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.

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Review Article

Survey of current trends in the abuse of psychotropic substances and plants in Japan

Ruri Kikura-Hanajiri *, Nahoko Uchiyama, Yukihiro Goda

National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya, Tokyo 158-8501, Japan

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A B S T R A C T

In recent years, many analogs of narcotics have been widely distributed as easily available psychotropic substances and have become a serious problem in Japan. To counter the spread of these non-controlled substances, the Pharmaceutical Affairs Law in Japan was amended in 2006 to establish a new category, "designated substances", to more strictly control these psychotropic substances. Fifty-one substances have been listed in this category as of December 2010. However, many new analogs have appeared, one after the other. Although the distribution of tryptamine-type designer drugs has decreased since the amendment of the law, the distribution of cathinone derivatives, as well as of phenetylamine-type and piperazine-type designer drugs, has increased. Moreover, non-controlled psychotropic plants have become popular in place of chemical psychotropic substances, which are now subject to stricter controls. Additionally, since 2008, new herbal products containing synthetic cannabinoids (for example, a brand named "Spice") have appeared. Sixteen synthetic cannabinoids, classified into four groups, have been detected in products purchased up to December 2010 via Japanese-based websites. The distribution of products containing the psychoactive substances described above (so-called "legal highs" in European countries) is a worldwide problem. In this review, we survey current trends in the abuse of psychotropic substances and plants in Japan, focusing especially on synthetic cannabinoids, cathinone derivatives and psychotropic plants.

1. Introduction

In the last 10 years, many analogs of narcotics have been widely distributed in Japan as easily available psychotropic substances and have become a serious problem [1–23]. Products, ranging from herbal mixtures to synthetic drugs, that are sold for "decorative purposes" or as "herbal incense" or "research chemicals" are available in various forms via the Internet or at video stores. In Japan, these products are called "dappo drugs" or "iho drugs". They are not controlled under the Narcotics and Psychotropics Control Law in Japan because their pharmacological effects have not yet been proven scientifically. Although these substances should be controlled by the Pharmaceutical Affairs Law, enforcement is difficult because these drugs are sold as non-pharmaceuticals, such as reagents. To fight their distribution, the Ministry of Health, Labor and Welfare of Japan amended the Pharmaceutical Affairs Law in 2006 by establishing a new category of "designated substances" (Shitei-Yakubutsu). In April 2007, 31 compounds (11 tryptamines, 11 phenethylamines, 6 alkyl nitrites, 2 piperazines and salvinorin A) and 1 plant (*Salvia divinorum*) were first listed as designated substances (three of them were then designated as narcotics and removed from the category of designated substances). After that first designation, many new analogs have appeared, one after the other, in the illegal drug market [11–23]. Up to September 2010, 22 compounds (1 tryptamine, 13 phenethylamines (including 5 cathinone derivatives), 2 piperazines, 5 synthetic cannabinoids and 1 other) have been newly added to this category.

Before 2007, the major psychotropic substances distributed in the Japanese illegal drug market were tryptamine-type derivatives (such as 5-MeO-DIPT; *N*,*N*-diisopropyl-5-methoxytryptamine, "Foxy"), phenetylamine-type derivatives (such as 2C series, for example, 2C-T-7; 2-(2,5-dimethoxy-4-propylsulfanylphenyl)ethanamine) and piperazine-type derivatives (such as BZP; 1-benzylpiperazine) [1–3,5–10]. Alkyl nitrites, such as isobutyl nitrite and isopentyl nitrite ("Rush"), were also widely distributed in Japan [4,9]. After these compounds were listed as narcotics or designated substances, they quickly disappeared from the market; instead, cathinone derivatives have been widely distributed, as well as phenetylamine-type and piperazine-type designer drugs [11–15,23]. Although the tryptamine-type designer drugs have been decreasing, non-controlled psychotropic plants have become popular in place of chemical psychotropic substances, which are now subject to stricter controls. Additionally, in recent years new herbal products containing synthetic cannabinoids (for example, "Spice") have appeared [15–22]. Similarly, the distribution of these non-

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* Corresponding author. Address: Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya, Tokyo 158-8501, Japan. Tel.: +81 3 3700 8764; fax: +81 3 3707 6950.
E-mail address: kikura@nihs.go.jp (R. Kikura-Hanajiri).

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controlled psychoactive products, so-called "legal highs" in European countries, is a serious problem in other countries [24–29].

In this review, we survey current trends in the abuse of psychotropic substances and plants in Japan, focusing especially on synthetic cannabinoids, cathinone derivatives and psychotropic plants.

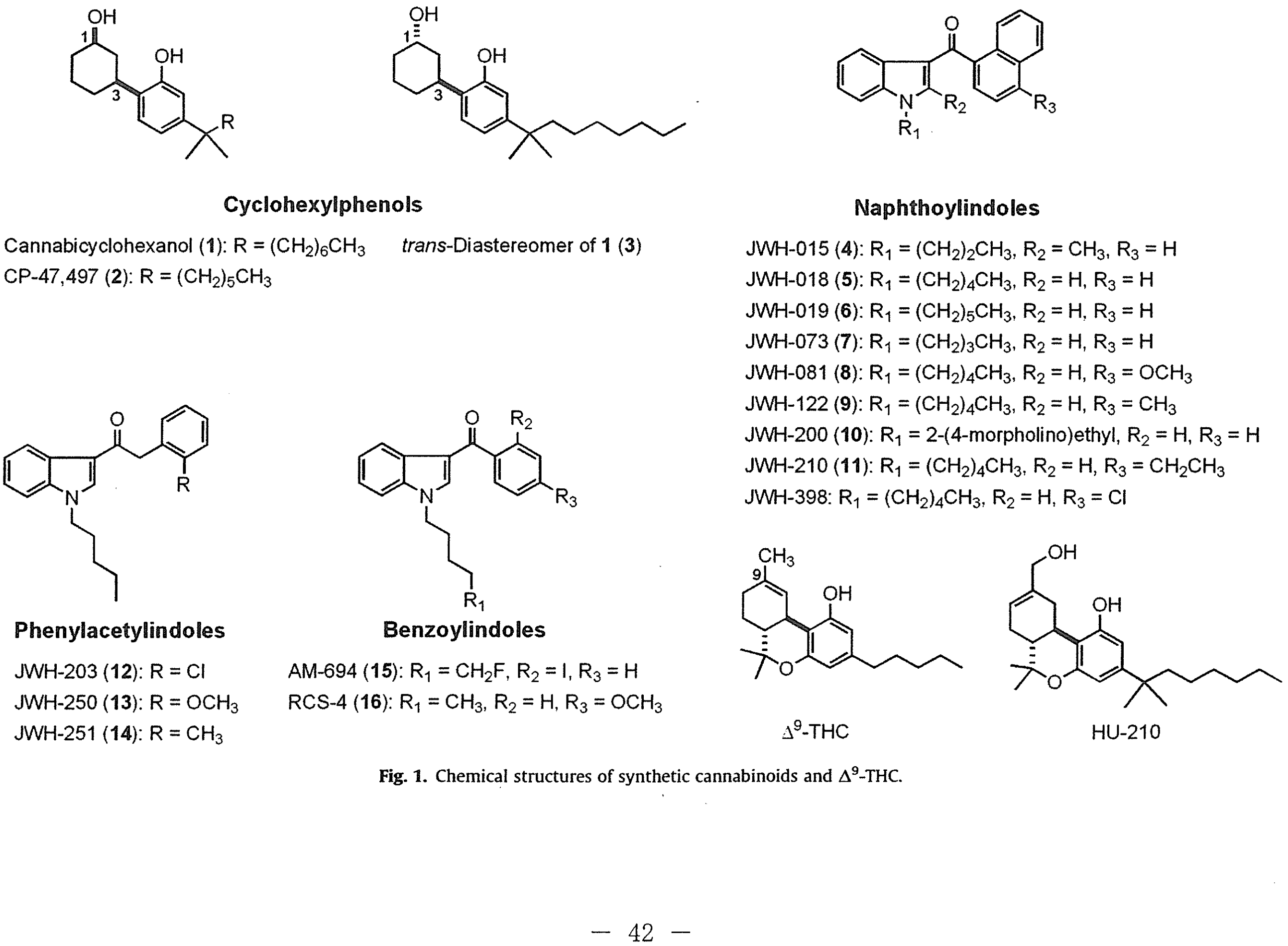
2. Survey of newly distributed designer drugs in Japan

2.1. Synthetic cannabinoids

Since 2006, many psychotropic herbal products have been globally marketed on the Internet under names such as "Spice" and "herbal blends" for their expected cannabis-like effects, and such substances have appeared in Japan since 2008 [15,24]. These herbal products have been distributed in the form of dried leaves or solids (resins). Recently, powdery products have also begun to appear. In early 2009, it was reported that these herbal products contained synthetic cannabinoids as psychoactive ingredients [15,16,30]. These compounds have been synthesised as cannabimimetic substances and were reported to have affinity actions on cannabinoid CB_1 and/or CB_2 receptors [31,32]. The CB_1 receptor, located in the central nervous system, mediates the psychoactive effects associated with cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC), the major active component of cannabis. The CB_2 receptor is expressed mostly in peripheral immune cells. Four groups of synthetic cannabinoids have detected as psychoactive ingredients in herbal products available in Japan since 2009 [15–18,20–22] (compounds 1–16, Fig. 1). Some of these compounds have also found in herbal products in Germany [30,33,34].

The first group of the synthetic cannabinoids consists of cyclohexylphenols such as cannabicyclohexanol (CCH) and CP-47,497, as shown in Fig. 1. These compounds were first found as a new type of designer drugs: synthetic cannabinoids in herbal products [15,30]. The cyclohexylphenols were synthesised as cannabimimetic analogs by Pfizer Inc. during the 1970s and 1980s [31,35–38]. CCH and CP-47,497 have respectively 10- and 4-fold more potent affinity to CB_1 and/or CB_2 receptors than does Δ^9-THC [31,39]. CCH was detected together with its *trans*-diastereomer in many herbal products [30,17], and these cyclohexylphenols have two chiral centers in their structures. Chiral analyses of the herbal products revealed that CCH and the *trans*-diastereomer existed as mixtures of enantiomers [20]. The second and third groups consist of naphthoylindoles (JWH-015, JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-210) and phenylacetylindoles (JWH-203, JWH-250, JWH-251), respectively (Fig. 1). These JWH compounds have been synthesised mainly by Huffman et al. since the 1990s [32]. Although the chemical structures of the JWH compounds differ greatly from those of Δ^9-THC, the JWH compounds have a higher affinity to CB_1 and/or CB_2 receptors and have activities more potent than that of Δ^9-THC [32,40,41]. Only JWH-015 acts as a selective CB_2 receptor agonist [42]. Since January 2009, CP-47,497, its three homologs (including CCH) and JWH-018 have been controlled, first in Germany [43] and later in other countries [24]. In Japan, CCH, CP-47,497, JWH-018, JWH-073 and JWH-250 have been controlled as designated substances under the Pharmaceutical Affairs Law as of December 2010. The fourth group consists of benzoylindoles such as AM-694 and RCS-4 (Fig. 1). AM-694 was reported to have potent binding activity to CB_1 and CB_2 receptors [44]. However, RCS-4 has not been reported to have any biologic activity.

The classical cannabinoid HU-210 (Fig. 1) was identified in herbal products in the US and the UK [24]. In addition, JWH-398 was found in the UK and Germany in October 2009 (Fig. 1) [24]. Both compounds were reported as cannabinoid receptor agonists [32,41]. However, these compounds have not been detected or reported in Japan. HU-210 has been controlled in several European countries since 2009 and in the US since March 2010 [24,45].



Cannabicyclohexanol (**1**): R = $(CH_2)_6CH_3$
CP-47,497 (**2**): R = $(CH_2)_5CH_3$
trans-Diastereomer of **1** (**3**)

JWH-015 (**4**): $R_1 = (CH_2)_2CH_3$, $R_2 = CH_3$, R_3 = H
JWH-018 (**5**): $R_1 = (CH_2)_4CH_3$, R_2 = H, R_3 = H
JWH-019 (**6**): $R_1 = (CH_2)_5CH_3$, R_2 = H, R_3 = H
JWH-073 (**7**): $R_1 = (CH_2)_3CH_3$, R_2 = H, R_3 = H
JWH-081 (**8**): $R_1 = (CH_2)_4CH_3$, R_2 = H, $R_3 = OCH_3$
JWH-122 (**9**): $R_1 = (CH_2)_4CH_3$, R_2 = H, $R_3 = CH_3$
JWH-200 (**10**): R_1 = 2-(4-morpholino)ethyl, R_2 = H, R_3 = H
JWH-210 (**11**): $R_1 = (CH_2)_4CH_3$, R_2 = H, $R_3 = CH_2CH_3$
JWH-398: $R_1 = (CH_2)_4CH_3$, R_2 = H, R_3 = Cl

JWH-203 (**12**): R = Cl
JWH-250 (**13**): R = OCH_3
JWH-251 (**14**): R = CH_3

AM-694 (**15**): $R_1 = CH_2F$, R_2 = I, R_3 = H
RCS-4 (**16**): $R_1 = CH_3$, R_2 = H, $R_3 = OCH_3$

Fig. 1. Chemical structures of synthetic cannabinoids and Δ^9-THC.

The packaging labels on some of these products indicate that they contain mixtures of several potentially psychoactive plants. However, the results of genetic analyses did not accord with the plant species named on 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 the listed plants [17,30].

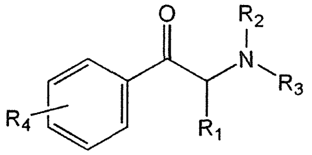
More than 140 different "Spice"-like products of herbal and chemical mixtures available in Japan have been surveyed since June 2008. CCH, CP-47,497 and JWH-018 were the most frequently detected adulterants in these products from June 2008 to October 2009 [17]. Oleamide (*cis*-9,10-octadecenoamide), which is an endogenous cannabinoid, was also detected in some products [14,30]. Soon after the Japanese law declared these three compounds as designated substances in November 2009, synthetic cannabinoids in herbal products were replaced by JWH-073, JWH-250, JWH-081, JWH-251, JWH-015 and JWH-200 [18,19]. Then, after the prohibition of two compounds (JWH-073 and JWH-250) in September 2010, novel cannabinoids such as JWH-122, JWH-019, JWH-210, JWH-203, AM-694 and RCS-4 began to appear in products [22]. This situation underscores the fact that various new synthetic cannabinoids will emerge to replace legally controlled cannabinoids.

2.2. Cathinone derivatives

Cathinone is one of the alkaloids that can be extracted from fresh leaves of *Catha edulis* (khat). Cathinone possesses a ketone oxygen at the β-carbon and represents the β-keto analog of amphetamine. In Japan, cathinone and its *N*-methyl derivative, methcathinone (ephedrone), have been controlled as narcotics, and pyrovalerone and amfepramone (diethylpropion) have been controlled as psychotropics under the Narcotics and Psychotropics Control Law (Fig. 2). Cathinone and methcathinone are amphetamine-like behavioral stimulants, with methcathinone being approximately 10 times more potent than cathinone [46]. Amfepramone and pyrovalerone have both been proposed as appetite suppressants, although they are not currently in clinical use.

The basic cathinone structure can be altered in some predictable ways, such as through a variation of the α-carbon substituent (R_1), *N*-alkylation (or inclusion of the nitrogen atom in a ring structure, typically pyrrolidine, R_2 and R_3) and additional functionality to the aromatic ring (ring substitution, R_4) (Fig. 2). It is noticed that multiple modifications may be present in a single derivative. Recently, there have been an increasing number of reports of cathinone-type designer drugs in EU countries, especially in the UK [47–52,25–27]. 4-Methylmethcathinone, well known as mephedrone, has been the most commonly used cathinone derivative in EU countries [47–52,25–27]. There are two reported fatalities in the EU in which 4-methylmethcathinone appears to be the sole cause of death, and there are at least another 37 deaths in which 4-methylmethcathinone has been detected in post-mortem samples [28]. The European Monitoring Centre for Drugs and Drug addiction (EMCDDA) submitted a Risk Assessment Report on 4-methylmethcathinone [28], and the EU made a Council decision to subject 4-methylmethcathinone to control measures in 2010 [29]. This compound is now controlled in a number of EU countries [28,52].

In Japan also, various cathinone-type designer drugs have appeared on the illegal drug market, one after another, as shown in Fig. 2. [6–8,11,13,23]. Methylone (bk-MDMA) was controlled first, in 2007, as a narcotic by the Narcotics and Psychotropics Control Law. Successively, bk-MDEA (ethylone) and bk-MBDB (butylone) were controlled as designated substances by the Pharmaceutical Affairs Law in January 2008. As a consequence of the β-keto substituent, it is also common practice for widely accepted amphetamine acronyms to be augmented with the prefix 'bk'. For example, ethylone, the cathinone analog of MDEA, is often referred to as 'bk-



Common name	R_1	R_2	R_3	R_4	Regulation category in Japan (as of December, 2010)
Cathinone	CH_3	H	H	H	Narcotic
Methcathinone (Ephedrone)	CH_3	CH_3	H	H	Narcotic
Ethcathinone	CH_3	CH_2CH_3	H	H	Designated substance
Amfepramone (Diethylpropion)	CH_3	CH_2CH_3	CH_2CH_3	H	Psychotropic
4-Methylmethcathinone (Mephedrone)	CH_3	CH_3	H	4-CH_3	Designated substance
4-Methylethcathinone	CH_3	CH_2CH_3	H	4-CH_3	
4-Fluoromethcathinone (Flephedrone)	CH_3	CH_3	H	4-F	
3-Fluoromethcathinone	CH_3	CH_3	H	3-F	
4-Methoxymethcathinone (Methedrone)	CH_3	CH_3	H	4-OCH_3	
Buphedrone	CH_2CH_3	CH_3	H	H	
Methylone (bk-MDMA)	CH_3	CH_3	H	3,4-methylenedioxy	Narcotic
Ethylone (bk-MDEA)	CH_3	CH_2CH_3	H	3,4-methylenedioxy	Designated substance
Butylone (bk-MBDB)	CH_2CH_3	CH_3	H	3,4-methylenedioxy	Designated substance
Pentylone	$CH_2CH_2CH_3$	CH_3	H	3,4-methylenedioxy	
MPPP	CH_3	pyrrolidinyl		4-CH_3	
Pyrovalerone	$CH_2CH_2CH_3$	pyrrolidinyl		4-CH_3	Psychotropic
MDPBP	CH_2CH_3	pyrrolidinyl		3,4-methylenedioxy	
MDPV (3,4-Methylenedioxypyrovalerone)	$CH_2CH_2CH_3$	pyrrolidinyl		3,4-methylenedioxy	Designated substance
Naphyrone (Naphthylpyrovalerone)	$CH_2CH_2CH_3$	pyrrolidinyl		(naphthyl structure)	

Fig. 2. Chemical structures of cathinone derivatives controlled under the laws in Japan and non-controlled compounds detected in products purchased via Japanese-based websites up to December 2010.

MDEA'. MDPV (3,4-methylenedioxypyrovalerone) and ethcathinone, the *N*-ethyl derivative of cathinone, were added to designated substances in January 2009. 4-Methylmethcathinone was additionally controlled in November 2009. Besides these compounds controlled as narcotics or designated substances, the following cathinones were detected in our analyses of products purchased up to December 2010 via Japanese-based websites [23]: 4-fluoromethcathinone (flephedrone), 3-fluoromethcathinone, 4-methoxymethcathinone (methedrone, bk-PMMA), 4-methylethcathinone, buphedrone, pentylone, MPPP and MDPBP (Fig. 2). In addition, a naphthyl analog of pyrovalerone, naphyrone (naphthylpyrovalerone), which has novel properties of cathinones, was detected in several products [23]. They were sold mostly as powder or liquid. The distribution of products containing these compounds has also been reported in Germany and in the UK [49–51,25,26,53]. As to 4-methoxymethcathinone, two fatalities involving this drug were reported in Sweden [54].

Specific field tests based on immunoassays for screening of the cathinone derivatives are not yet commercially available, although some chemical tests, using reagents such as the Simon's or Marquis reagents, may be used to indicate the presence of some cathinones. The color of β-carbonyl-methylenedioxyamphetamines, such as methylone, bk-MDEA and bk-MBDB, produced by the Marquis reagent was brilliant yellow, although typical methylendioxyamphetamines, such as MDMA and MDA, showed black, and β-carbonylamphetamines, such as cathinone and methcathinone, did not react with this reagent [10].

The cathinones act as central nervous system stimulants, although their potencies are generally lower than those of the amphetamine-type compounds. Studies of the effects of cathinones on monoamine neurotransmission in rat brain confirm their mechanism of action to be similar to that of amphetamines. Both groups of drugs bind to monoamine transporters for dopamine, serotonin and noradrenaline (norepinephrine) in the brain and promote the release of these monoamines [55,56]. As with the different amphetamines, individual cathinone derivatives vary in their relative potencies as inhibitors of the three monoamine transporters [55,56]. Little data are available on either the pharmacokinetics or pharmacodynamics of the cathinones. Research on the metabolism of bk-MBDB and bk-MDEA has suggested that *N*-dealkylation (demethylation) followed by *O*-methylation and β-keto reduction are major metabolic pathways [57].

As is the case with synthetic cannabinoids, medicinal chemistry has been used to produce synthetic cathinones. Therefore, it is possible that a variety of cathinone derivatives will appear in the global illegal drug markets.

3. Survey of psychotropic plants distributed in Japan

3.1. Typical psychotropic plants

In recent years, various products made from non-controlled psychotropic plants have become popular [58,59]. In 2002, "magic mushrooms" (containing strong hallucinogens, psilocin and/or psilocybin) were designated as a narcotic plant by the Narcotics and Psychotropics Control Law in Japan. Moreover, *S. divinorum* (containing a strong hallucinogen, salvinorin A) was listed as a designated substance by the Pharmaceutical Affairs Law in 2007. However, it is difficult to control other psychotropic plants that are distributed in the illegal drug markets because there is insufficient scientific information on their species, active compounds and pharmacology.

To survey current trends in the abuse of psychotropic plants, 127 kinds of plant products (purchased via Japanese-based websites from April 2004 to May 2007) were analyzed using LC–MS [59]. As a result of the analyses, typical hallucinogenic plant components were determined in 51 products, as follows: *N,N*-dimethyltryptamine (the genus *Phalaris* and *Psychotria viridis* et al.), mescaline ("San Pedro"; *Trichocereus pachano* and "Peyote"; *Lophophoria williamsii* et al.), salvinorin A (*S. divinorum*), lysergamide ("Hawaiian baby woodrose"; *Argyreia nervosa* and "Morning glory"; *Ipomea violacea* et al.), harmine and harmaline (*Banisteriopsis caapi* and the seeds of *Peganum harmala* et al.). Moreover, mitragynine ("Kratom"; *Mitragyna speciosa*), atropine and scoporamine (the genus Datura et al.) were detected in the products. One-third of the products were mixtures of several plant materials, and the compounds detected in some products were not consistent with the ingredients listed on the product labels. Moreover, there were products in which the contents of the active compounds were sufficient to produce hallucinogenic effects in humans. According to a survey in the UK [49], the top five products marketed as "legal highs" on UK-based websites from April 2009 to June 2009 by frequency were "*S. divinorum*", "Kratom", "Hawaiian Baby Woodrose Seeds", "Fly Agaric" (*Amanita muscaria*, a mushroom containing ibotenic acid and muscimol) and a herbal mixture called "Genie" (containing synthetic cannabinoids). This result was not inconsistent with the results of our survey.

For the simple and rapid screening of these psychotropic constituents in various plant products, a Direct Analysis in Real Time (DART) time-of-flight mass spectrometer (TOFMS) was suitable [60]. DART is a novel ionization technique that provides for the rapid ionization of small molecules under ambient conditions [60]. This analytical method provided molecular information about target compounds without time-consuming extraction and pre-treatment steps.

3.2. Mitragyna speciosa (Kratom)

Before considering the designation of psychotropic plants that are distributed in the illegal drug markets, it is necessary to clarify the contents of their active constituents (chemical analysis) and the genetic classification of these species (DNA analysis). We have carried out both chemical and DNA analyses of the commercial products of various psychotropic plants [61–69].

The leaves of a tropical plant, *M. speciosa* (known as "Kratom"), have been traditionally used as a substitute for opium in Thailand and Malaysia. Mitragynine, a major constituent of *M. speciosa*, has an opioid agonistic activity, and its derivative 7-hydroxymitragynine (a minor constituent) is much more potent than mitragynine or morphine (Fig. 3) [70]. Recently, many kinds of products containing this plant have been distributed as "incense" in the illegal drug market in Japan for their expected narcotic effects. As a result of our chemical and DNA analyses of commercial products of Kratom [67,68], mitragynine, 7-hydroxymitragynine and other alkaloids were detected in 11 of the 13 products using LC–MS. The contents of mitragynine in the products ranged from 1% to 6%,

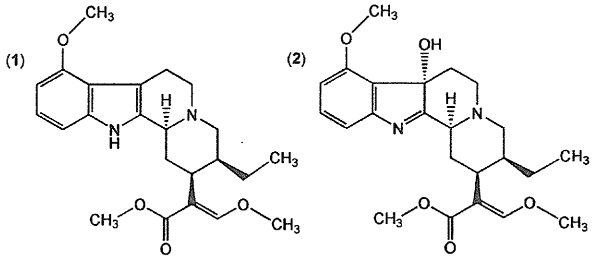


Fig. 3. Chemical structures of active compounds in *Mitragyna speciosa*, mitragynine (**1**) and 7-hydroxymitragynine (**2**).

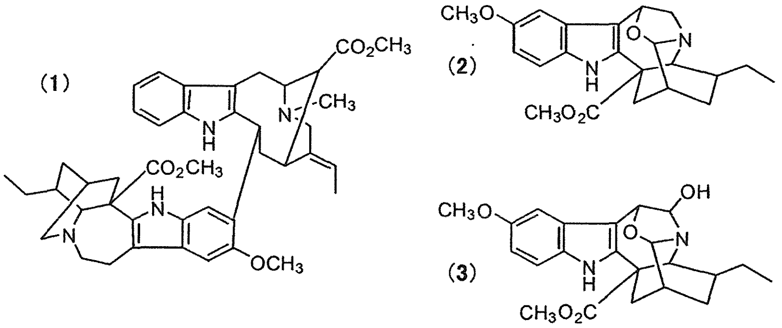


Fig. 4. Chemical structures of three indole alkaloids from *V. africana* root bark, which have potent antagonist activities against the CB1 receptor: voacamine (**1**), 3,6-oxidovoacangine (**2**) and 5-hydroxy-3,6-oxidovoacangine (**3**).

and those of 7-hydroxymitragynine ranged from 0.01% to 0.04%. The ITS sequence analysis of the products revealed that most of them were derived from *M. speciosa* or closely related plants, while others were made from the same tribe plant as *M. speciosa*. 7-Hydroxymitragynine has a highly potent narcotic activity, more potent than morphine. Therefore, *M. speciosa* abuse may be a matter of major concern.

3.3. *Voacanga africana*

Voacanga africana (Apocynaceae) is a small tropical African tree. The root bark and seeds of this tree contain a number of alkaloids, including ibogaine (a hallucinogenic/aphrodisiac compound in bark), tabersonine (a major constituent of seeds) and other voacanga alkaloids, traditionally used in Africa for religious purposes. Recently, some kinds of products containing this plant (root bark and seeds) have been distributed in the illegal drug market in the expectation of their hallucinogenic/aphrodisiac effects. As a result of LC/MS analyses of eight commercially available products [69], these products were classified into two chemical types: an ibogaine type and a tabersonine type. The samples of the ibogaine type contain ibogaine (0.05–0.6%) and other voacanga alkaloids; voacamine, voacamidine and voacangine, while those of the tabersonine type contain mainly tabersonine (0.6–1.6%). The sequence analyses of chloroplast DNA trnL-F region suggested that most of the products were derived from *V. africana* or closely related plants [69]. They were classified into four genotypes based on the nucleotide sequence of the trnL-F IGS region.

For the plant extracts and isolated alkaloids from *V. africana*, their agonistic or antagonistic activities on a dopamine (D_2) receptor, a serotonin ($5HT_{2A}$) receptor, kappa- and mu-opioid receptors and a CB_1 receptor were investigated using the aequorin/GPCR cell-based Ca^{2+} functional assay [71]. This revealed that the MeOH extract of *V. africana* root bark showed potent antagonistic activity against the CB_1 receptor. Takayama et al. isolated three active indole alkaloids from *V. africana* root bark, such as one iboga-vobasine-type bis-indole alkaloid, voacamine, and two iboga-type monomer alkaloids, 3,6-oxidovoacangine and 5-hydroxy-3,6-oxidovoacangine [72] (Fig. 4). These compounds exhibited relatively potent CB_1 receptor antagonistic activities compared with rimonabant, a typical antagonist. Rimonabant was an anorectic antiobesity medicine first approved as the CB_1 receptor antagonist. However, the approval of this drug was officially withdrawn by the European Medicines Agency in 2009 due to the risk of serious psychiatric problems and even suicide. Concerning this fact, there is a possibility that the roots of *V. africana*, which contain these compounds with relatively potent CB_1 antagonist activities, could cause unexpected psychiatric problems in the abusers.

4. The future

The recently distributed designer drugs, such as synthetic cannabinoids and cathinone derivatives, were partially produced by medicinal chemistry. Until now, their numerous analogs have been synthesised during the development of new medicines affecting the central nervous system, and only some of them have appeared as designer drugs in the illegal drug market. Therefore, it is possible that other analogs that have strong activity levels will appear one after the other. In fact, the actual composition in terms of synthetic additives in the psychotropic products is dynamically changing and rapidly responding to the newly implemented control measures.

There is little information on the pharmacology, toxicology, and safety of the newly distributed psychotropic substances and plants for humans, but there may be possibilities of serious health damage to their abusers. Thus, continuous monitoring of herbal and chemical products in the illegal drug market is needed. In view of the worldwide trend in herbal or chemical products (including adulterated products) an international cooperation system for sharing analytical information is required to prevent their worldwide spread.

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Methylone and Monoamine Transporters: Correlation with Toxicity

Chiharu Sogawa[a], Norio Sogawa[a], Kazumi Ohyama[b], Ruri Kikura-Hanajiri[c], Yukihiro Goda[c], Ichiro Sora[d] and Shigeo Kitayama[a,*]

[a]Department of Dental Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8525, Japan; [b]RI Research Center, Okayama University Dental School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8525, Japan; [c]Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; [d]Department of Biological Psychiatry, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

Abstract: Methylone (2-methylamino-1-[3,4-methylenedioxyphenyl]propane-1-one) is a synthetic hallucinogenic amphetamine analog, like MDMA (3,4-methylenedioxy- methamphetamine), considered to act on monoaminergic systems. However, the psychopharmacological profile of its cytotoxicity as a consequence of monoaminergic deficits remains unclear. We examined here the effects of methylone on the transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT), using a heterologous expression system in CHO cells, in association with its cytotoxicity. Methylone inhibited the activities of DAT, NET, and SERT, but not GABA transporter-1 (GAT1), in a concentration-dependent fashion with a rank order of NET > DAT > SERT. Methylone was less effective at inhibiting DAT and NET, but more effective against SERT, than was methamphetamine. Methylone alone was not toxic to cells except at high concentrations, but in combination with methamphetamine had a synergistic effect in CHO cells expressing the monoamine transporters but not in control CHO cells or cells expressing GAT1. The ability of methylone to inhibit monoamine transporter function, probably by acting as a transportable substrate, underlies the synergistic effect of methylone and methamphetamine.

Keywords: Methylone, neurotransmitter transporter, uptake, cocaine, methamphetamine, MDMA.

INTRODUCTION

Various illegal and/or restricted "designer drugs" have been created by modifying amphetamines. Methylone (2-methylamino-1-[3,4-methylenedioxy- phenyl]propane-1-one) is one such synthetic hallucinogenic amphetamine analogue, which resembles MDMA (3,4-methylenedioxymethamphetamine) but differs structurally by the presence of a ketone at the benzylic position [1, 2]. This compound has been newly placed under legal control as a drug of abuse in Japan [3].

Because of its structural similarity to MDMA, methylone is thought to act on monoaminergic systems. A behavioral study by Dal Cason *et al.* [4] found that methylone substituted for MDMA in rats trained to discriminate MDMA from saline. In studies of its pharmacology in vitro methylone was threefold less potent than MDMA at inhibiting platelet serotonin transporter (SERT) and as potent as MDMA in inhibiting transporters for dopamine (DAT) and norepinephrine (NET), but only weakly inhibited the vesicular monoamine transporter [5, 6]. However, there have been few pharmacological investigations of methylone in animal models, or studies about its mechanism of action.

In addition, the cytotoxicity of MDMA is considered a consequence of monoaminergic deficits through the drug's effects on the plasmalemmmal and vesicular monoamine transporters [7, 8]. Again, methylone may resemble MDMA in cytotoxic profiles. However, Nakagawa *et al.* [9] reported that methylone did not exhibit cytotoxic effects on isolated rat hepatocytes in contrast to MDMA and its analogues. The possibility can not be excluded that methylone is cytotoxic in some circumstances, since Nagai *et al.* [6] observed that like MDMA, it reduced mitochondrial membrane potential.

Recently, we examined the effects of 5-methoxy-N, N-diisopropyltryptamine (5-MeO-DIPT), known as Foxy, on monoamine neurotransmitter transporters, including DAT, NET and SERT, using a heterologous expression system in COS-7 cells and rat brain synaptosomes, in association with its cytotoxicity [10]. In the present study, we used the same strategy to evaluate the relationship between the effects of methylone and methamphetamine on monoamine transporters and cell toxicity.

The results indicated an ability of methylone to inhibit monoamine transporter function, and cause damage synergistcally with methamphetamine in cells heterologously expressing monoamine transporters, suggesting that the transport of these drugs underlies their cytotoxicity.

MATERIALS AND METHODS

Materials

Methylone was synthesized at the Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of

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*Address correspondence to this author at the Department of Dental Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Shikata 2-5-1, Okayama 700-8525, Japan; Tel: +81-86-235-6660; Fax: +81-86-235-6664;
E-mail: shigeok@md.okayama-u.ac.jp

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Health Sciences (Tokyo, Japan). Its structure and purity were confirmed by melting point (degradation, 225°C), TLC, GC-MS and ^{1}H-NMR analyses [11]. Other chemicals used were purchased from commercial sources. [^{3}H]Dopamine (DA) (1.29 TBq/mmol), [^{3}H]serotonin (5-HT) (1.04 TBq/mmol), and [^{3}H]GABA (1.2 TBq/mmol) were obtained from PerkinElmer Life Science, Inc. (Boston, MA, USA), and [^{3}H] norepinephrine (NE) (1.18 TBq/mmol) from GE healthcare Bioscience, Inc. (Buckinghamshire, UK).

Cell Culture and Expression

Chinese hamster ovary (CHO) cells were cultured at 37°C under 5 % CO_2 / 95 % air in Minimum Essential Medium-alpha (α-MEM) supplemented with 10 % fetal calf serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml fungisone.

For the preparation of cell lines stably expressing transporters, CHO cells at subconfluence were transfected with cDNA of rat DAT (rDAT), NET (rNET), or SERT (rSERT), or mouse GABA transporter-1 (mGAT1) using FuGENE6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's directions. The cells were then diluted sequentially, seeded in 96-well plates, and selected using G418. The cell lines were confirmed to stably express the transporters based on the uptake of each tritium-labeled ligand, and designated CHO/rDAT, CHO/rNET, CHO/rSERT and CHO/mGAT1, respectively.

Uptake and Release Assay

The uptake assay using radio-labeled ligands was performed, as described previously [10, 12]. Cells were washed three times with an oxygenated Krebs Ringer HEPES-buffered solution (KRH; 125 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, and 20 mM HEPES, pH 7.3) and incubated for 10 min at 37°C with 10 nM of [^{3}H]DA or other radio-labeled ligand. Nonspecific uptake was determined in mock-transfected cells and also in each plate in the presence of 100 µM cocaine for monoamine and 1 mM nipecotic acid for GABA. Data were analyzed using Eadie-Hofstee plots with Prism 5 (GraphPad Software, Inc., San Diego, CA). Statistical analyses were performed using the unpaired Student's t-test.

Reverse transport (release) was analyzed, as described [10]. Cells loaded with [^{3}H]substrate were incubated with or without the drug under investigation at 37°C for 2 min, and separated from the incubation solution. The radioactivity retained in the cells and also in the separated solution was measured by liquid scintillation counting. Statistical tests were performed using an analysis of variance (Kruskal-Wallis test) with pairwise comparisons using Dunn's multiple comparison test.

Cell Toxicity Assay

The amount of lactate dehydrogenase (LDH) released into the culture medium was measured for the evaluation of methylone's toxicity, as described previously [12] with some modifications. Briefly, cells cultured in 96-well culture plates were washed and incubated without phenol red in α-MEM supplemented with 1% BSA and different concentrations of methylone and/or methamphetamine for 24 h. The amount of LDH released into the medium was measured colorimetrically (Wako, Tokyo, Japan). Statistical analyses were performed using the Kruskal-Wallis test and Dunn's multiple comparison test.

RESULTS

Effects of Methylone on the Uptake of Substrate in CHO Cells Stably Expressing Monoamine and GABA Transporters

The effect of methylone on the transport of monoamines was examined in CHO cells stably expressing the rat monoamine transporters, rDAT, rNET and rSERT, in comparison with that on the mouse GABA transporter, mGAT1. Simultaneous incubation with [^{3}H]DA, [^{3}H]NE, or [^{3}H]5HT and methylone caused a decrease in the uptake of [^{3}H]substrate in a concentration-dependent fashion, although the effects differed between transporters in contrast to the effects of methamphetamine (Fig. **1**). The rank order of the transporters in terms of the potency with which they were inhibited by methylone was NET > DAT >> SERT (Fig. **1** and Table **1**). Methylone inhibited SERT more, DAT and NETless, than methamphetamine. However, it had no effect on GAT transport activity at concentrations up to 1 mM (Fig. **1**), while nipecotic acid, an inhibitor of neuronal GABA transporter such as GAT1, inhibited [^{3}H]GABA uptake in a concentration-dependent manner (data not shown).

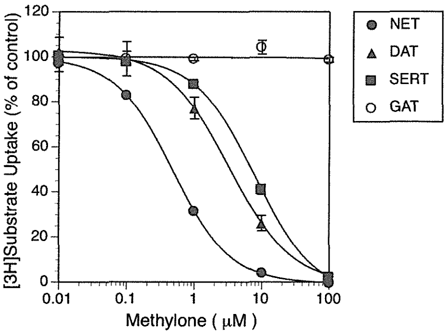

Fig. (1). Effects of methylone on the uptake of substrates in CHO cells stably expressing monoamine and GABA transporters. Cells were incubated with [^{3}H]substrates at 10 nM in the absence or presence of methylone at various concentrations. Specific uptake was expressed as a percentage of the control, and values represent the mean ± SEM, n=3. Control uptake in the absence of drugs was 4715 ± 182, 4961 ± 170, 13964 ± 1135, and 798 ± 114 dpm/well for DAT, NET, SERT and GAT, respectively.

Table 1. Effects of Methylone and Methamphetamine on the Uptake of Monoamines in CHO Cells Stably Expressing rat DAT, NET and SERT

Transporter	IC50 (µM)	
	Methylone	Methamphetamine
DAT	2.84 ± 0.36	0.65 ± 0.06
NET	0.48 ± 0.03	0.16 ± 0.00
SERT	8.42 ± 1.01	27.62 ± 2.87

Values represent the mean ± SEM for three experiments each performed in triplicate.

Next, we analyzed the effect of methylone on uptake kinetically. Table **2** summarizes the effects on [^{3}H]substrates in comparison with those of methamphetamine. Methylone increased the K_m value without changing the V_{max} for the uptake of [^{3}H]NE, indicating competitive inhibition similar to metamphetamine (Table **2**). On the other hand, it showed uncompetitive inhibition of [^{3}H]DA and [^{3}H]5-HT, tending to decrease the V_{max} while increasing the K_m, as methamphetamine did.

Effects of Methylone and Methamphetamine on the Reverse Transport of [^{3}H]substrates

To further characterize the effects of methylone on the monoamine transporters, we examined its influence on the reverse transport of [^{3}H]substrates through DAT, NET and SERT in comparison with methamphetamine, since unlike cocaine, methamphetamine induces the release of monoamines *via* a reversal of transport [13]. Methylone elicited the release of [^{3}H]DA, [^{3}H]NE and [^{3}H]5-HT from the cells expressing rDAT, rNET and rSERT, respectively, similar to methamphetamine (Fig. **2**). In addition, the combination of methylone and methamphetamine did not cause a further increase in the release of [^{3}H]substrates.

Cytotoxicity Elicited by Methylone and/or Methamphetamine

Initially, we examined the effect of methylone and methamphetamine on cell viability using a MTT-based WST-1 assay previously applied to Foxy [10]. However, we found no changes with methylone and/or methamphetamine at any concentrations tested (data not shown). Therefore, we used a LDH release assay to evaluate methylone's toxicity.

Table 2. Changes in the Transport Kinetics Induced by Methylone and Methamphetamine in CHO Cells Stably Expressing the Monoamine Transporters

	K_m (μM)	V_{max} (fmol/μg protein/min)
DAT		
Control	1.73 ± 0.55	34.12 ± 7.64
Methylone 5μM	2.33 ± 0.67	26.68 ± 8.70
Methamphetamine 1μM	2.06 ± 0.83	24.18 ± 9.89
NET		
Control	0.25 ± 0.03	3.63 ± 0.31
Methylone 0.5μM	0.58 ± 0.10*	4.54 ± 1.01
Methamphetamine 0.2μM	0.71 ± 0.20	4.69 ± 0.49
SERT		
Control	0.27 ± 0.04	40.68 ± 7.23
Methylone 10μM	1.12 ± 0.25*	36.95 ± 5.68
Methamphetamine 30μM	0.86 ± 0.21*	31.41 ± 4.63

Values represent the mean ± SEM for three experiments each performed in triplicate. *P<0.05 vs control.

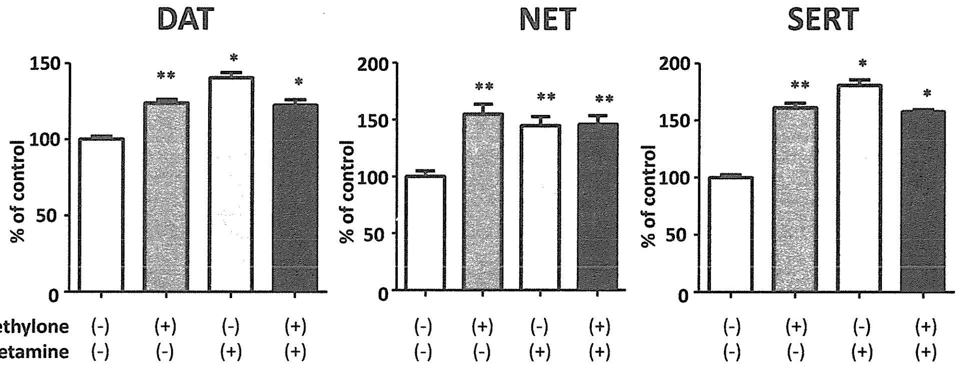

Fig. (2). Effects of methylone and methamphetamine on the reverse transport of [^{3}H]substrates through the monoamine transporters. CHO cells stably expressing rDAT, rNET, or rSERT were preloaded with a [^{3}H]substrate at 20 nM for 30 min. They were then washed with KRH buffer, and incubated for 2min in fresh medium containing 100 μM methylone and/or 100 μM methamphetamine. Values represent the mean ± SEM, n=8-11, and are expressed as a percentage of the control (without drugs). *P< 0.05, **P< 0.01 vs control.

Fig. (**3**) shows the cytotoxicity of methylone and methamphetamine in CHO cells stably expressing rDAT, rNET and rSERT, together with mGAT1 as a control. Methylone did not induce the release of LDH from any cell line except that expressing SERT (Fig. **3**). Furthermore, in combination with methamphetamine it caused a significant increase in the release of LDH in the cells stably expressing the monoamine transporters but not in the control CHO cells or cells expressing GAT (Fig. **3**).

DISCUSSION

The present study demonstrated that methylone acts as a non-selective inhibitor of the monoamine neurotransmitter transporters DAT, NET and SERT, with a rank order in terms of potencies of NET > DAT >> SERT. Kinetic analyses revealed the characteristics of the inhibition to resemble those by methamphetamine. In addition, methylone induced a reversal of transport similar to methamphetamine. Methamphetamine, like amphetamine, is transported by DAT and NET, and probably SERT [13]. The present study demonstrated that methylone had similar properties to methamphetamine not only in inhibiting the uptake by transporters but also in reversing the direction of transport, suggesting that it too is likely to be a transportable inhibitor. However, methylone itself was cytotoxic only at high concentrations, though in combination with methamphetamine it had a significant effect on cells expressing the monoamine transporters but not the control CHO cells or cells expressing GAT. These results suggest that the transport of methylone through monoamine transporters underlies the cytotoxicity.

The effectiveness of methylone and methamphetamine in inhibiting the uptake of substrates by monoamine transporters demonstrated here was well consistent with previous findings [5, 6]. Cozzi *et al.* [5] found that methylone inhibited DAT more than NET, while Nagai *et al.* [6] and ourselves observed a more potent effect on NET than DAT. On the other hand, Nagai *et al.* [6] reported that methylone inhibited DAT and SERT equally, while Cozzi *et al.* [5] and ourselves found the effect to be weaker at SERT than DAT. The discrepancy may be due to the different preparations or concentrations of labeled substrates used.

The concentrations of methylone in the brain at dosages at which the drug is abused, 100 – 200 mg [14], are unknown. Experiments with rats demonstrated the intraperitoneal administration of methylone at 5 mg/kg to be followed by a rapid increase in the plasma concentration ranging from 700 to 1500 ng/mL within 15 or 30 min [11]. These values seem compatible with those for MDMA [7]. Therefore, the present findings suggest that methylone may inhibit monoamine transporters in the CNS at concentrations relevant to its abuse.

The present study demonstrated cytotoxicity at high concentrations, as assessed from the amount of LDH released in

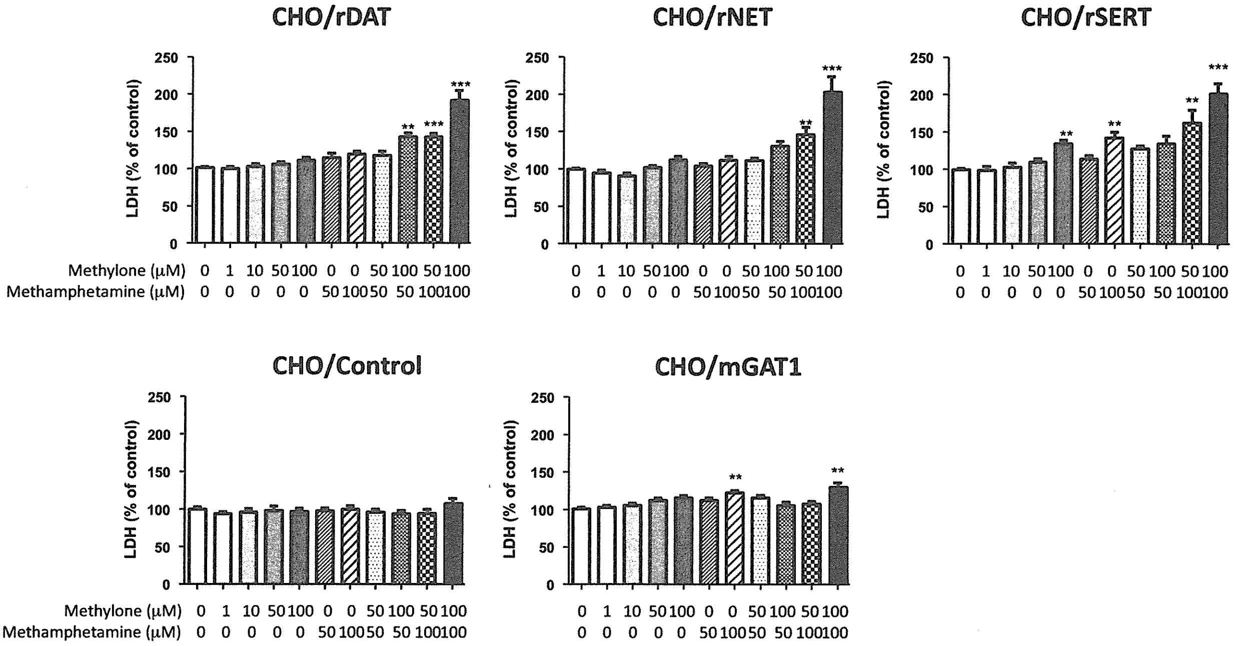

Fig. (3). Effect of methylone and methamphetamine on viability of CHO cells stably expressing monoamine and GABA transporters. Cells were seeded on 96-well culture plates and cultured for 24 h prior to treatment with the drug under examination. They were then incubated with various concentrations of methylone and/or methamphetamine for 24h, after which the amount of LDH released into the medium was determined. Values are expressed as a percentage of the control (absence of drugs), and represent the mean ± SEM, n=6-16. **P<0.01, ***P<0.001 vs control.

CHO cells. This effect was observed in cells expressing the monoamine transporters, especially those expressing SERT, but not cells expressing mGAT1, suggesting a relationship with the transport of methylone, although the affinity of the transporter for methylone does not explain the potency of methylone's toxicity. In addition, the present study demonstrated that methylone and methamphetamine combined had a supra-additive effect on the release of LDH in CHO cells expressing monoamine transporters. The cytosolic accumulation of monoamines or methamphetamine may cause oxidative stress, resulting in cell death [15]. Therefore, one may assume that methylone modulates the toxic effects of other monoaminergic agents, such as methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA), through interaction at monoamine transporters. Recently, Shimizu *et al.* [14] reported a case study of a 27-year-old male who took methylone and 5-MeO-MIPT after ingesting a drug powder called pure methylone obtained via the internet, suggesting that substance-related disorders may be complicated by the combined use of psychoactive drugs. According to recent analyses, "ecstacy" and other designer drugs consist of mixtures of MDMA and other substances [16, 17]. Therefore, it is important to note their cytotoxicity when taken simultaneously. Further study is needed to clarify this issue.

In summary, we investigated the effects of methylone on monoamine transporters. The ability of methylone to inhibit transporter function, and damage cells heterologously expressing monoamine transporters, suggests that the transport of methylone underlies its cytotoxicity.

ACKNOWLEDGEMENTS

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Enzyme-assisted synthesis of the glucuronide conjugate of psilocin, an hallucinogenic component of magic mushrooms

Takuji Shoda,* Kiyoshi Fukuhara, Yukihiro Goda and Haruhiro Okuda

An enzyme-assisted synthesis of psilocin glucuronide (PCG), a metabolite excreted in the urine of magic mushroom (MM) users, is described. In the presence of Aroclor 1254 pretreated rat liver microsomes, psilocin and the cofactor UDPGA were incubated for 20 h. Purification by HPLC gave PCG in 19% yield (3.6 mg). The compound structure was characterized by MS and NMR. The milligram amounts of PCG produced by this method will allow the direct identification and quantification of PCG in the urine of MM users. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: psilocin; glucuronide; enzyme-assisted synthesis; uridine 5′-diphospho-glucuronosyltransferase

Introduction

Psilocybin (PB) and psilocin (PC) are hallucinogenic compounds found in *Psilocybe* mushrooms, also called 'magic mushrooms' (MM). The structure of these compounds is similar to the neurotransmitter serotonin. The hallucinogenic effect of PB and PC is attributed to their agonist activities to serotonin 5-HT$_{2A}$ receptors.[1–3] In Japan, the possession, cultivation, and intake of MM containing PB and PC have been prohibited by the Narcotics and Psychotropic Control Law since 2002.

Several studies on the metabolism of PB and PC have been reported. The metabolic pathway for PB and PC is shown in Figure 1. After ingestion of MM, PB is rapidly dephosphorylated to PC by an intrinsic phosphatase.[4] PC is metabolized by conjugation with glucuronic acid to PC glucuronide (PCG).[5] Direct analysis by liquid chromatography-mass spectrometry (LC-MS) of urine from MM users has determined that psilocin is mostly excreted as PCG.[6] Thus, the detection of PCG in urine is useful for unequivocal proof of MM use. However, PCG reference material is not currently available. In addition, the preparation of PCG has not been described in the literature. The development of a synthetic method for the preparation of PCG is important for forensic analysis.

Enzyme-assisted synthesis offers an effective way of preparing glucuronides. The advantage of biocatalyzed reactions is that the formation of solely the biologically relevant β-anomer conjugate can be expected. Usually, enzyme-assisted synthesis uses liver microsomes as a general source of glucuronidation enzymes, and this has made it possible to synthesize glucuronides of several types of substrates, for example, COMT inhibitors,[7] silybin,[8] hydroxypyrene,[9] propofol,[10] buprenorphine,[10] anthraflavic acid,[10] octylgallate,[10] anabolic-androgenic steroids,[11,12] dobutamine,[13] losartan,[14] and benzodiazepines.[15] Recently, we reported the enzyme-assisted synthesis of the glucuronide of 4-hydroxy-3-methoxymethamphetamine, one of the metabolites of methylenedioxymethamphetamine (MDMA).[16]

This paper describes the synthesis of PCG by an enzyme-assisted synthesis. The synthesized product was characterized by NMR and MS. PCG was readily prepared in milligram amounts, quantities sufficient for *in vitro* enzyme kinetic studies or reference standards required for *in vivo* pharmacokinetics and metabolism studies, as well as for forensic analysis.

Experimental

General information

UDPGA and alamethicin were purchased from Sigma-Aldrich (St Louis, MO, USA). Psilocin was synthesized according to literature procedures.[17] All other reagents and solvents were purchased from Wako Pure Chemical (Osaka, Japan), Tokyo Kasei Kogyo (Tokyo, Japan), and Kanto Chemical (Tokyo, Japan) and were used without purification. Aroclor 1254-induced male Sprague-Dawley rat liver microsomes (Lot No. ADM) were purchased from Charles River Laboratories (Wilmington, MA, USA).

Analytical HPLC was performed using a CBM-20A system controller, LC-20A pump, SPD-M20A UV/Vis photodiode array detector, and CTO-10AC column oven (Shimadzu, Kyoto, Japan) equipped with a CAPCELL PAK C18 MGII 5 μm, 4.6 × 250 mm column (Shiseido, Tokyo, Japan). The mobile phases were A: 0.1% trifluoroacetic acid (TFA)/H$_2$O and B: 0.1% TFA/CH$_3$CN. The gradient was programmed to begin the analysis at 5:95 A/B and linearly increase to 30:70 A/B over 15 min at a flow rate of 1 ml/min. Preparative HPLC was performed using an SSC-6600 gradient controller, SSC-3465 pump, SSC-5410 UV/Vis detector and SSC-3465 column oven equipped with a SenshuPak PEGASIL ODS column 5 μm, 20 × 250 mm (Senshu Kagaku, Tokyo, Japan). The mobile phases were A: 0.1% TFA/H$_2$O and B: 0.1% TFA/CH$_3$CN. The gradient was programmed to begin the analysis at 5:95 A/B and linearly increase to 30:70 A/B over 20 min at a flow rate of 10 ml/min. The gradient condition was LC-MS was performed using a Dual λ Absorbance Detector 2487

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* Correspondence to: Takuji Shoda, National Institute of Health Sciences; 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. E-mail: tsho@nihs.go.jp

National Institute of Health Sciences; 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

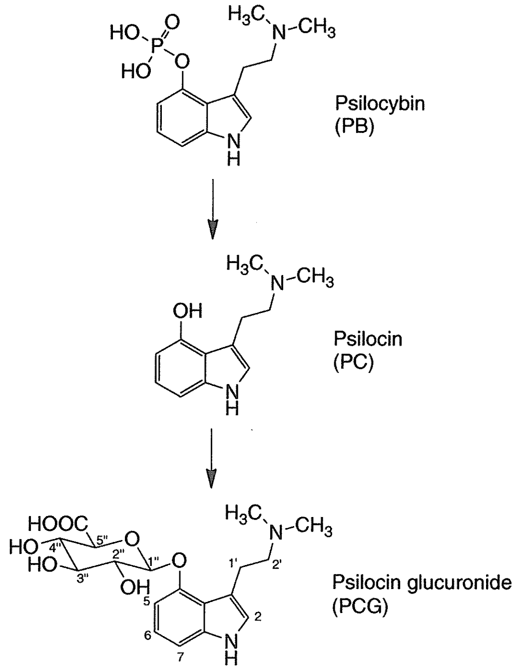

Figure 1. Metabolic pathway for psilocybin and psilocin.

(Waters, Milford, MA, USA), micromass ZQ (Waters) and an Alliance model 2695 (Waters) equipped with a CAPCELL PAK C18 MGII 5 μm, 4.6 × 250 mm column (Shiseido, Tokyo, Japan). The mobile phases were composed of A: 0.1% $HCOOH/H_2O$ and B: CH_3CN. The gradient was programmed to begin the analysis at 5 : 95 A/B and linearly increase to 30 : 70 A/B over 15 min at a flow rate of 1.0 ml/min. The NMR spectra were recorded on a Varian AS 400 Mercury spectrometer and a JEOL ECA-600. Assignments were made *via* ^1H NMR, ^{13}C NMR, ^1H-^1H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond connectivity (HMBC), and rotating frame nuclear overhauser effect (ROE) spectra. Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)-propionate-2,2,3,3,-d_4 (δ scale). High-resolution mass spectra were obtained on an LTQ Orbitrap equipped with an electrospray ionization source.

Enzyme-assisted synthesis of PCG

A mixture of 10 ml of buffer solution containing 50 mM of Tris-HCl (pH 7.5), 8 mM of $MgCl_2$, 25 μg/ml of alamethicin, 5 mM of UDPGA, and 5 mM of PC was stirred in a 37 °C water bath. The reaction was started by the addition of 200 μl of Aroclor 1254-induced rat liver microsomes (24 mg protein/ml) and was gently stirred for 20 h under protection from light. The reaction was stopped with 5 ml of 10% $HClO_4$. The precipitated proteins were removed by centrifugation (3000 rpm, 10 min, 4 °C), and the supernatant was filtered. The filtrate was purified by preparative HPLC, and after evaporation of the fraction containing the product, PCG was obtained as a white solid (3.6 mg, yield 19%).

Results and discussion

The enzyme-assisted synthesis of PCG was carried out using a 10-ml reaction volume. The incubation condition was based on our recent report.[16] The reaction mixture contained PC (5 mM), Uridine 5′-diphosphoglucuronic acid (UDPGA, 5 mM), alamethicin (25 μg/ml) and Aroclor 1254-induced rat liver microsomes (0.48 mg protein/ml). Alamethicin, a pore-forming peptide, was added to the reaction system to activate UGT activity in the liver microsomes.[18]

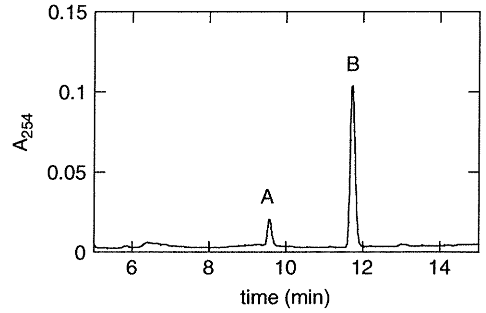

Figure 2. HPLC chromatogram after enzyme-assisted synthesis of PCG. A: PCG; B: PC.

Treatment of rats with Aroclor 1254, a mixture of polychlorinated biphenyls, increased the UGT activity of the rat liver microsomes.[19] The reaction mixture was incubated for 20 h at 37 °C with gentle shaking under protection from light. After protein precipitation and centrifugation, the supernatant was analyzed by HPLC. A representative chromatogram of the reaction mixture is shown in Figure 2. The new peak at retention time 9.6 min (peak A) was found together with the PC peak at 11.6 min (peak B). The purification of peak A was carried out by preparative HPLC. After removal of the eluent, 3.6 mg of white solid was obtained from the initial 10.2 mg of PC.

The most abundant protonated $[M+H]^+$ (*m/z* 381) and deprotonated $[M-H]^-$ (*m/z* 379) molecules recorded in the positive and negative electrospray ionization mass spectra, respectively, indicated the correct molecular weights for PCG, and the observed mass obtained by high-resolution MS (*m/z* 381.1661) corresponded well with the calculated mass for $[M + H]^+$ of PCG (*m/z* 381.1656). ^1H and ^{13}C NMR spectra in D_2O were fully assigned on the basis of ^1H-^1H COSY, HMQC, and HMBC spectra (Table 1). One-dimensional ^1H NMR was used to determine the anomer composition of the glucuronic acid moiety in the PCG. The anomeric proton H1″ was identified by its characteristic chemical shift at 5.28 ppm, and the coupling constant between H1″ and H2″ was 7.6 Hz, which is consistent with a β-configuration. In addition, the irradiation of the H1″ proton at 5.28 ppm resulted in ROE on H5 of the indole proton at 6.70 ppm. This signal indicated that adduct formation could only have occurred at the indole hydroxyl group. Thus, the MS and NMR spectral data confirm the structure of this compound to be PCG.

We also examined the chemical synthesis of PCG by typical procedures for preparing glucuronides, such as the Koenigs-Knorr and the Williamson ether syntheses. However, all attempts with these procedures failed to produce PCG. To date, the enzyme-assisted synthesis is the only successful method for preparing the glucuronide of PC. This method allows the synthesis of the stereochemically pure β-anomer of PCG in milligram amounts (3.6 mg, yield 19%), which is sufficient for use as reference material in forensic analysis.

It has been reported that PC was decomposed during incubation in the presence of microsomes, presumably due to nonenzymatic oxidation.[20] The enzyme-assisted synthesis in this study was

Table 1. ^{1}H and ^{13}C chemical shift assignments of psilocin glucuronide in D_2O

Position	^{1}H^a δ (*m*, *J* in Hz)	^{13}C^b δ
NCH_3	2.74 (3H, s), 2.76 (3H, s)	41.6
1′	3.15-3.28 (2H, m)	24.7
2′	3.36-3.46 (2H, m)	61.8
2	7.12 (1H, s)	127.2
3		110.9
3a		119.7
4		153.5
5	6.70 (1H, d, 7.6)	106.3
6	7.05 (1H, dd, 7.6, 8.4)	125.9
7	7.10 (1H, d, 8.4)	110.1
7a		141.5
1″	5.28 (1H, d, 7.6)	102.7
2″, 3″, 4″	3.49-3.59 (3H, m)	74.6, 75.9, 78.7
5″	3.90 (1H, d, 7.6)	79.2
6″		178.2

^{a} 400 MHz.
^{b} 125 MHz.

carried out under protection from light, resulting in the formation of glucuronide as the sole product without any degradation products which were not detected in HPLC chromatogram as shown in Figure 2. Protecting the reaction mixture from light is enough to synthesize the glucuronide without accompanying the degradation of PC.

The lower yield of PCG than that of 4-hydroxy-3-methoxymethamphetamine glucuronide[16] might be attributed to low capacities of the microsomes to catalyze the glucuronidation of PC. Our preliminary data indicate that the yield of PCG using *β*-Naphthoflavone-induced rat liver microsomes as a biocatalyst is 1.3 times higher than that using Aroclor 1254-induced rat liver microsomes (data not shown). Therefore, the optimization of the incubation conditions for large-scale synthesis is under consideration.

In conclusion, we have demonstrated an efficient method for the preparation of PCG by an enzyme-assisted synthesis. The PCG synthesized by this method makes it possible to directly identify and quantify PCG in the urine of MM users. Further studies for the identification of MM intake using PCG as a reference material are now in progress.

Acknowledgement

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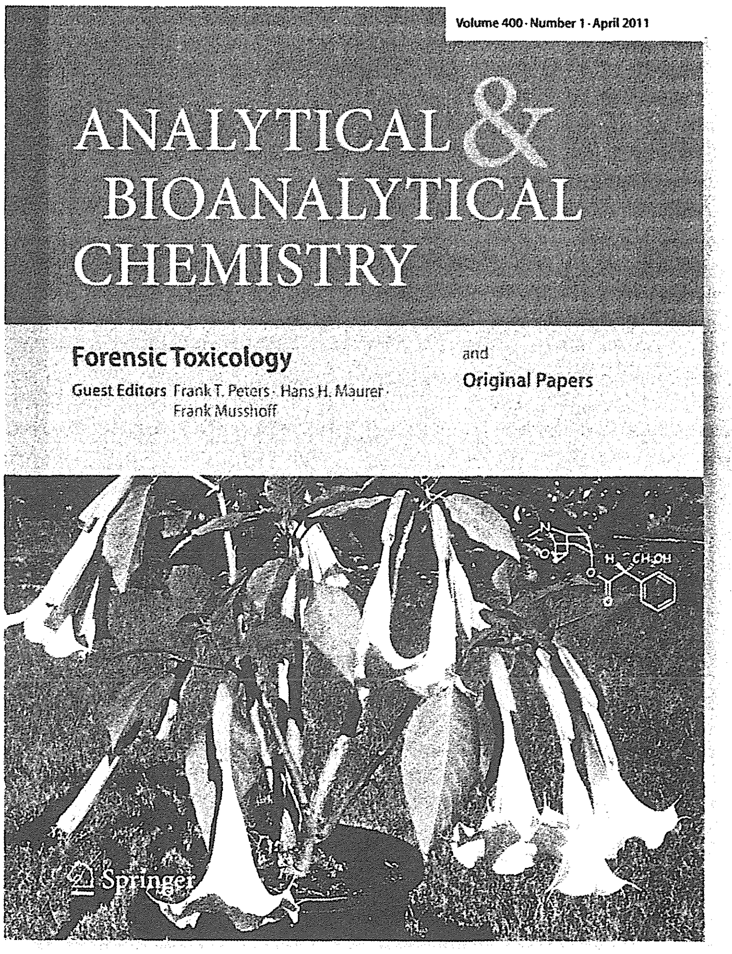

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ORIGINAL PAPER

Chiral analyses of dextromethorphan/levomethorphan and their metabolites in rat and human samples using LC-MS/MS

Ruri Kikura-Hanajiri · Maiko Kawamura ·
Atsuko Miyajima · Momoko Sunouchi · Yukihiro Goda

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Abstract In order to develop an analytical method for the discrimination of dextromethorphan (an antitussive medicine) from its enantiomer, levomethorphan (a narcotic) in biological samples, chiral analyses of these drugs and their *O*-demethyl and/or *N*-demethyl metabolites in rat plasma, urine, and hair were carried out using LC-MS/MS. After the i.p. administration of dextromethorphan or levomethorphan to pigmented hairy male DA rats (5 mg/kg/day, 10 days), the parent compounds and their three metabolites in plasma, urine and hair were determined using LC-MS/MS. Complete chiral separation was achieved in 12 min on a Chiral CD-Ph column in 0.1% formic acid–acetonitrile by a linear gradient program. Most of the metabolites were detected as being the corresponding *O*-demethyl and *N*, *O*-didemethyl metabolites in the rat plasma and urine after the hydrolysis of *O*-glucuronides, although obvious differences in the amounts of these metabolites were found between the dextro and levo forms. No racemation was observed through *O*- and/or *N*-demethylation. In the rat hair samples collected 4 weeks after the first administration, those differences were more clearly detected and the concentrations of the parent compounds, their *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites were 63.4, 2.7, 25.1, and 0.7 ng/mg for the dextro forms and 24.5, 24.6, 2.6, and 0.5 ng/mg for the levo forms, respectively. In order to fully investigate the differences of their metabolic properties between dextromethorphan and levomethorphan, DA rat and human liver microsomes were studied. The results suggested that there might be an enantioselective metabolism of levomethorphan, especially with regard to the *O*-demethylation, not only in DA rat but human liver microsomes as well. The proposed chiral analyses might be applied to human samples and could be useful for discriminating dextromethorphan use from levomethorphan use in the field of forensic toxicology, although further studies should be carried out using authentic human samples.

Keywords Levomethorphan · Dextromethorphan · Chiral analysis · Biological samples · LC-MS/MS · Enantioselective metabolism

Introduction

Dextromethorphan is widely used all over the world as an over-the-counter antitussive medicine. It produces little or no central nervous system depression at therapeutic doses, but it has dissociative effects similar to ketamine and phencyclidine in large doses as an *N*-methyl-*D*-aspartate receptor antagonist [1–6]. To obtain its hallucinogenic effect, young people abuse this drug by large doses and many fatalities from overdoses have been reported [7, 8]. In

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R. Kikura-Hanajiri (✉) · M. Kawamura · Y. Goda
Division of Pharmacognosy, Phytochemistry and Narcotics,
National Institute of Health Sciences,
1-18-1, Kamiyoga, Setagaya,
Tokyo 158-8501, Japan
e-mail: kikura@nihs.go.jp

A. Miyajima
Division of Medical Devices,
National Institute of Health Sciences,
1-18-1, Kamiyoga, Setagaya,
Tokyo 158-8501, Japan

M. Sunouchi
Division of Pharmacology, National Institute of Health Sciences,
1-18-1, Kamiyoga, Setagaya,
Tokyo 158-8501, Japan

contrast, its enantiomer, levomethorphan, is a potent narcotic analgesic [9] (Fig. 1), and an *O*-demethyl compound of levomethorphan, levorphanol, is known to have stronger opioid pharmacological effects [9, 10]. Levomethorphan is strictly controlled in the world as a narcotic and is never used for therapeutic purposes.

In humans, as shown in Fig. 2, it has been reported that dextromethorphan is primarily metabolized to dextrorphan via *O*-demethylation by cytochrome P450 2D6 (CYP2D6), which is polymorphically expressed in humans, who can be classified as poor, intermediate, and extensive metabolizers [11, 12]. Dextromethorphan is *N*-demethylated via an additional route to 3-methoxymorphinan (3-MEM), primarily mediated by CYP3A4 in human liver microsomes [11, 13]. Dextrorphan and 3-MEM are then demethylated to 3-hydroxymorphinan (3-HM) via CYP3A4 and CYP2D6, respectively. Moreover, dextrorphan and 3-HM are glucuronized to their *O*-glucuronides and these are mainly excreted into human urine [14, 15].

A variety of analytical methods for the determination of dextromethorphan and its metabolites in biological samples have been reported using capillary electrophoresis (CE) [16, 17], HPLC [18–22], GC-MS [23–26], and LC-MS (/MS) [15, 27–30]. However, there is little information regarding the metabolic properties of levomethorphan. Although a chiral separation method of dextromethorphan and levomethorphan using HPLC with fluorometric detection [22] or using CE [17] has been reported, there has been no report that describes a simultaneous determination of dextromethorphan, levomethorphan, and their metabolites in biological samples after administration of these drugs. Considering the possibility of the adulteration or substitution of dextromethorphan with levomethorphan due to its chemical similarities for illegal purposes, it is necessary to establish the enantiometric separation of dextromethorphan and levomethorphan in biological samples.

In order to develop an analytical method for the discrimination of dextromethorphan from levomethorphan in biological samples, we first investigated chiral analyses of these drugs and their *O*-demethyl and/or *N*-demethyl metabolites in plasma, urine, and hair samples of rats administered with each enantiomer, using LC-MS/MS. In addition, detailed metabolic properties of these drugs were investigated using rat and human liver microsomes.

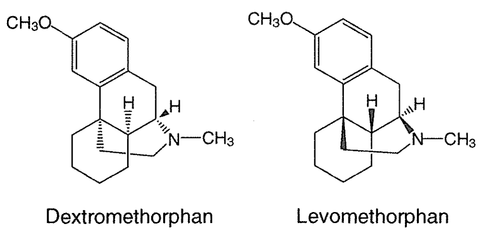

Fig. 1 Chemical structures of dextromethorphan and its enantiomer, levomethorphan

Experimental

Materials

Levomethorphan was obtained from Cerilliant (Round Rock, TX, USA). Dextromethorphan hydrobromide, dextrorphan tartrate, (+)-3-HM hydrobromide, (+)-3-MEM hydrochloride, and levallorphan tartrate (used as internal standard, IS) were from Sigma-Aldrich (St. Louis, MO, USA). Levorphanol tartrate was given by Professor T. Nagano (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan). A β-glucuronidase solution (EC 3.2.1.31, 103,000 units/mL, Source: *Helix pomatia*) was purchased from Wako Chemicals (Osaka, Japan). A solid-phase extraction column (OASIS HLB, 3 cc, 60 mg) was obtained from Waters (Milford, MA, USA), and the membrane filter (Ultrafree-MC, 0.45 μm) was from Millipore Corporation (Bedford, MA, USA).

Liver microsomes from individual male dark agouti (DA) rats (*n*=4, 6 weeks old, around 125 g mean weight) were prepared by ultracentrifugation as described [31, 32]. The concentrations of microsomal protein were estimated using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Human liver microsomes (50-donor pool, 20 mg/mL) were purchased from BD Biosciences (Woburn, MA, USA). Nicotinamide adenine dinucleotide phosphate (NADP) and glucose 6-phosphate (G-6-P) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) and G-6-P dehydrogenase (G-6-PDH) was from Roche Diagnostics (Indianapolis, IN, USA). All other chemicals and solvents were of an analytical reagent grade or HPLC grade (Wako Chemicals, Osaka, Japan).

Instrumentation

The UPLC analysis was performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA). The separations were achieved using a Chiral CD-Ph column (150×2.1 mm i.d., 5 μm) from Shiseido (Tokyo, Japan). The column temperature was maintained at 30 °C, and the following gradient system was used with a mobile phase A (0.1% formic acid) and mobile phase B (0.1% formic acid/acetonitrile) delivered at 0.25 mL/min: 80% A/20% B (2 min hold)—70% A/30% B (15 min). The mobile phase was used as a wash solvent to avoid any carry-over from previous injections. The autosampler was maintained at 4 °C, and the injection volume was 2 μL. Quantitation was achieved by MS/MS detection in a positive ion mode using a Quattro Premier XE mass