with its *trans*-diastereomer (**2**) [1]; the other was 1-naphthalenyl(1-pentyl-1*H*-indol-3-yl)methanone (JWH-018, **3**) [2] (Fig. 1). Compound **1**, which is a non-classical cannabinoid, was first synthesized by Pfizer Inc. in 1979 [3] and reported as a potent cannabinoid analog in the 1990s [4–8]. In consideration of its general properties, this compound was renamed cannabicyclohexanol with the agreement of Pfizer Inc. Compound **3**, which is an aminoalkyl naphthoyl indole derivative, was first synthesized by Huffman et al. in 1998 and reported as a potent cannabinoid receptor agonist possessing *in vivo* pharmacological cannabinoid analog activity [9–12]. Auwärter et al. also reported the identification of these compounds from some herbal products around the same time [13]. In January 2009, Germany's Health Minister announced that compounds **1** and **3** and their homologs had been identified as active components in a mislabeled mixture of herbs. Control of these compounds was begun in Germany immediately thereafter (on 22 January 2009) [14]; Austria, France and other countries initiated legal actions to ban or otherwise control these synthetic cannabinoids over the preceding months [15].

In this study, we report the analysis and identification of several synthetic cannabinoids as adulterants in herbal products using gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS). In addition, the results of a survey of herbal products being sold on the Japanese market are described.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetonitrile, betamethasone valerate (internal standard, IS) and α-tocopherol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Authentic cannabicyclohexanol (**1**) and JWH-018 (**3**) were isolated from herbal

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doi:10.1016/j.forsciint.2010.01.004

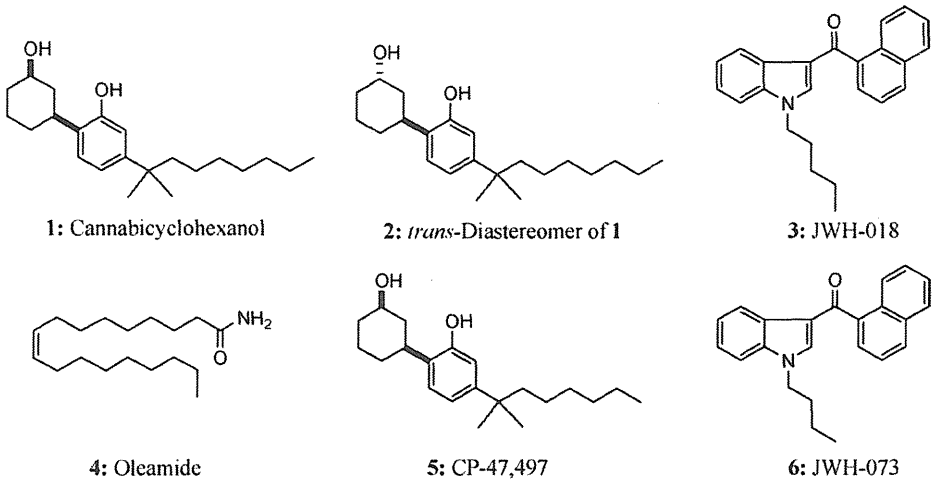

Fig. 1. Structures of detected cannabimimetic compounds.

products and identified in our previous studies [1,2]. Oleamide (**4**) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). CP-47,497 (**5**) and JWH-073 (**6**) were purchased from Cayman Chemical (Ann Arbor, MI). All other common chemicals and solvents were of analytical reagent grade or HPLC-grade.

2.2. Samples

Forty-six herbal products being sold in Japan for their expected cannabis-like effects were purchased via the Internet from June 2008 to June 2009. All products had different names and were contained in different packages. Thirty-nine products were in the form of dried leaves and 7 products were in the form of cigarettes. In most cases, the labels on the packages indicated that the products contained between 1 and 3 g of a mixture of plants.

2.3. Instrumentation

The sample solutions were qualitatively and quantitatively analyzed by using an ultra-performance liquid chromatography-electrospray ionization-mass spectrometer (UPLC-ESI-MS), consisting of an ACQUITY UPLC system equipped with a Single Quadrupole Detector (SQD) mass detector and a photo diode array (PDA) (Waters, Milford, MA). The sample solutions were separated using an ACQUITY UPLC HSS T3 column (2.1 mm i.d. × 100 mm, 1.8 μm; Waters) protected by a Van Guard column (2.1 mm i.d. × 5 mm, 1.8 μm; Waters) at 40 °C. The following gradient system was used with a mobile phase A (0.1% formic acid in water) and a mobile phase B (0.1% formic acid in acetonitrile) delivered at 0.3 ml/min: A:B 50:50 (0–3 min) followed by −20:80 (5–10 min). The injection volume was 1 μl. The wavelength of the PDA detector for screening was set from UV–vis 190 to 500 nm. ESI mass analysis was carried out in positive mode. Nitrogen gas was used for desolvation at a flow rate of 650 l/h at 350 °C. The capillary voltage was 3000 V, and the cone voltage was 30 V. MS data were recorded in the full scan mode (*m/z* 50–500). For qualitative analysis of cannabicyclohexanol (**1**) and JWH-018 (**3**), the protonated molecular peaks ([M+H$^+$]) of these compounds and IS were monitored in the scan mode. The monitoring ions were as follows: cannabicyclohexanol (**1**) (*m/z* 333), JWH-018 (**3**) (*m/z* 342) and betamethasone valerate (IS, *m/z* 477).

MS analysis was also performed by GC-MS in electron impact (EI) mode at 70 eV electron energy. The GC-MS analysis was performed on a Hewlett-Packard 6890N GC with a 5975 mass selective detector using a capillary column (HP1-MS capillary; 30 m × 0.25 mm i.d., 0.25 μm film thickness) at 0.7 ml/min and helium gas as a carrier. The injector temperature was 200 °C and splitless injection was employed with a split value on-time of 1.0 min. The initial column temperature was 80 °C (held for 1 min), and was increased at a rate of 5 °C/min to 190 °C (held for 15 min) followed by 10 °C/min to 310 °C (held for 5 min). The mass selective detector was kept at 280 °C. Data were obtained in full scan mode with a scan range of *m/z* 40–550. The analysis was performed under the established methods and conditions as used in the analysis of designated drugs (Shitei-Yakubutsu) controlled by the Pharmaceutical Affairs Law of Japan described in our previous report [16]. In the case of oleamide (**4**), the relative content was calculated from the area of the target molecular ion peak to that of the standard sample (1 mg/ml methanol) following GC-MS.

2.4. Standard solutions

For qualitative analysis, standard solutions were prepared for each compound (cannabicyclohexanol (**1**), JWH-018 (**3**), oleamide (**4**), CP-47,497 (**5**), JWH-073 (**6**) and α-tocopherol) at a concentration of 1.0 or 0.1 mg/ml in methanol.

2.5. Calibration curves

The concentrations of cannabicyclohexanol (**1**) and JWH-018 (**3**) in the samples were calculated using the peak area ratios of **1** versus IS at 275 nm, and those of **3** at 314 nm versus IS at 240 nm, respectively. Cannabicyclohexanol (**1**) and JWH-018 (**3**) were diluted with methanol to prepare calibration solutions containing 10, 25, 50, 100, 250 and 500 μg/ml. The solutions also included IS (betamethasone valerate) at 100 μg/ml.

2.6. Precision and accuracy of the method

The precision and accuracy of the method were evaluated by analyzing triplicates of the standard solutions containing 10, 50, 500 μg/ml of each compounds. Accuracy, expressed as bias, was calculated as difference between the amounts of each compound added and recovered.

2.7. Preparation of sample solution

For quantitative analysis, the product (10 mg) was crushed into powder and extracted with 1 ml of methanol including IS (100 μg/ml) under ultrasonication for 10 min. After centrifugation (5 min at 3000 rpm), the solution was passed through a centrifugal filter (Ultrafree-MC, 0.45 μm filter unit; Millipore, Bedford, MA). For qualitative analysis, the product (50 mg) was crushed into powder and extracted with 2 ml of methanol under ultrasonication for 10 min. After evaporation to dryness, the extract was dissolved with 200 μl of methanol and the solution was filtered through the centrifugal filter. If necessary, the solution was diluted with methanol to a suitable concentration.

3. Results

3.1. Analyses of herbal products obtained from the Japanese market

Forty-six herbal products currently being sold in Japan for their expected cannabis-like effects were purchased via the Internet. These products appeared in primarily two forms i.e., as bits of dried leaves or as cigarettes (Table 1).

GC-MS and LC-MS analyses indicated that most of the products contained the two major compounds (**1** and **3**) (Figs. 1 and 2a–p) and these compounds were reported in our previous studies [1,2]. Oleamide (*cis*-9,10-octadecenoamide, **4**), which shows cannabinoid-like behavioral effects [17,18], was also detected at 42.96 min in some products by GC-MS analysis, and this was also in agreement with a previous study (Figs. 1 and 2a and e) [13]. Compound **4** was not clearly detected by LC-MS analysis. Compound **2**, which is detected at 47.30 min, was considered as *trans*-diastereomer of **1** based on comparison with the previously determined mass spectrum of reported data of the compound (**2**) (Figs. 1 and 2a, c, f, h, l, and o) [13]. Furthermore, it was revealed that 37 products contained α-tocopherol by the direct comparison of the GC-MS data to those of the authentic sample (Table 1).

Table 1
List of detected compounds in the herbal products.[a].

Product no.	Form	Cannabicyclohexanol (**1**) (mg/g)	Ratio of the *cis*-form (**1**)[b]	JWH-018 (**3**) (mg/g)	Oleamide (**4**)[c] (mg/g)	Other compounds	
1	Dried leaf (cutting)	3.86 ± 0.72	++	n.d.[d]	n.d.	CP-47,497 (**5**)	α-Tocopherol
2	Dried leaf (cutting)	15.17 ± 1.96	++	n.d.	n.d.	α-Tocopherol	
3	Dried leaf (cutting)	16.93 ± 0.23	+++	n.d.	n.d.	α-Tocopherol	
4	Dried leaf (cigarette of no. 2)	15.17 ± 1.96	++	n.d.	n.d.	α-Tocopherol	
5	Dried leaf (cigarette of no. 3)	12.49 ± 1.26	+	<1.00	n.d.	α-Tocopherol	
6	Dried leaf (cutting)	10.89 ± 0.22	++	<1.00	n.d.	α-Tocopherol	
7	Dried leaf (cutting)	8.55 ± 0.89	++	n.d.	n.d.	α-Tocopherol	
8	Dried leaf (cutting)	4.11 ± 0.12	++	16.37 ± 0.75	n.d.	α-Tocopherol	
9	Dried leaf (cutting)	3.92 ± 0.07	++	12.23 ± 0.04	n.d.	α-Tocopherol	
10	Dried leaf (cutting)	n.d.	n.d.	16.85 ± 1.37	n.d.	α-Tocopherol	
11	Dried leaf (cutting)	5.96 ± 1.00	++	15.96 ± 1.82	n.d.	α-Tocopherol	
12	Dried leaf (cutting)	4.23 ± 0.42	+++	n.d.	n.d.	α-Tocopherol	
13	Dried leaf (cigarette of no. 12)	3.85 ± 0.92	++	n.d.	n.d.	α-Tocopherol	
14	Dried leaf (cutting)	9.77 ± 0.15	+++	n.d.	n.d.	α-Tocopherol	
15	Dried leaf (cutting)	7.95 ± 1.38	+	n.d.	n.d.		
16	Dried leaf (cutting)	6.46 ± 0.40	+	n.d.	n.d.	α-Tocopherol	
17	Dried leaf (cutting)	6.59 ± 0.70	++	n.d.	n.d.	α-Tocopherol	
18	Dried leaf (cigarette of no. 17)	6.87 ± 0.64	++	n.d.	n.d.	α-Tocopherol	
19	Dried leaf (cigarette)	3.33 ± 0.51	++	n.d.	n.d.	α-Tocopherol	
20	Dried leaf (cutting)	5.14 ± 0.93	++	<1.00	n.d.	α-Tocopherol	
21	Dried leaf (cutting)	11.42 ± 0.30	+++	<1.00	n.d.	α-Tocopherol	
22	Dried leaf (cigarette)	10.27 ± 0.30	++	<1.00	n.d.	α-Tocopherol	
23	Dried leaf (cutting)	n.d.	n.d.	11.92 ± 0.89	188.56	α-Tocopherol	
24	Dried leaf (cutting)	n.d.	n.d.	13.54 ± 0.50	141.58	α-Tocopherol	
25	Dried leaf (cutting)	n.d.	n.d.	10.22 ± 0.84	75.33	α-Tocopherol	
26	Dried leaf (cutting)	n.d.	n.d.	10.08 ± 0.56	n.d.	α-Tocopherol	
27	Dried leaf (cutting)	n.d.	n.d.	12.77 ± 0.91	n.d.	α-Tocopherol	
28	Dried leaf (cutting)	n.d.	n.d.	27.91 ± 1.09	210.90		
29	Dried leaf (cutting)	n.d.	n.d.	14.63 ± 0.79	203.98		
30	Dried leaf (cutting)	n.d.	n.d.	n.d.	n.d.		
31	Dried leaf (cutting)	n.d.	n.d.	n.d.	n.d.		
32	Dried leaf (cutting)	3.03 ± 0.42	+	n.d.	n.d.		
33	Dried leaf (cutting)	3.78 ± 0.85	+	n.d.	n.d.		
34	Dried leaf (cutting)	6.71 ± 0.28	+++	n.d.	n.d.	α-Tocopherol	
35	Dried leaf (cutting)	7.70 ± 0.40	+++	n.d.	n.d.	α-Tocopherol	
36	Dried leaf (cutting)	6.65 ± 0.84	+++	n.d.	n.d.	α-Tocopherol	
37	Dried leaf (cutting)	7.50 ± 0.27	+++	<1.00	n.d.	α-Tocopherol	
38	Dried leaf (cutting)	8.09 ± 0.12	+	n.d.	n.d.	α-Tocopherol	
39	Dried leaf (cutting)	5.13 ± 0.16	+	n.d.	n.d.	α-Tocopherol	
40	Dried leaf (cutting)	6.64 ± 0.40	+	n.d.	n.d.	α-Tocopherol	
41	Dried leaf (cutting)	n.d.	n.d.	11.81 ± 1.55	157.86		
42	Dried leaf (cutting)	n.d.	n.d.	35.90 ± 1.05	n.d.		
43	Dried leaf (cutting)	7.65 ± 0.51	+	n.d.	n.d.	α-Tocopherol	
44	Dried leaf (cutting)	1.09 ± 0.24	+++	2.03 ± 0.62	17.64	α-Tocopherol	
45	Dried leaf (cutting)	5.60 ± 0.85	+	n.d.	n.d.	JWH-073 (**6**)	α-Tocopherol
46	Dried leaf (cigarette of no. 27 + no. 45)	2.98 ± 0.60	+	7.59 ± 1.26	n.d.	JWH-073 (**6**)	α-Tocopherol

[a] Data given as mean ± standard deviation, $n = 3$.
[b] The number of '+' indicates a ratio of the *cis*-form (**1**) as estimated from signal intensities in the corresponding GC-MS chromatograms. +++: >90%, ++: <90% ~ 80%, +: <80% ~ 65%.
[c] Relative content of oleamide (**4**) in product.
[d] Not detected.

3.2. Identification of unknown peaks in several samples

In the GC-MS chromatogram of product no. 1, minor unknown peaks at 46.26 min (**5**) and 46.07 min (**7**) were detected with major peaks at 47.45 and 47.30 min corresponding to compounds **1** and **2**, respectively (Fig. 3a). These peaks (**5** and **7**) showed two specific major EI-MS signals at *m/z* 215 and 233 (Fig. 3b and c) the same as those from compounds **1** and **2** (Fig. 2b and c), with a putative molecular ion peak at *m/z* 318. In addition, the other minor EI-MS signals of the unknown peaks are quite similar to those of compounds **1** and **2**. This data suggested that the side chains of **5** and **7** have one methylene unit less than compounds **1** and **2**. This estimation was supported by the fact that a corresponding peak showing a UV spectrum very similar to that of compound **1** (Figs. 3g and 2k) and having a quasi-molecular ion peak at *m/z* 319.4 (Fig. 3i) existed in the LC-PDA and LC-MS chromatograms at 7.0 min of the product no. 1 (Fig. 3e and f). Therefore, we purchased authentic CP-47,497 and analyzed it to compare the data to those of our unknown peaks. The results shown in Fig. 3d, h, and j are determinately identical of those of the peak of **5**. Therefore, the peak of **5** is identified as CP-47,497, namely (1*RS*,3*SR*)-3-[2-hydroxy-4-(2-methyloctan-2-yl)phenyl]cyclohexan-1-ol, and the peak of **7** is deduced as its *trans*-diastereomer. Compound **5** was reported to be a potent cannabinoid receptor-binding substance [4–6] and has been identified as an adulterant in herbal products being sold in Germany [13]. It is worth noting that other detected peaks (**8**–**10**; Fig. 3e and f) in the LC-ESI-MS chromatograms showed UV and ESI mass spectra very similar to those of **5** (data not shown); however, these peaks were not identified.

Similar reasoning and analyses were used to determine the unknown peak of **6** in the chromatograms of products nos. 45 and 46. First, it was deduced that the peak was a desmethylene

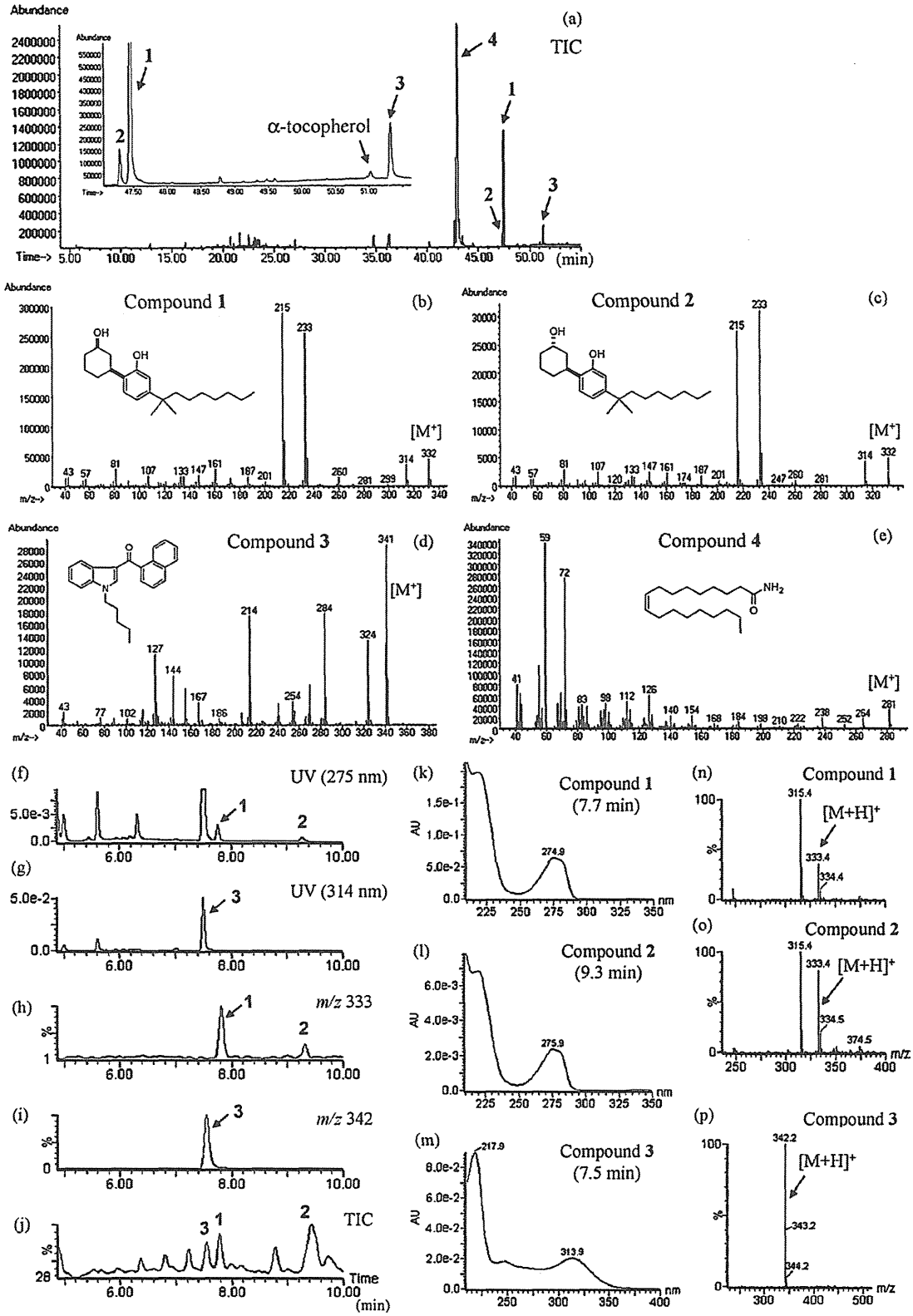

Fig. 2. GC-MS chromatogram of the extract of the product no. 44 (a), and EI mass spectra of the detected peaks at 47.45 min (b, **1**), 47.30 min (c, **2**), 51.31 min (d, **3**) and 42.96 min (e, **4:** oleamide). UPLC-UV (f and g) and MS chromatograms of the extract of the product no. 44. Mass chromatograms of *m/z* 333 (**1** and **2**) (h), *m/z* 342 (**3**) (i) and total ion chromatogram (j). UV spectra (k–m) and ESI mass spectra (n–p) of each peak are shown.

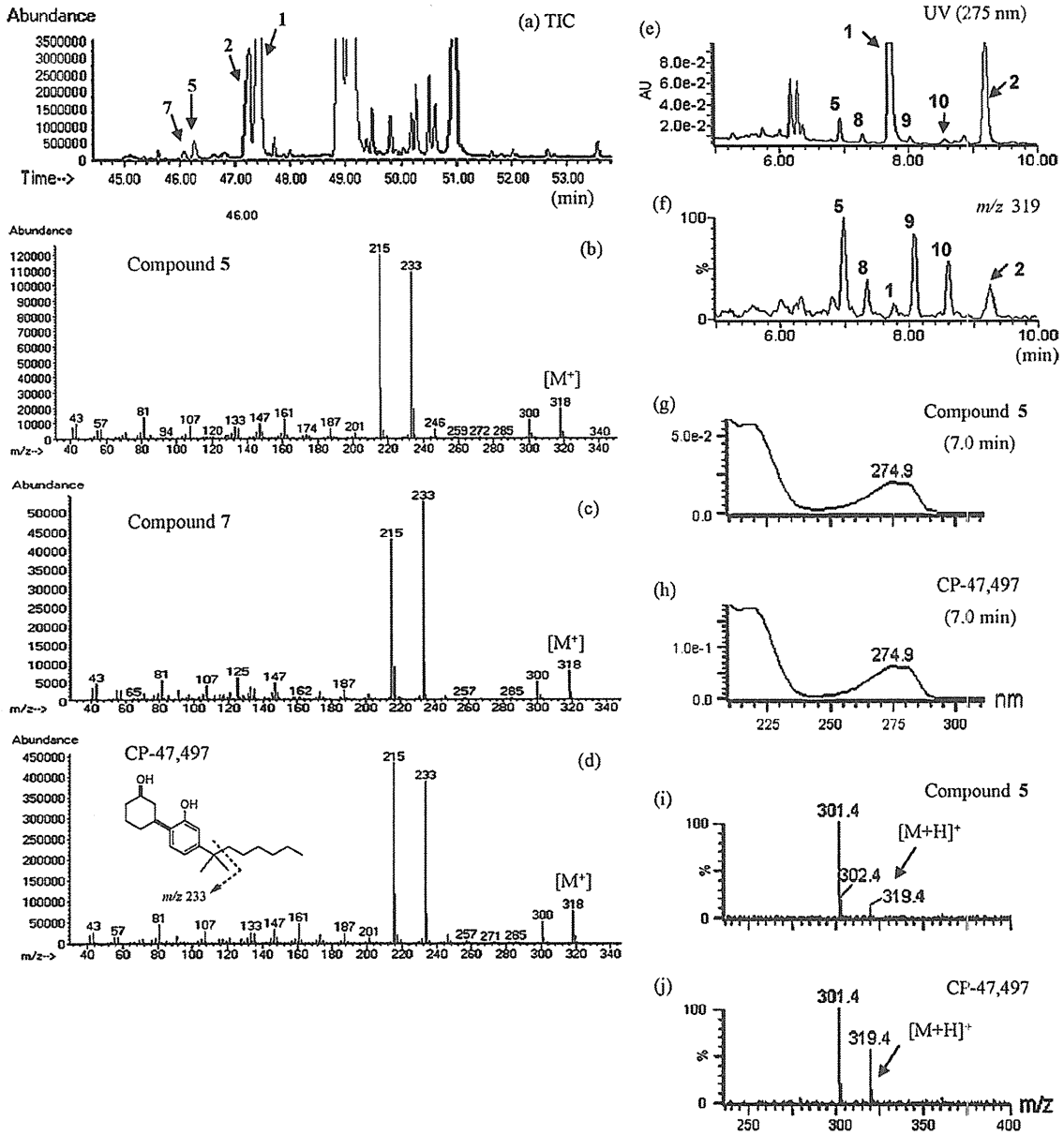

Fig. 3. GC-MS chromatograms of the extract of the product no. 1 (a). EI mass spectra of the detected peaks at 46.26 min (b, **5**), 46.07 min (c, **7**: putative diastereomer of **5**) and the standard of CP-47,497 (RT: 46.25 min, d). UPLC-UV (e) and mass chromatogram of *m/z* 319 (f) of the extract of the product no. 1. UV and ESI mass spectra of the detected peak at 7.0 min of the product no. 1 (**5**) (g and i) and the standard of CP-47,497 (h and j).

compound of **3** on the basis of GC-MS and LC-MS data (Fig. 4a, b, d–f, and h) and finally was determined to be JWH-073, namely 1-naphthalenyl(1-butyl-1*H*-indol-3-yl)methanone (**6**) by direct comparison of the data (Fig. 4c, g and i) to those of the authentic, purchased sample. Compound **6** has also been reported as a synthetic cannabinoid receptor agonist [9,10]. Most recently, Lindigkeit et al. reported the identification of **6** in herbal products available on the German market [19].

3.3. Validation of the method

As shown in Table 2, the calibration curves were linear over concentration range 10–500 μg/ml for product with good coefficients of determination of $r^2 \geq 0.997$. The precision of the compounds ranged from 2.3 to 11.9% and accuracy ranged from −6.9 to 4.4% (Table 2). These data were generally satisfactory.

3.4. Quantitative analysis of the synthetic cannabinoids

Compounds **1**, **3** or **4** were found in 44 of the 46 products (Table 1). Most of the products contained either one or two of these compounds. Twenty-three of the products contained only compound **1** and four products contained only compound **3**. Ten products contained compounds **1** and **3**, and six products contained compounds **3** and **4** (Table 1). Only product no. 44 contained all three compounds (**1**, **3** and **4**) (Table 1; Fig. 2). The concentration of **1** in these products ranged from 1.09 to 16.93 mg/g, and that of **3** ranged from 2.03 to 35.90 mg/g. The relative concentration of **4** in these products ranged from 7.64 to 210.90 mg/g, and was higher than the concentrations of **1** and **3** except in the case of product no. 44 (Table 1). Thus the results showed that the concentrations of compounds **1**, **3** and **4** varied considerably among the products.

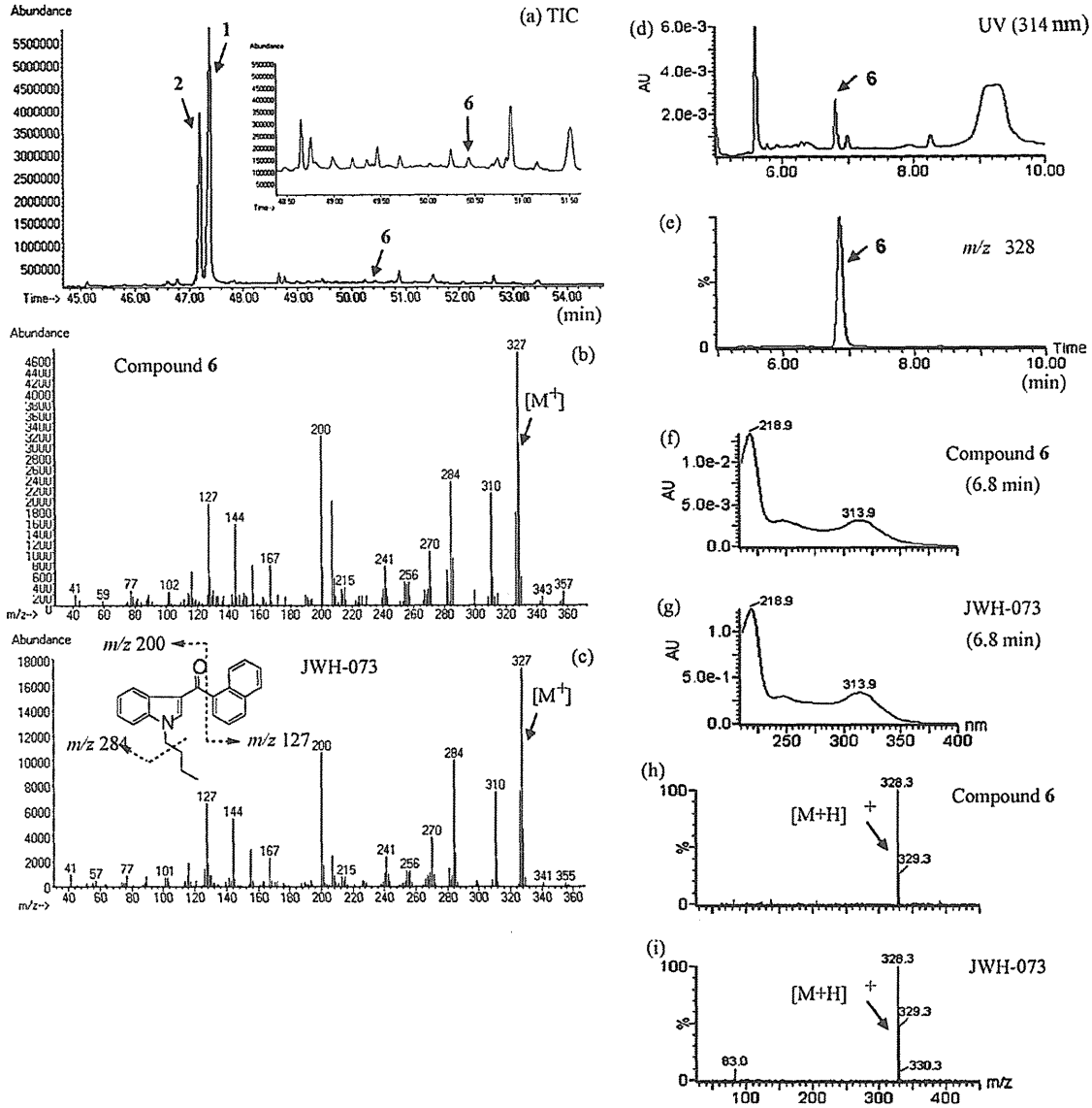

Fig. 4. GC-MS chromatogram of the extract of the product no. 45 (a), EI mass spectrum of the detected peak at 50.43 min (b, **6**) and the standard of JWH-073 (RT: 50.45 min, c). UPLC-UV (d) and mass chromatogram of *m/z* 328 (e) of the extract of the product no. 45. UV and ESI mass spectra of the detected peak at 6.8 min of the product No. 45 (**6**) (f and h) and the standard of JWH-073 (g and i).

4. Discussion

Compound **1** has been reported as a cannabimimetic agent that produces more potent effects than those of typical cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC). Compton et al. reported that compound **1** showed an 8.5-fold more potent affinity for the CB receptor (Ki = 4.7 nM) and **5** had a 4.3-fold more potent affinity for the CB receptor (Ki = 9.5 nM) than did Δ^9-THC (Ki = 40.7 nM) [4]. Additionally, they reported that compounds **1** and **5** were approximately 5-fold and 2-fold more potent than Δ^9-THC, respectively, and showed similar efficacy from the viewpoint of pharmacological activity [5]. In the case of compound **1**, the effective dose in 50% of subjects (ED_{50}) values for induction of locomotor activity, tail-flick latency, hypothermia and ring

Table 2
Precision and accuracy of the LC-UV analysis of cannabicyclohexanol (**1**) and JWH-018 (**3**).

Compounds	Linear range (μg/ml)	Linearity	Concentration (μg/ml)	Precision (%)	Accuracy (%)
Cannabicyclohexanol (**1**)	10–500	$y = 0.0122x + 0.0522$, $r^2 = 0.9994$	10	5.3	−5.9
			50	4.4	3.7
			500	2.3	−6.9
JWH-018 (**3**)	10–500	$y = 0.0133x + 0.0414$, $r^2 = 0.9970$	10	7.6	4.4
			50	11.9	−1.2
			500	2.5	0.4

immobility ranged from 0.1 to 0.3 mg/kg when the compound was administered intravenously (i.v.) to mice [5]. In the present study, the concentration of **1** was 1.09–16.93 mg/g in those products that contained it (Table 1). Thus, approximately 0.2–8 mg of the products might be enough to show pharmacological effects in mice (30 g weight). Compound **2** (*trans*-diastereomer of **1**), which was detected in 34 products together with **1**, may be produced as a by-product of the synthesis procedure of **1**, which is a sodium borohydride reduction of the ketone precursor [20]. The ratio of the *cis*-form (**1**) is shown in Table 1. Nine products contained more than 90% ratio of *cis*-form (**1**), 14 products contained from 80% to 90% ratio of *cis*-form (**1**) and 11 products contained from 65% to less than 80% ratio of *cis*-form (**1**). There has been no report on the pharmacological effects of **2**. However, Melvin et al. reported that the *trans*-diastereomer of **5** has weak cannabinoid-like effects, such as anagogic activity [20], and thus **2** may have similar effects. Compounds **3** and **6** have approximately four times greater affinity for the CB_1 receptor (Ki = 9 ± 5 nM and 8.9 ± 1.8 nM, respectively) than Δ^9-THC (Ki = 41 ± 2 nM) [9]. Furthermore, **3** produced potent cannabinoid effects on locomotor activity, tail-flick latency, hypothermia and ring immobility. The ED_{50} values of **3** ranged from 0.4 to 3.9 μmol/kg (0.1–1.3 mg/kg) when administered i.v. to mice [9]. In the present study, the concentration of **3** was 2.03–35.90 mg/g in those products that contained it (Table 1). Thus, approximately 0.1–20 mg of the products might be enough to show pharmacological effects in mice (30 g weight). Oleamide (**4**), which is an endogenous sleep-inducing cannabinoid, exhibits weak cannabinoid-like behavioral responses without binding significantly to CB receptors (Ki = 1.14 μM for CB_1) and behaves as a full agonist at rat and human CB_1 receptors [17,18,21]. This may be the reason why **4** was present in a higher concentration that compounds **1** or **3**.

In our successive studies, we found that several cannabimimetic compounds, including cannabicyclohexanol and JWH-018, were ingredients in more than 40 commercial herbal products. At present, there is little information about the pharmacology, toxicology and safety profile of these compounds. However, since the amounts of synthetic cannabinoids added varied considerably (Table 1) and some of these compounds may be active at low doses, there is a possibility of serious health problems.

We also investigated the origins of the plant species in each product using genetic analyses of the same herbal products. High homology sequences corresponding to *Glycyrrhiza glabra*, *Astragalus membranaceus* and *Verbascum thapsus* were detected. The details of the genetic results are in preparation. The packaging labels on some of these products indicate that they contain mixtures of several potentially psychoactive plants. However, the results of genetic analysis did not accord with the plant species named in the labels. Therefore, it might be that the plant materials were included mainly as diluents for the synthetic compounds, since no reliable psychoactive effects have been reported for these plants.

Over the past few decades, a number of analogs of classical cannabinoids Δ^9-THC have been synthesized based on its partially reduced dibenzopyran structure, and their structure–activity relationships have been studied [22,23]. In the 1980s, a group at Pfizer Inc. explored the development of analgesics using potent synthetic non-classical cannabinoids, which lack the dibenzopyran structure present in traditional cannabinoids but exhibit typical cannabinoid pharmacology [4–8,20,24–26]. On the other hand, Huffman and Dai reported that cannabimimetic indoles have high affinity for a cannabinoid brain receptor and exhibit typical cannabinoid pharmacology *in vivo* [27]. The latter authors also described the structure–activity relationships of indole-, pyrrole- and indene-derived cannabinoids [9–12,28]. After the discovery of cannabinoid receptors (CB_1 and CB_2) and their endogenous cannabinoids, their physiological roles were elucidated to some extent. A number of cannabinoid analogs were then newly synthesized, including not only THC derivatives but also indole-, pyrrole-, indene-, and pyrazole-derivatives, and their pharmacological activities applicable to the treatment of various diseases were studied [21,29]. Monitoring and surveillance analyses are a first-step in the regulation of abused compounds. In addition, rapid and reliable international exchange of information will be needed to reduce the incidence of drug abuse worldwide.

Acknowledgment

Part of this work was supported by a Health and Labor Sciences Research Grant from the Ministry of Health, Labour, and Welfare, Japan.

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