Legal Medicine 13 (2011) 109–115

Contents lists available at ScienceDirect

Legal Medicine

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

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

ARTICLE INFO

Article history:
Received 1 February 2011
Accepted 2 February 2011
Available online 5 March 2011

Keywords:
Designer drugs
Synthetic cannabinoids
Cathinone derivatives
Psychotropic plants

ABSTRACT

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.

© 2011 Elsevier Ireland Ltd. All rights reserved.

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-

* 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).

1344-6223/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.legalmed.2011.02.003

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].

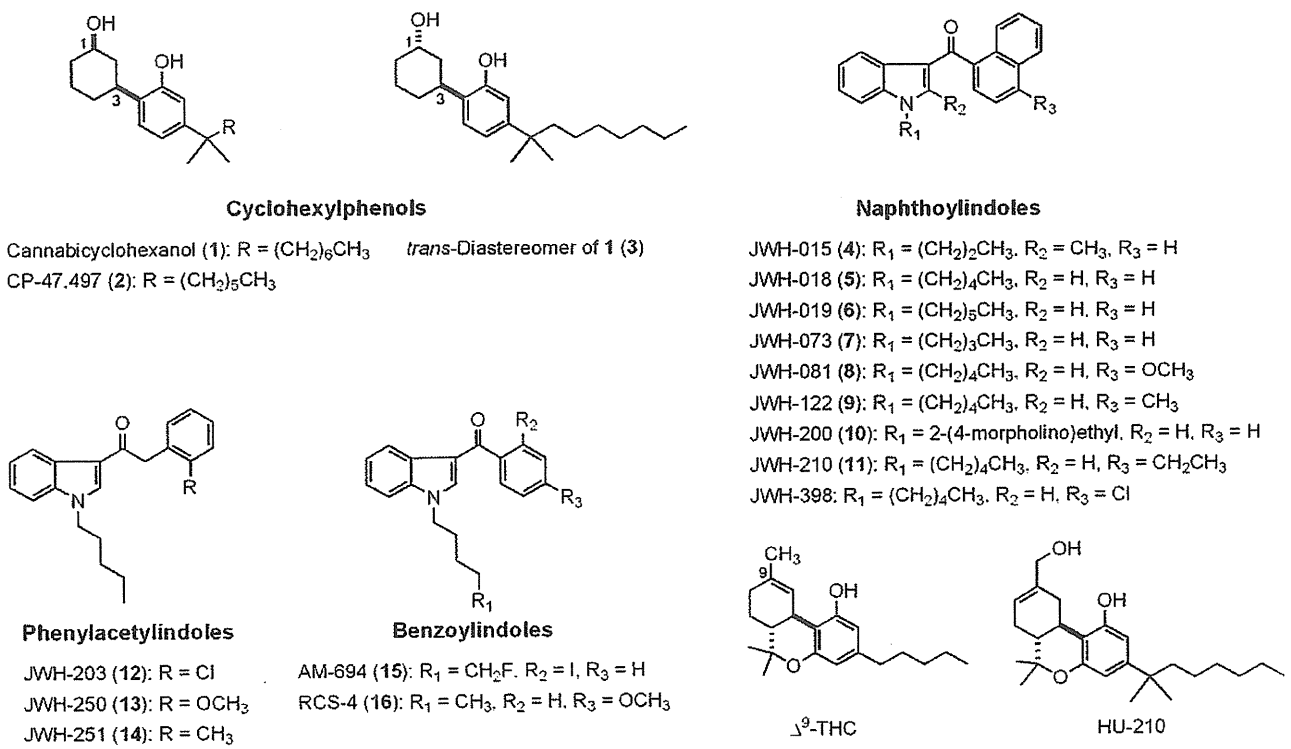


Fig. 1. Chemical structures of synthetic cannabinoids and Δ^9-THC.

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].

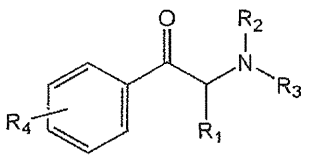
More than 140 different "Spice"-like products of herbal and chemical mixtures available in Japan have been surveyed since June 2008. CCH, CP-47,497 and JWH-018 were the most frequently detected adulterants in these products from June 2008 to October 2009 [17]. Oleamide (*cis*-9,10-octadecenoamide), which 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 MDPDP (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%,

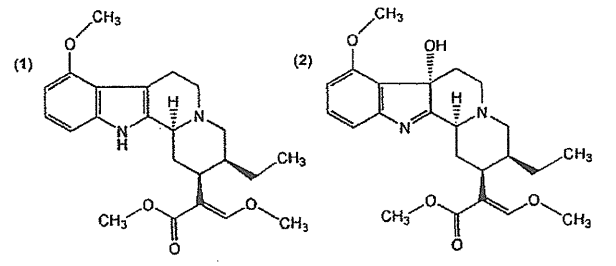


Fig. 3. Chemical structures of active compounds in *Mitragyna speciosa*, mitragynine (1) and 7-hydroxymitragynine (2).

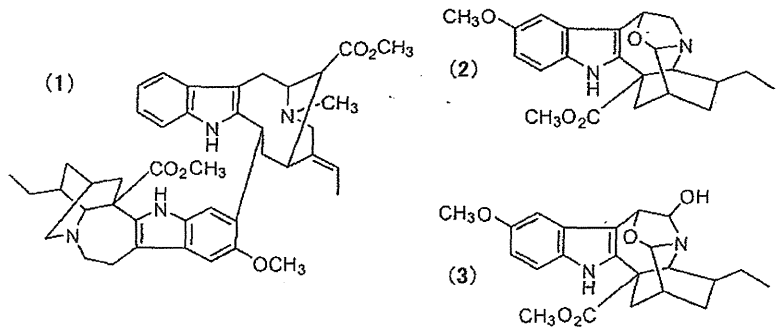


Fig. 4. Chemical structures of three indole alkaloids from *V. africana* root bark, which have potent antagonist activities against the CB1 receptor: voacamine (1), 3,6-oxidovoacangine (2) and 5-hydroxy-3,6-oxidovoacangine (3).

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.

References

[1] Nagashima M, Seto T, Takahashi M, Miyake H, Yasuda I. Screening method of the chemical illegal drugs by HPLC-PDA. Ann Rep Tokyo Metr Inst Pub Health 2004;55:67–71.
[2] Kikura-Hanajiri R, Hayashi M, Saisho K, Goda Y. Simultaneous determination of 19 hallucinogenic tryptamines/ß-calbolines and phenethylamines using GC–MS and LC-ESI–MS. J Chromatogr B 2005;825:29–37.
[3] Seto T, Takahashi M, Nagashima M, Suzuki J, Yasuda I. The identifications and the aspects of the commercially available uncontrolled drugs purchased between Apr. 2003 and Mar. 2004. Ann Rep Tokyo Metr Inst Pub Health 2005;56:75–80.
[4] Suzuki J, Takahashi M, Seto T, Nagashima M, Okumoto C, Yasuda I. Qualitative method of nitrite inhalants. Ann Rep Tokyo Metr Inst Pub Health 2006;57:115–20.
[5] Matsumoto T, Kikura-Hanajiri R, Kamakura H, Kawamura N, Goda Y. Identification of *N*-Methyl-4-(3, 4-methylenedioxyphenyl)butan-2-amine, distributed as MBDB. J Health Sci 2006;52:805–10.
[6] Takahashi M, Suzuki J, Nagashima M, Seto T, Yasuda I. Newly detected compounds in uncontrolled drugs purchased in Tokyo between April 2006 and March 2007. Ann Rep Tokyo Metr Inst Pub Health 2007;58:83–7.
[7] Kikura-Hanajiri R, Kawamura M, Saisho K, Kodama Y, Goda Y. The disposition into hair of new designer drugs: methylone, MBDB and methcathinone. J Chromatogr B 2007;855:121–6.
[8] Uchiyama N, Kikura-Hanajiri R, Kawahara N, Goda Y. Analysis of designer drugs detected in the products purchased in fiscal year 2006. Yakugaku Zasshi 2008;128:1499–505.
[9] Kikura-Hanajiri R, Kawamura M, Uchiyama N, Ogata J, Kamakura H, Saisho K, et al. Analytical data of designated substances (Shitei-Yakubutsu) controlled by the pharmaceutical affairs law in Japan, part I: GC–MS and LC–MS. Yakugaku Zasshi 2008;128:971–9.
[10] Uchiyama N, Kawamura M, Kamakura H, Kikura-Hanajiri R, Goda Y. Analytical data of designated substances (Shitei-Yakubutsu) controlled by the

pharmaceutical affairs law in Japan, part II: color test and TLC. Yakugaku Zasshi 2008;128:981–7.
[11] Nagashima M, Seto T, Takahashi M, Suzuki J, Mori K, Ogino S. Analyses of uncontrolled drugs purchased from April 2007 to March 2008 and newly found compounds. Ann Rep Tokyo Metr Inst Pub Health 2008;59:71–7.
[12] Uchiyama N, Miyazawa N, Kawamura M, Kikura-Hanajiri R, Goda Y. Analysis of newly distributed designer drugs detected in the products purchased in fiscal year 2008. Yakugaku Zasshi 2010;130:263–70.
[13] Nagashima M, Takahashi M, Suzuki J, Seto T, Mori K, Ogino S. Analyses of uncontrolled drugs purchased Apr. 2008–Mar. 2009. Ann Rep Tokyo Metr Inst Pub Health 2009;60:81–4.
[14] Kikura-Hanajiri R, Kawamura M, Miyajima A, Sunouchi M, Goda Y. Determination of a new designer drug, *N*-hydroxy-3,4-methylenedioxymethamphetamine and its metabolites in rats using ultra-performance liquid chromatography–tandem mass spectrometry. Forensic Sci Int 2010;198:62–9.
[15] Uchiyama N, Kikura-Hanajiri R, Kawahara N, Haishima Y, Goda Y. Identification of a cannabinoid analog as a new type of designer drug in a herbal product. Chem Pharm Bull 2009;57:439–41.
[16] Uchiyama N, Kikura-Hanajiri R, Kawahara N, Goda Y. Identification of a cannabimimetic indole as a designer drug in a herbal product. Forensic Toxicol 2009;27:61–6.
[17] Uchiyama N, Kikura-Hanajiri R, Ogata J, Goda Y. Chemical analysis of synthetic cannabinoids as designer drugs in herbal products. Forensic Sci Int 2010;198:31–8.
[18] Uchiyama N, Kawamura M, Kikura-Hanajiri R, Goda Y. Identification and quantitative analyses of two cannabimimetic phenylacetylindoles, JWH-251 and JWH-250, and four cannabimimetic naphthoylindoles, JWH-081, JWH-015, JWH-200 and JWH-073, as designer drugs in illegal products. Forensic Toxicol 2011;29:25–37.
[19] Nakajima J, Takahashi M, Seto T, Suzuki J. Identification and quantitation of cannabimimetic compound JWH-250 as an adulterant in products obtained via the internet. Forensic Toxicol 2011;29:51–5.
[20] Uchiyama N, Kikura-Hanajiri R, Shoda T, Fukuhara, K, Goda Y. Isomeric analysis of synthetic cannabinoids detected as designer drugs. Yakugaku Zasshi, submitted for publication.
[21] Miyasaka K, Kawamura M, Uchiyama N, Kikura-Hanajiri R, Goda Y. Yakugaku Zasshi, Analytical data of synthetic cannabinoids distributed as psychotropic substances, Yakugaku Zasshi, submitted for publication.
[22] Uchiyama N, Kikura-Hanajiri R, Goda Y. Identification of cannabimimetic indoles as designer drugs in illegal products, in preparation.
[23] Uchiyama N, Kikura-Hanajiri R, Goda Y. Analysis of newly distributed designer drugs-Cathinone derivatives-detected in products purchased in fiscal year 2010, in preparation.
[24] EMCDDA action on new drugs briefing paper: understanding the 'Spice' phenomenon (a report from an EMCDDA expert meeting, 6 March 2009, Lisbon), updated version: 13 August 2009.
[25] ACMD. Consideration of the cathinones, 2010.
[26] ACMD. Consideration of the naphthylpyrovalerone analogs and related compounds, 2010.
[27] EMCDDA–Europol 2009 Annual Report on the implementation of Council Decision 2005/387/JHA. In accordance with Article 10 of Council Decision 2005/387/JHA on the information exchange, risk-assessment and control of new psychoactive substances, 2010.
[28] EMCDDA. Risk assessment report of a new psychoactive substance: 4-methylmethcathinone (mephedrone). In accordance with Article 6 of Council Decision 2005/387/JHA on information exchange, risk assessment and control of new psychoactive substances, 2010.
[29] Official Journal of the European Union. Council decision of 2 December 2010 on submitting 4-methylmethcathinone (mephedrone) to control measures (2010/759/EU) 8.12.2010.
[30] Auwärter V, Dresen S, Weinmann W, Müller M, Pütz M, Ferreirós N. 'Spice' and other herbal blends: harmless incense or cannabinoid designer drugs? J Mass Spectrom 2009;44:832–7.
[31] Compton DR, Rice KC, De Costa BR, Razdan RK, Melvin LS, Johnson MR, Martin BR. Cannabinoid structure-activity relationships: correlation of receptor binding and in vivo activities. J Pharmacol Exp Ther 1993;265:218–26.
[32] Huffman JW. Cannabimimetic indoles, pyrroles, and indenes: structure-activity relationships and receptor interactions. In: Reggio PH, editor. The cannabinoid receptors. New York: Humana Press; 2009. p. 49–94.
[33] Lindigkeit R, Boehme A, Eiserloh I, Luebbecke M, Wiggermann M, Ernst L, et al. Spice: a never ending story? Forensic Sci Int 2009;191:58–63.
[34] Dresen S, Ferreirós N, Pütz M, Westphal F, Zimmermann R, Auwärter V. Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds. J Mass Spectrom 2010;45:1186–94.
[35] Compton DR, Johnson MR, Melvin LS, Martin BR. Pharmacological profile of a series of bicyclic cannabinoid analogs: classification as cannabimimetic agents. J Pharmacol Exp Ther 1992;260:201–9.
[36] Melvin LS, Milne GM, Johnson MR, Subramaniam B, Wilken GH, Howlett AC. Structure-activity relationships for cannabinoid receptor-binding and analgesic activity: studies of bicyclic cannabinoid analogs. Mol Pharmacol 1993;44:1008–15.
[37] Thomas BF, Compton DR, Martin BR. Characterization of the lipophilicity of natural and synthetic analogs of delta 9-tetrahydrocannabinol and its relationship to pharmacological potency. J Pharmacol Exp Ther 1990;255:624–30.
[38] Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, et al. Behavioral, biochemical, and molecular modeling evaluations of cannabinoid analogs. Pharmacol Biochem Behav 1991;40:471–8.
[39] Showalter VM, Compton DR, Martin BR, Abood ME. Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands. J Pharmacol Exp Ther 1996;278:989–99.
[40] Huffman JW, Mabon R, Wu MJ, Lu J, Hart R, Hurst DP, et al. 3-Indolyl-1-naphthylmethanes: new cannabimimetic indoles provide evidence for aromatic stacking interactions with the CB_1 cannabinoid receptor. Bioorg Med Chem 2003;11:539–49.
[41] ACMD report on the major cannabinoid agonists: consideration of the major cannabinoid agonist, 2009.
[42] Aung MM, Griffin G, Huffman JW, Wu M, Keel C, Yang B, et al. Influence of the N-1 alkyl chain length of cannabimimetic indoles upon CB_1 and CB_2 receptor binding. Drug Alcohol Depend 2000;60:133–40.
[43] Zweiundzwanzigste Verordnung, zur Änderung betäubungsmittelrechtlicher Vorschriften, Germany. BGBl I Nr. 3 vom 21.01.2009, 22. BtMÄndV vom 19. Januar 2009, S. 49–50.
[44] Makriyannis A, Deng H. WO patent 200128557. Cannabimimetic indole derivatives, granted 2001-06-07.
[45] Vardakou I, Pistos C, Spiliopoulou Ch. Spice drugs as a new trend: mode of action, identification and legislation. Toxicol Lett 2010;197:157–62.
[46] Glennon RA, Yousif M, Naiman N, Kalix P. Methcathinone: a new and potent amphetamine-like agent. Pharmacol Biochem Behav 1987;26:547–51.
[47] Davies S, Wood DM, Smith G, Button J, Ramsey J, Archer R, et al. Purchasing 'legal highs' on the Internet – is there consistency in what you get? QJM 2010;103:489–93.
[48] Gibbons S, Zloh M. "Legal highs" on the net-Evaluation of UK-based Websites, products and product information. An analysis of the 'legal high' mephedrone. Bioorg Med Chem Lett 2010;20:4135–9.
[49] Schmidt MM, Sharma A, Schifano F, Feinmann C, "Legal highs" on the net-Evaluation of UK-based Websites, products and product information. Forensic Sci Int 2010. doi:10.1016/j.forsciint.2010.06.030.
[50] Brandt SD, Sumnall HR, Measham F, Cole J. Analyses of second-generation 'legal highs' in the UK: initial findings. Drug Test Anal 2010;2:377–82.
[51] Brandt SD, Freeman S, Sumnall HR, Measham F, Cole J. Analysis of NRG 'legal highs' in the UK: identification and formation of novel cathinones. Drug Test Anal 2010. doi:10.1002/dta.204.
[52] Vardakou I, Pistos C, Spiliopoulou C. Drugs for youth via Internet and the example of mephedrone. Toxicol Lett 2010. doi:10.1016/j.toxlet.2010.12.014.
[53] Wikström M, Thelander G, Nyström I, Kronstrand R. Two fatal intoxications with the new designer drug methedrone (4-methoxymethcathinone). J Anal Toxicol 2010;34:594–8.
[54] Springer D, Peters FT, Fritschi G, Maurer HH. Studies on the metabolism and toxicological detection of the new designer drug 4′-methyl-alpha-pyrrolidinopropiophenone in urine using gas chromatography-mass spectrometry. J Chromatogr B 2002;773:25–33.
[55] Foley KF, Van Dort ME, Sievert MK, Ruoho AE, Cozzi NV. Stereospecific inhibition of monoamine uptake transporters by meta-hydroxyephedrine isomers. J Neural Transm 2002;109:1229–40.
[56] Nagai F, Nonaka R, Satoh H, Kamimura K. The effects of non-medically used psychoactive drugs on monoamine neurotransmission in rat brain. Eur J Pharmacol 2007;559:132–7.
[57] Zaitsu K, Katagi M, Kamata HT, Kamata T, Shima N, Miki A, et al. Determination of the metabolites of the new designer drugs bk-MBDB and bk-MDEA in human urine. Forensic Sci Int 2009;188:131–9.
[58] Hamano T, Shioda H, Nakajima J, Yasuda I. Analysis of psychotropic components in commercial botanical drugs. Ann Rep Tokyo Metr Inst Pub Health 2006;57:121–6.
[59] Kawamura M, Kikura-Hanajiri R, Goda Y. Survey of current trends in the abuse of psychotropic plants using LC–MS. Jpn J Food Chemistry 2008; 15:73–8.
[60] Kawamura M, Kikura-Hanajiri R, Goda Y. Simple and rapid screening for psychotropic natural products using direct analysis in real time (DART)-TOFMS. Yakugaku Zasshi 2009;129:719–25.
[61] Maruyama T, Shirota O, Kawahara N, Yokoyama K, Makino Y, Goda Y. Discrimination of psychoactive fungi (commonly called "magic mushrooms") based on the DNA sequence of the internal transcribed spacer region. Shokuhin Eiseigaku Zasshi 2003;44:44–8.
[62] Maruyama T, Yokoyama K, Makino Y, Goda Y. Phylogenetic relationship of psychoactive fungi based on the rRNA gene for a large subunit and their identification using the TaqMan assay. Chem Pharm Bull 2003;51:710–4.
[63] Maruyama T, Kawahara N, Fukiharu T, Yokoyama K, Makino Y, Goda Y. DNA and chemical analyses of commercial fly agaric-related products. Shokuhin Eiseigaku Zasshi 2005;46:49–54.
[64] Maruyama T, Kawahara N, Yokoyama K, Makino Y, Fukiharu T, Goda Y. Phylogenetic relationship of psychoactive fungi based on rRNA gene for a large subunit and their identification using the TaqMan assay (II). Forensic Sci Int 2006;163:51–8.
[65] Maruyama T, Kamakura H, Kikura-Hanajiri R, Goda Y. Authentication and ultra performance liquid chromatography (UPLC)/MS analysis of magic mint, *Salvia divinorum* and its related plants. Yakugaku Zasshi 2008;128:179–83.
[66] Kikuchi H, Uchiyama N, Ogata J, Kikura-Hanajiri R, Goda Y. Chemical constituents and DNA sequence analysis of a psychotropic herbal product. Forensic Toxicol 2010;28:1–7.

[67] Kikura-Hanajiri R, Kawamura M, Maruyama T, Kitajima M, Takayama H, Goda Y. Simultaneous analysis of mitragynine, 7-hydroxymitragynine and other alkaloids in the psychotropic plant "Kratom" (*Mitragyna speciosa*) by LC-ESI-MS. Forensic Toxicol 2009;27:67–74.
[68] Maruyama M, Kawamura M, Kikura-Hanajiri R, Takayama H, Goda Y. The botanical origin of Kratom (*Mitragyna speciosa*; Rubiaceae) available as abused drugs in the Japanese markets. J Nat Med 2009;63(3):340–4.
[69] Kikura-Hanajiri R, Maruyama T, Miyashita A, Goda Y. Chemical and DNA analyses for the products of a psychoactive plant, *Voacanga Africana*. Yakugaku Zasshi 2009;129:975–82.
[70] Takayama H. Chemistry and pharmacology of analgesic indole alkaloids from the rubiaceous plant, *Mitragyna speciosa*. Chem Pharm Bull 2004;52: 916–28.
[71] Stables J, Green A, Marshall F, Fraser N, Knight E, Sautel M, et al. A bioluminescent assay for agonist activity at potentially any G protein-coupled receptor. Anal Biochem 1997;252:115–26.
[72] Kitajima M, Iwai M, Kikura-Hanajiri R, Goda Y, Iida M, Yabushita H, Takayama H. Discovery of indole alkaloids with cannabinoid CB1 receptor antagonistic activity. Bioorg Med Chem Lett, accepted for publication.

Bioorganic & Medicinal Chemistry Letters 21 (2011) 1962–1964

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Discovery of indole alkaloids with cannabinoid CB1 receptor antagonistic activity

Mariko Kitajima [a], Masumi Iwai [a], Ruri Kikura-Hanajiri [b], Yukihiro Goda [b], Mitsuru Iida [c], Hisatoshi Yabushita [c], Hiromitsu Takayama [a,*]

[a] Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan
[b] National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[c] Otsuka Pharmaceutical Co., Ltd, 224-18 Hiraishi Ebisuno, Kawauchi-cho, Tokushima 771-0182, Japan

ARTICLE INFO

Article history:
Received 6 January 2011
Revised 8 February 2011
Accepted 9 February 2011
Available online 13 February 2011

Keywords:
Cannabinoid
CB1 receptor
Antagonist
Indole alkaloid
Iboga
Voacanga

ABSTRACT

Three indole alkaloids, voacamine (**1**), 3,6-oxidovoacangine (**2**), and a new alkaloid, 5-hydroxy-3,6-oxidovoacangine (**3**), isolated from *Voacanga africana* were found to exhibit potent cannabinoid CB1 receptor antagonistic activity. This is the first example of CB1 antagonists derived from natural alkaloids.
© 2011 Elsevier Ltd. All rights reserved.

The endocannabinoid system is involved in a wide variety of psychological and physiological processes. The cannabinoid (CB) receptors are G-protein-coupled receptors (GPCRs) and two subtypes are known: CB1 and CB2.[1] The CB1 receptor is distributed throughout the body, mainly expressed in the brain, and involved in memory, cognitive process, pain, and appetite. It is also one of the targets of drug development for the treatment of obesity, metabolic syndrome, obesity-related cardiovascular disorder, substance abuse, and cognitive impairment. The therapeutic potential of CB1 receptor antagonists has been demonstrated.[1] In this Letter, we disclose three indole alkaloids possessing CB1 receptor antagonistic activity: voacamine (**1**), 3,6-oxidovoacangine (**2**), and a new alkaloid, 5-hydroxy-3,6-oxidovoacangine (**3**), isolated from *Voacanga africana* (Fig. 1).

The screening of plant extracts and isolated alkaloids for agonistic or antagonistic activity on the CB1 receptor was performed using the aequorin/GPCR cell-based Ca^{2+} functional assay.[2] CP55940 or rimonabant was used as the positive control for agonist or antagonist. As a result, we found that the MeOH extract of *V. africana* root bark showed potent activity. This prompted us to clarify the active principle in *V. africana*. Separation of the crude base that was prepared from *V. africana* root bark resulted in the isolation of three active indole alkaloids: one iboga-vobasine-type bis-indole alkaloid, voacamine (**1**),[3] and two iboga-type monomer alkaloids, 3,6-oxidovoacangine (**2**)[4] and 5-hydroxy-3,6-oxidovoacangine (**3**).[5] These compounds exhibited relatively potent CB1 receptor antagonistic activity with IC_{50} of 0.041, 0.199, and 0.141 μM, respectively, compared with that of rimonabant (IC_{50} 0.004 μM). These are a new class of small molecules possessing CB1 receptor antagonistic activity. Figure 2 shows that their effects were concentration-dependent. Interestingly, well-known coexisting alkaloids, such as voacangine (**4**), vobasine (**5**), and tabersonine (**6**), did not show the activity.

Among the three alkaloids, 5-hydroxy-3,6-oxidovoacangine (**3**) is a new alkaloid and its structure was deduced by spectroscopic analysis as follows. Compound **3** was found to have the molecular formula $C_{22}H_{26}N_2O_5$ from HREIMS [*m/z* 398.1844 (M^+)], which indicated that **3** has an extra oxygen atom compared to 3,6-oxidovoacangine (**2**). The UV spectrum exhibited a characteristic 5-methoxyindole chromophore. Its NMR spectra were similar to those of 3,6-oxidovoacangine (**2**) except for the existence of a low-field oxygenated methine group at δ_H 5.16 and δ_C 92.7 in the ^1H and ^{13}C NMR spectra, respectively, instead of a methylene group due to C-5. HMBC correlation of the proton at δ 5.16 to the carbon at δ 59.0 due to C-21, together with the above data, indicated that the hydroxyl group was attached to C-5 (Fig. 3). As a result of the rigid character of the skeleton, the relative configuration at C-3, C-6, C-14, C-16, and C-21 was restricted, that is, 3*S**, 6*R**, 14*R**, 16*S**, and 21*S**. The NOE correlation of H-5 to H-21 suggested that

* Corresponding author. Tel./fax: +81 (43) 290 2901.
E-mail address: htakayam@p.chiba-u.ac.jp (H. Takayama).

0960-894X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.02.036

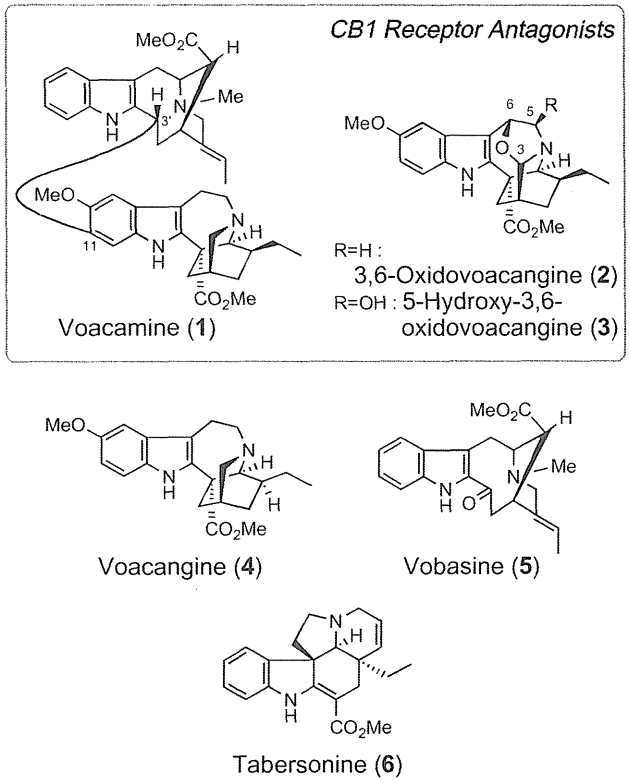


Figure 1. Structures of indole alkaloids **1**–**3** with CB1 receptor antagonistic activity and alkaloids **4**–**6** from *Voacanga africana*.

both protons were in *cis* relationship and the hydroxyl group at C-5 had *R** configuration. The absolute configuration at C-16 was deduced to be *S* from the negative Cotton effect that appeared at approximately 280 nm in the CD spectrum, which was the same as that of voacangine (**4**), whose absolute configuration is known.[6] Therefore, new alkaloid **3** was deduced to be 5-hydroxy-3,6-oxidovoacangine.

In conclusion, we have found three cannabinoid CB1 receptor antagonists, voacamine (**1**), 3,6-oxidovoacangine (**2**), and 5-hydroxy-3,6-oxidovoacangine (**3**), from *V. africana*. As far as we know, these are the first examples of natural alkaloids having CB1 receptor antagonistic activity. Further studies of the medicinal chemistry using these structurally and biologically unique alkaloids are under way in our laboratories.

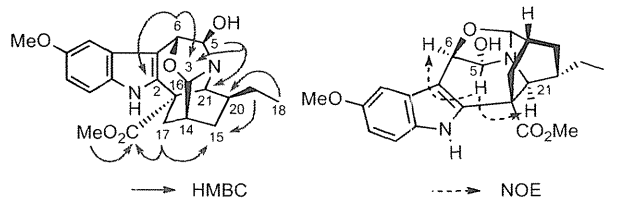


Figure 3. Selected HMBC and NOE correlations of 5-hydroxy-3,6-oxidovoacangine (**3**).

Acknowledgement

This work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan.

References and notes

1. For recent reviews on cannabinoid receptor agonists/antagonists, see: (a) Thomas, B. F. *Drug Dev. Res.* **2009**, *70*, 527; (b) Janero, D. R.; Makriyannis, A. *Expert Opin. Emerg. Drugs* **2009**, *14*, 43; (c) Jagerovic, N.; Fernandez-Fernandez, C.; Goya, P. *Curr. Top. Med. Chem.* **2008**, *8*, 205; (d) Muccioli, G.; Lambert, D. M. *Expert Opin. Ther. Patents* **2006**, *16*, 1405. and references cited therein.
2. Stables, J.; Green, A.; Marshall, F.; Fraser, N.; Knight, E.; Sautel, M.; Milligan, G.; Lee, M.; Rees, S. *Anal. Biochem.* **1997**, *252*, 115.
3. (a) Achenbach, H.; Schaller, E. *Chem. Ber.* **1976**, *109*, 3527; (b) Medeiros, W. L. B.; Vieira, I. J. C.; Mathias, L.; Braz-Filho, R.; Leal, K. Z.; Rodrigues-Filho, E.; Schripsema, J. *Magn. Reson. Chem.* **1999**, *37*, 676; (c) Agwada, J.; Patel, M. B.; Hesse, M.; Schmid, H. *Helv. Chim. Acta* **1972**, *55*, 1849.
4. (a) Ghorbel, N.; Damak, M.; Ahond, A.; Philogène, E.; Poupat, C.; Potier, P.; Jacquemin, H. *J. Nat. Prod.* **1981**, *44*, 717; (b) Morfaux, A. M.; Mulamba, B.; Richard, B.; Delaude, C.; Massiot, G.; Le Men-Oliver, L. *Phytochemistry* **1982**, *21*, 1767.
5. The root bark of *Voacanga africana* (608 g dry weight) was extracted with MeOH to give a MeOH extract (70.12 g). The crude base (15.5 g) was prepared from the MeOH extract by a conventional method. The $CHCl_3$ extract was separated by silica gel open column chromatography with a MeOH/$CHCl_3$ gradient. The fraction eluted with 3% MeOH/$CHCl_3$ was purified by repeated chromatography to afford 5-hydroxy-3,6-oxidovoacangine (**3**, 1.4 mg). Compound **3**: UV (MeOH) λ_{max} nm (log ε) 285.0, 203.5; ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.53 (1H, br s, NH), 7.14 (1H, d, J = 8.8 Hz, H-12), 7.02 (1H, d, J = 2.5 Hz, H-9), 6.80 (1H, dd, J = 8.8, 2.5 Hz, H-11), 5.27 (1H, s, H-6), 5.24 (1H, d, J = 3.8 Hz, H-3), 5.16 (1H, s, H-5), 3.88 (3H, s, CO_2Me), 3.84 (3H, s, 10-OMe), 3.69 (1H, br d, J = 2.7 Hz, H-21), 2.44 (1H, br d, J = 14.0 Hz, H-17), 2.30 (1H, m, H-14), 2.14 (1H, br ddd, J = 14.0, 3.4, 3.4 Hz, H-17), 1.92 (1H, m, H-15), 1.57 (1H, m, H-19), 1.42 (1H, m, H-19), 1.30 (1H, m, H-20), 1.08 (1H, m, H-15), 0.89 (3H, dd, J = 7.1, 7.1 Hz, H_3-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 175.0 (CO_2Me), 154.7 (C-10), 139.0 (C-2), 128.8 (C-13), 127.1 (C-8), 112.7 (C-7), 112.6 (C-11), 111.7 (C-12), 99.5 (C-9), 93.4 (C-3), 92.7 (C-5), 74.9 (C-6), 59.0 (C-21), 55.9 (10-OMe), 53.0 (CO_2Me), 52.9 (C-16), 38.4 (C-20), 32.4 (C-17), 29.7 (C-15), 29.5 (C-14), 26.6 (C-19), 11.7 (C-18); EIMS

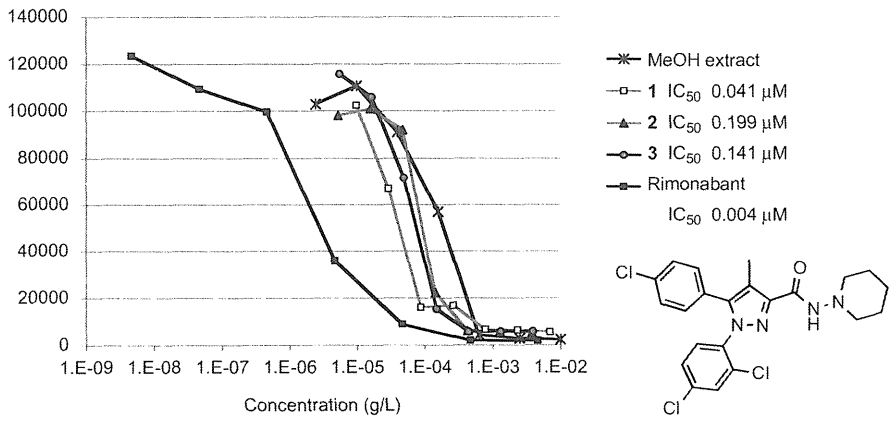


Figure 2. CB1 receptor antagonistic activity of MeOH extract of *Voacanga africana*, alkaloids **1**–**3**, and rimonabant.

m/z (%) 398 ([M]$^+$, 27), 75 (100); HREIMS *m/z* 398.1844 (calcd for $C_{22}H_{26}N_2O_5$ 398.1841); CD (MeOH, 24 °C, *c* 0.29 mmol/L) $\Delta\varepsilon$ (λ nm) 0 (356), −0.24 (320), 0 (306), −0.54 (281), 0 (265), +0.15 (255), 0 (251), −0.26 (241), 0 (231), +2.38 (217).

6. (a) Kutney, J. B.; Brown, R. T.; Piers, E. *Can. J. Chem.* **1966**, *44*, 637; (b) Bláha, K.; Koblicová, Z.; Trojánek, J. *Tetrahedron Lett.* **1972**, *27*, 2763.

Anal Bioanal Chem (2011) 400:165–174
DOI 10.1007/s00216-011-4707-y

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

Received: 30 September 2010 / Revised: 7 January 2011 / Accepted: 22 January 2011 / Published online: 11 February 2011
© Springer-Verlag 2011

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

Published in the special issue *Forensic Toxicology* with Guest Editors Frank T. Peters, Hans H. Maurer, and Frank Musshoff.

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

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

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.

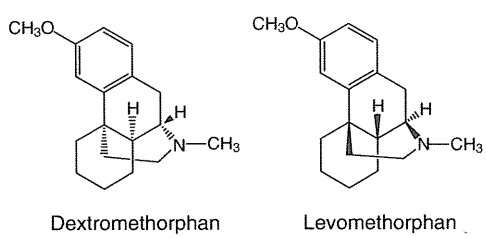


Fig. 1 Chemical structures of dextromethorphan and its enantiomer, levomethorphan

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

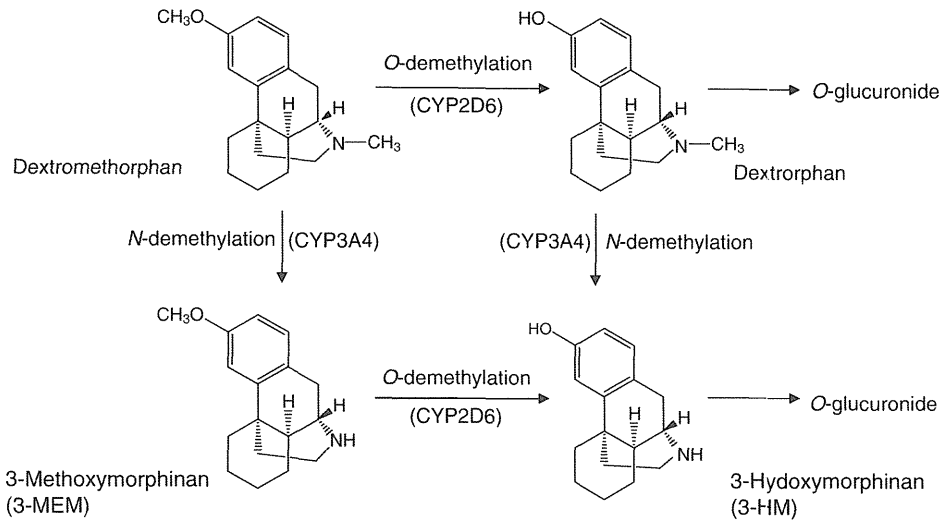


Fig. 2 Main metabolic pathway of dextromethorphan in humans

spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface. Quantification was performed using multiple reaction monitoring of the transitions of precursor ions to product ions with each cone voltage and collision energy as shown in Table 1. The optimal MS parameters obtained were as follows: capillary 3.0 kV, source temperature 120 °C, and desolvation temperature 350 °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).

Since the standard compounds of (−)-3-MEM and (−)-3-HM were not available, these peaks were confirmed by comparison of their retention times and mass fragmentations with those of the standard compounds of the dextro forms ((+)-3-MEM and (+)-3-HM) using an ODS column. The analyses were performed using an Acquity HSS T3 column (100×2.1 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 MS parameters were the same as for the analyses using the chiral column described above.

Animal experiments

The animal experimental model was designed as shown in our previous reports [33, 34]. All experiments were carried out with the approval of the Committee for Animal Care and Use of the National Institute of Health Sciences, Japan. Dextromethorphan hydrobromide (dissolved in an isotonic sodium chloride solution, 2.5 mg/mL, rat 1–3) or levomethorphan (dissolved in a mixed solution of 5% Emulphor™ EL-620/5% ethanol/90% isotonic sodium chloride solution, 2.5 mg/mL, rat 4–6) was administered to male 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 ten 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. Urine samples were collected 0–24, 24–48, and 48–72 h after the last administration and stored at −20 °C. 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.

Extraction of parent compounds and their metabolites from rat plasma and urine samples

For the quantitative analysis of *O*-demethyl and *N*, *O*-didemethyl metabolites in the rat plasma and urine samples, the analytes were measured as free compounds after the hydrolysis of *O*-glucuronides. The optimal condition of the hydrolysis was evaluated, with the peak of putative *O*-glucuronide at nearly 2 min (m/z 434→258) on the MRM chromatogram disappearing from rat plasma and urine samples after the hydrolysis.

A 25-μL plasma sample with 50 μL of added 10 mM ammonium formate buffer (pH 5.0) was reacted with 20 μL of the β-glucuronidase solution at 37 °C for 20 h. To precipitate the proteins in the plasma, 40 μL of the IS methanol solution and 100 μL of methanol were poured into each tube, and the mixtures were then vigorously mixed. The

Table 1 Analytical conditions of LC-MS/MS using the Chiral CD-Ph column

Compounds	Retention time min	Precursor ions *m/z*	Cone voltage V	Product ions *m/z*	Collision energy eV
Dextromethorphan	10.6	272	40	171	45
Dextrorphan	6.1	258	45	157	40
(+)-3-MEM	8.1	258	40	170	35
(+)-3-HM	3.9	244	30	156	35
Levomethorphan	11.3	272	40	171	45
Levorphanol	5.5	258	45	157	40
(−)-3-MEM	9.8	258	40	170	35
(−)-3-HM	4.5	244	30	156	35
Levallorphan (IS)	7.5	284	40	157	40

mixed solution was centrifuged at 1,200×*g* for 3 min and filtered prior to the injection for the LC-MS/MS analysis.

To a 50-μL urine sample (20 μL for 0–24 h samples) was added 100 μL of the β-glucuronidase solution, 1 mL of 10 mM ammonium formate buffer (pH 5.0) and 50 μL of the IS aqueous solution, respectively. The mixed solution was incubated at 37 °C with gentle shaking. After an OASIS HLB column was pre-activated with 2 mL of methanol and distilled water, the reaction mixture was applied to the column. Following the wash of the column with 2 mL of distilled water, 1 mL of methanol was passed through the column to elute the target drugs. A 2-μL of the solution was automatically injected into the UPLC-MS/MS.

Extraction of parent compounds and their metabolites from rat hair samples

Hair samples were washed three times with 0.1% sodium dodecyl sulfate under ultrasonication, followed by washing three times with water under the same condition. 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 mL of methanol/5 M hydrochloric acid mixed solution (20:1) containing 50 μL of the IS methanol solution for 1 h under ultrasonication. 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 distilled water. The solution was treated with an OASIS HLB column and analyzed as described above.

Linearity, precision, and recovery of the analytical method for the rat samples

An individual standard solution of 1.0 mg/mL of each drug, dextromethorphan, levomethorphan, dextrorphan, 3-hydroxymorphinan, 3-methoxymorphinan, and levorphanol, was prepared in methanol and stored at 4 °C. The IS solutions of 1 μg/mL of levallorphan in methanol for the analysis of hair samples and those of 1 μg/mL of levallorphan in distilled water for plasma and urine samples were also prepared.

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, 1, 2, 4, 20, 40, 200, and 400 ng/mL of the target drugs for the rat plasma, 0, 5, 10, 50, 100, 500, 1,000 2,500, 5,000, and 10,000 ng/mL for the urine samples and 0, 0.1, 0.5, 1.0, 5.0, 10, 25, and 50 ng/mg for the hair samples were prepared just before analysis. The limit of quantitation (LOQ) of each drug was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance, while the limit of detection (LOD) was defined as concentrations in a sample matrix resulting in peak areas with signal-to-noise ratios (*S/N*) of 3.

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 2, 20, and 200 ng/mL for the rat plasma samples and 5, 500, and 5,000 ng/mL for the urine samples, respectively. For the hair analyses, the control samples spiked with the standard solutions each containing 0.1, 5, and 50 ng/mg of the targeted drugs were evaluated. The recoveries of the four analytes from the rat samples were determined using each sample spiked with the analytes at a concentration of 80 ng/mL for the plasma, 500 ng/mL for the urine, and 10 ng/mg for the hair, respectively. To determine the recoveries, the responses of the analytes in the standard solutions and in the extracts from the rat control samples were compared. For the quantitative analysis of (−)-MEM and (−)-HM, the calibration curves of (+)-MEM and (+)-HM were used.

Demethylation of dextromethorphan/levomethorphan in rat and human liver microsomes

For the *in vitro* experiments with rat and human liver microsomes, the reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.4) with an NADPH generating system (1.3 mM NADP, 3.3 mM G-6-P, 0.4 U/mL G-6-PDH, 3.3 mM $MgCl_2$), 50 μM substrate (dextromethorphan or levomethorphan), and 0.5 mg protein/mL microsomes (rat or human liver microsomes) in a final volume of 200 μL. Dextromethorphan and levomethorphan were dissolved in methanol, and the final concentration of the organic solvent was 0.1%. The incubation was started by adding the microsomal fraction and then continued for 0, 5, 10, or 20 min. The reaction was terminated by adding an equal volume of a mixed organic solution of 50% acetonitrile and 50% methanol, including 10 μM levallorphan (IS), and vigorous shaking. At the same time, a reaction mixture without the microsomal fraction was also incubated as an enzyme-free control. The mixture was centrifuged at 3,500×*g* for 3 min at 4 °C, and the supernatant was filtered prior to the injection for the LC-MS/MS analysis. The *in vitro* experiments for kinetic analyses were also performed as described above, except that 2, 5, 10, 50, 100, and 150 μM of substrates were incubated with the rat and human liver microsomes for 10 min. Each experiment was performed in duplicate and kinetic parameters were calculated with Eadie–Hofstee plots.

The results of the *in vitro* experiments were each evaluated by three consecutive analyses. The amounts of dextromethorphan/levomethorphan and their metabolites were calculated on the basis of calibration curves made by spiking known amounts of these compounds into the reaction mixture without the microsomal fraction.

Results

Chiral separation of dextromethorphan/levomethorphan and their metabolites

Complete chiral separation of dextromethorphan, levomethorphan, and their metabolites was achieved in 12 min on a Chiral CD-Ph column in 0.1% formic acid–acetonitrile by a linear gradient program. The retention time of each compound was as follows: the parent compounds (dextro/levo forms, 10.6/11.3 min) and their metabolites of *O*-demethyl (6.1/5.5 min), *N*-demethyl (8.1/9.8 min), and *O*, *N*-didemethyl (3.9/4.5 min) as shown in Table 1. Figure 3 shows LC-MS/MS total ion current chromatograms (MRM mode) of the extract from plasma (30 min after the first administration), urine (0–24 h after the last administration), and hair (collected 4 weeks after the first administration) of rats administered with dextromethorphan or levomethorphan. Under the chromatographic conditions used, there was no interference with any of the compounds or the internal standard by any extractable endogenous materials in the rat samples. The peaks 7 (9.8 min, *m/z* 258→170) and 8 (4.5 min, *m/z* 244→156) on the chromatograms shown in Fig. 3 were identified as those of (−)-3-MEM and (−)-3-HM when the mass fragmentations of these peaks were considered, although the standard compounds of these two metabolites were not available. These peaks were also confirmed by comparison of their retention times and mass fragmentations with those of the standard compounds of the dextro forms ((+)-3-MEM and (+)-3-HM) using an ODS column.

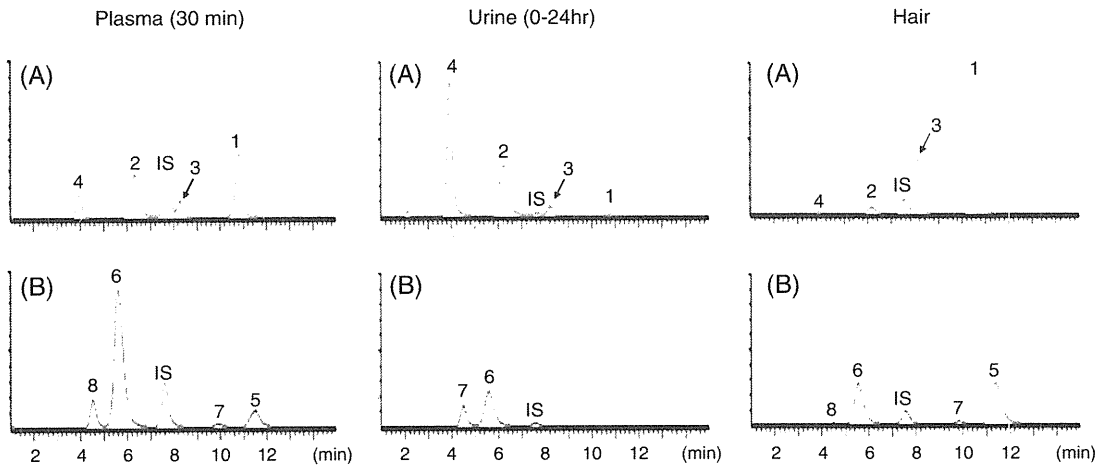


Fig. 3 LC-MS/MS total ion current (TIC) chromatograms (MRM mode) of the extracts from plasma, urine, and hair of rats administered with (**A**) dextromethorphan and (**B**) levomethorphan using a chiral column. *1* Dextromethorphan, *2* Dextrorphan, *3* (+)-3-MEM, *4* (+)-3-HM, *5* Levomethorphan, *6* Levorphanol, *7* (−)-3-MEM, *8* (−)-3-HM

Table 2 Validation of results of the LC-MS/MS analyses of dextromethorphan/levomethorphan and their metabolites in rat plasma, urine and hair samples (n=5)

Samples		Compounds	LOD (S/N>3)	LOQ (S/N>10)	Linear ranges	Recoveries (%)	Precision (%) (n=5)			Accuracy (%) (n=5)		
				(ng/mL)		80 ng/mL	2.0 ng/mL	20 ng/mL	200 ng/mL	2.0 ng/mL	20 ng/mL	200 ng/mL
Plasma (50 μL)	Dextro	Dextromethorphan	0.8	1.0	1.0-400	106.1	22.1	9.3	1.5	-19.2	5.5	-0.2
		Dextrorphan	0.4	0.8		81.7	10.2	3.8	1.5	10.2	2.2	-3.6
		(+)-3-MEM	0.8	1.0		110.5	15.0	3.2	2.5	23.5	2.1	2.6
		(+)-3-HM	0.8	1.0		92.5	15.7	6.1	1.8	13.7	-8.3	2.9
	Levo	Levomethorphan	0.8	1.0	1.0-400	100.8	8.6	4.9	2.5	21.6	-4.4	-5.7
		Levorphanol	0.8	1.0		90.7	15.9	4.1	2.3	-10.6	-5.6	-3.6
				(ng/mL)		500 ng/mL	5.0 ng/mL	500 ng/mL	5000 ng/mL	5.0 ng/mL	500 ng/mL	5000 ng/mL
Urine (100 μL)	Dextro	Dextromethorphan	1.0	2.5	5.0-10000	90.2	9.7	0.8	2.6	-4.8	-5.2	-4.8
		Dextrorphan	1.0	2.5		106.1	23.6	4.6	3.2	-17.9	11.1	-3.3
		(+)-3-MEM	2.5	5.0		102.5	19.7	6.1	4.2	10.4	-5.8	2.7
		(+)-3-HM	2.5	5.0		91.3	24.6	5.1	2.6	1.6	-9.9	1.5
							10 ng/mL			10 ng/mL		
	Levo	Levomethorphan	1.0	5.0	10-10000	94.6	10.9	9.5	2.6	-4.3	-17.0	-2.2
		Levorphanol	1.0	5.0		93.1	4.8	4.5	4.6	18.6	-8.0	6.8
				(ng/mg)		10 ng/mg	0.1 ng/mg	5.0 ng/mg	50 ng/mg	0.1 ng/mg	5.0 ng/mg	50 ng/mg
Hair (10 mg)	Dextro	Dextromethorphan	0.025	0.05	0.1-50	84.2	11.5	4.5	2.8	4.6	18.8	-6.6
		Dextrorphan	0.025	0.05		99.8	6.4	2.6	2.7	3.7	15.4	-3.5
		(+)-3-MEM	0.025	0.05		83.8	18.6	3.9	1.5	4.7	0.6	-2.2
		(+)-3-HM	0.025	0.1		91.4	11.2	6.2	2.8	4.6	18.8	-6.6
	Levo	Levomethorphan	0.025	0.1	0.1-50	98.1	9.9	9.8	5.5	0.1	-2.3	-5.1
		Levorphanol	0.025	0.05		112.2	8.8	2.8	4.2	11.9	1.3	-3.8

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

The calibration curves were linear over the concentration range 1.0–400 ng/mL for rat plasma, 5.0–10,000 ng/mL (compounds of dextro forms) and 10.0–10,000 ng/mL (compounds of levo forms) for rat urine, and 0.1–50 ng/mg for rat hair, with good correlation coefficients of $r^2 \geq 0.996$, respectively. The LOD of each drug was 0.4 or 0.8 ng/mL for the plasma, 1.0 or 2.5 ng/mL for the urine, and 25 pg/mg for the hair samples. The recoveries and the precision and accuracy data from the analytical procedures for the rat samples (n=5), spiked with a standard solution of the targeted compounds, are shown in Table 2.

Determination of dextromethorphan/levomethorphan and their metabolites in DA rat plasma, urine, and hair samples

It has been reported that a female DA rat lacks the CYP2D1 enzyme, which is known to be related to *O*-demethylation of dextromethorphan in the SD rat; it is therefore used as a model animal for the poor metabolizer phenotype of dextromethorphan [35–37]. As such, the metabolic data from female DA rats may not reflect the "normal" situation. On the other hand, pigmented hairy rats appear to be suitable for the investigation of analytical methods of basic drugs in hair samples, compared with albino rats (SD or Wistar rats) because pigmentation (the melanin contents) is one of the most important factors regarding the incorporation of basic drugs into hair, as described before [38]. Therefore, thus far, we have studied the analytical properties of various drugs in hair samples using the pigmented hairy male DA rats, avoiding female DA rats.

After the i.p. administration of dextromethorphan or levomethorphan to pigmented hairy male DA rats, the parent compounds and their three metabolites in the plasma, urine, and hair were determined using LC-MS/MS. The optical purities of the resulting metabolites were unchanged in any rat biological sample, and no racemation was observed through *O*- and/or *N*-demethylation (Fig. 3). In the rat plasma ($AUCs_{0-360\ min}$) and urine samples (total excretions for 0–72 h) after the hydrolysis of *O*-glucuronides, most metabolites were detected as being the corresponding *O*-demethyl and *N*, *O*-didemethyl compounds, as shown in Table 3. However, obvious differences in the amounts of these metabolites were found between the dextro and levo forms. After administration of dextromethorphan, dextrorphan and (+)-3-HM were the major metabolites in the plasma (59.4 and 64.3 mg/L·min) and urine (106.1 and 226.9 μg/mL). However, *O*-demethyl metabolites (levorphanol) were mainly detected in the plasma (197.1 mg/L·min) and urine (210.5 μg/mL) after administration of levomethorphan (Table 3).

In the hair samples, the differences in the amounts of the metabolites are more clearly detected. After administration of dextromethorphan, the parent compound and the *N*-demethyl metabolite ((+)-3-MEM) were mainly detected at 63.4 and 25.1 ng/mg, respectively, although the *O*-demethyl metabolite of dextromethorphan (dextrorphan) was detected at only 2.70 ng/mg, which was nearly one tenth of the level

Table 3 Rat plasma $AUCs_{0-360min}$, total excretion into rat urine, and concentrations in rat hair of dextromethorphan/levomethorphan and their metabolites

Administrations	Targeted compounds	Plasma $AUC_{0-360min}$ (mg/L·min)	Urine Total excretion 0–72 h (μg)	Hair Concentration (ng/mg)
Dextromethorphan (rat 1–3)	Dextromethorphan	23.8±17.6	2.13±1.05	63.4±4.6
	Dextrorphan	59.4±16.3	106.1±15.3	2.70±0.04
	(+)-3-MEM	3.10±2.15	6.95±0.68	25.1±1.9
	(+)-3-HM	64.3±31.3	226.9±51.3	0.70±0.11
Levomethorphan (rat 4–6)	Levomethorphan	6.90±5.12	0.59±0.61	24.5±5.3
	Levorphanol	197.1±48.2	210.5±36.2	24.6±2.4
	(−)-3-MEM	1.47±0.64	0.13±0.06	2.57±0.71
	(−)-3-HM	51.5±9.6	39.0±5.9	0.49±0.09

of levorphanol. In contrast, after the administration of levomethorphan, the parent compound and the *O*-demethyl metabolite (levorphanol) were mainly detected at 24.5 and 24.6 ng/mg, respectively, with a small amount of the *N*-demethyl metabolite ((−)-3-MEM). The *N*, *O*-didemethyl metabolites (3-HM) were hardly detected in either sample (Table 3). The ratios of the parent compounds, their *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites in the hair samples were 100:4:40:1 for the dextro forms and 100:100:11:2 for the levo forms, respectively.

The rat plasma AUCs, total excretions into rat urine and concentrations in rat hair of dextromethorphan or levomethorphan, and their metabolites are summarized in Fig. 4. The metabolic ratios of dextromethorphan/levomethorphan, *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites in rat plasma ($AUC_{0-360\ min}$) and hair (collected 4 weeks after the first administration) were 1:3:0.1:3 and 1:0.04:0.4:0.01 for the dextro forms and 1:29:0.2:7 and 1:1:0.1:0.02 for the levo forms, respectively. It is of interest that the concentrations of dextromethorphan and levome-

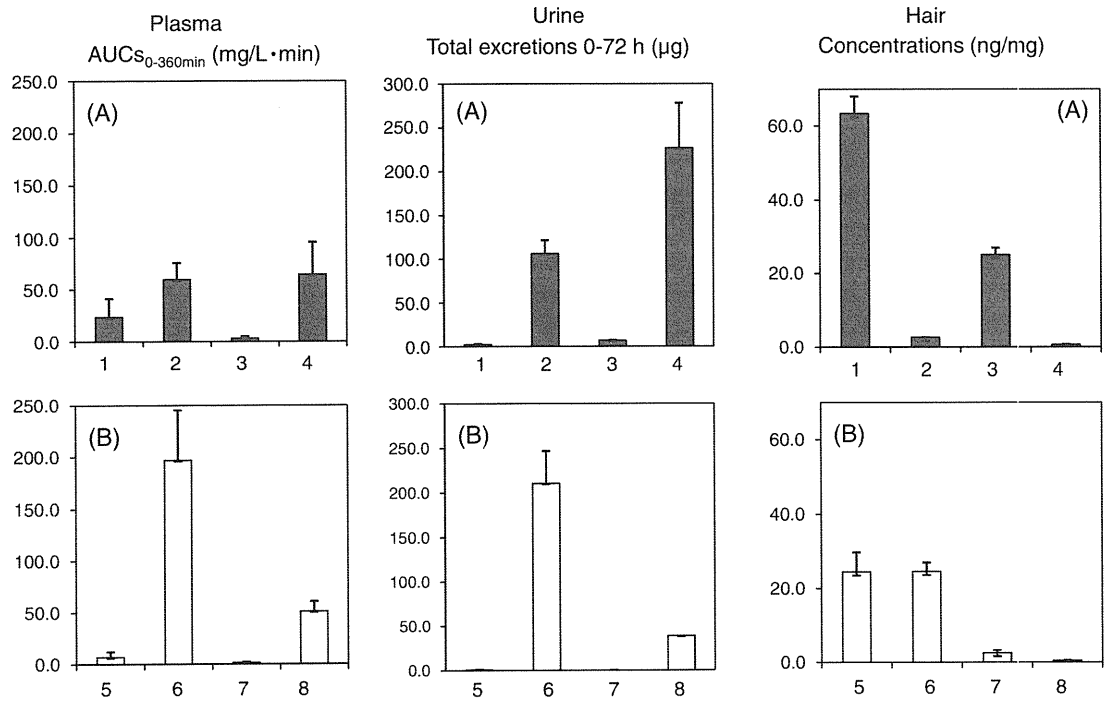


Fig. 4 Rat plasma $AUCs_{0-360min}$, total excretions into rat urine, and concentrations in rat hair of parent compounds and their metabolites after administration of (**A**) dextromethorphan and (**B**) levomethorphan. *1* Dextromethorphan, *2* Dextrorphan, *3* (+)-3-MEM, *4* (+)-3-HM, *5* Levomethorphan, *6* Levorphanol, *7* (−)-3-MEM, *8* (−)-3-HM

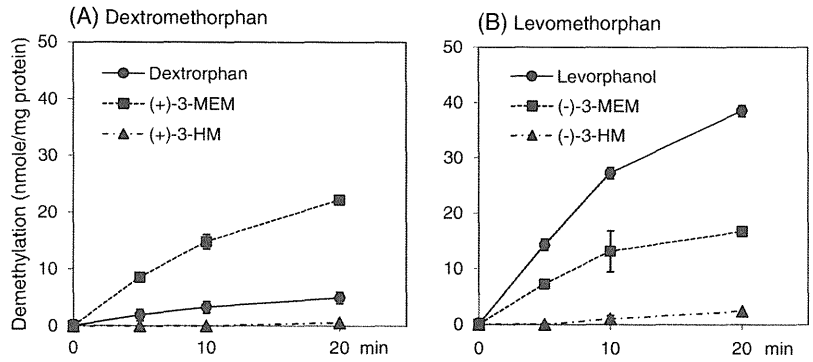


Fig. 5 Demethylation of (**A**) dextromethorphan and (**B**) levomethorphan in DA rat liver microsomes

thorphan in the rat hair were obviously high compared with those in the plasma, while those of their *O*-demethyl and *N*, *O*-didemethyl metabolites in the hair (which mostly existed as very hydrophilic metabolites, *O*-glucuronides in the plasma) were extremely low considering their high plasma AUCs.

Demethylation of dextromethorphan/levomethorphan in DA rat liver microsomes

In order to fully investigate the differences of the metabolic properties between dextromethorphan and levomethorphan, DA rat liver microsomes were studied. Figure 5 shows the *O*- and/or *N*-demethylation of dextromethorphan/levomethorphan in the rat liver microsomes.

The optical purities of the resulting metabolites were unchanged in the liver microsomes, and no racemation was observed through *O*- and/or *N*-demethylation. After 20-min incubation, 4.8% of dextromethorphan and 45% of levomethorphan were transformed to each *O*-demethyl metabolite, and 22% and 19% of the parent compounds were transformed to each *N*-demethyl metabolite. The *N*-demethylation was preferred over *O*-demethylation for dextromethorphan. In contrast, *O*-demethylation was preferred over *N*-demethylation for levomethorphan and the *O*-demethylation of levomethorphan was performed at levels 9.4 times that of dextromethorphan after 20-min incubation. The *N*-demethylation of levomethorphan was almost the same as that of dextromethorphan. Table 4 shows kinetic parameters for *O*-demethylation of dextromethorphan and levomethorphan by the DA rat microsomes. The V_{max} value for levomethorphan (3.8±0.3 nmol/min/mg protein) was 5.9 times higher than that of dextromethorphan (0.65±0.03 nmol/min/mg protein). The K_m values for levomethorphan and dextromethorphan were 22.1±5.0 and 44.1±4.0 μM, respectively. These results suggest that there might be an enantioselective *O*-demethylation of levomethorphan in the DA rat liver microsomes. This enantioselective metabolism might be the cause of the different amounts of the metabolites observed in the rat plasma, urine, and hair after administration of dextromethorphan and levomethorphan.

Demethylation of dextromethorphan/levomethorphan in pooled human liver microsomes

In order to investigate whether the enantioselective metabolism could be observed in humans as well as in DA rats, the pooled human liver microsomes were examined. Figure 6 shows the *O*- and/or *N*-demethylation of dextromethorphan/levomethorphan in the human liver microsomes.

The optical purities of the resulting metabolites were unchanged also in the human liver microsomes, and no racemation was observed through *O*- and/or *N*-demethylation. After 20-min incubation, 3.3% of dextromethorphan and 11% of levomethorphan were transformed to each *O*-demethyl metabolite and 2.5% and 7.1% of the parent compounds were transformed to each *N*-demethyl metabolite. The total amounts of the three metabolites from levomethorphan were higher than those from dextromethorphan in human (3.1 times) microsomes. Kinetic parameters for *O*-demethylation of dextromethorphan and

Table 4 Kinetic parameters for *O*-demethylation of dextromethorphan/levomethorphan by DA rat and human liver microsomes

	DA rat liver microsomes		Human liver microsomes	
	Dextromethorphan	Levomethorphan	Dextromethorphan	Levomethorphan
V_{max} (nmol/min/mg protein)	0.65±0.03	3.8±0.3[a]	0.26±0.03	0.58±0.02[a]
K_m (μM)	44.1±4.0	22.1±5.0[a]	4.5±0.9	8.9±1.7[a]

[a] Significantly different from dextromethorphan ($p<0.01$)

levomethorphan in the human liver microsomes are listed in Table 4. The V_{max} value for levomethorphan (0.58±0.02 nmol/min/mg protein) was 2.2 times higher than that of dextromethorphan (0.26±0.03 nmol/min/mg protein). The K_m values for levomethorphan and dextromethorphan were 8.9±1.7 and 4.5±0.8 μM, respectively. There could also be an enantioselective metabolism of levomethorphan in human liver microsomes.

Discussion

In this study, we first investigated the analytical methods of dextromethorphan/levomethorphan and their metabolites in biological samples using DA male rats. As a result, chiral separation of dextromethorphan, levomethorphan, and their metabolites in biological samples was achieved in 12 min on a Chiral CD-Ph column. The optical purities of the resulting metabolites were unchanged in all rat biological samples, and no racemation was observed through *O*- and/or *N*-demethylation. The proposed chiral analyses might be applied to human samples and could provide useful information for discriminating dextromethorphan use from levomethorphan use, considering the possibility of the adulteration or substitution of dextromethorphan with levomethorphan for illegal purposes. However, for application to forensic toxicological purposes, further studies should be carried out using authentic human samples.

The concentrations of dextromethorphan and levomethorphan in the rat hair were obviously high compared with those of metabolites in the plasma and urine samples in this study. In our previous study [38], we determined the melanin affinity and lipophilicity of 20 abused drugs and these values were compared with the ratio of drug concentration in hair to plasma AUC as an index of the incorporation tendency into hair. As a result, the combination of melanin affinity (basicity) and lipophilicity showed a high correlation with the incorporation tendency into hair. Parent compounds can be detected relatively easily in hair in comparison with their hydrophilic metabolites. Actually, it has been reported that cocaine is detected in hair at a much higher concentration than its metabolite, benzoylecgonine, although cocaine is rapidly hydrolyzed to benzoylecgonine and disappears from plasma [39]. Considering those reports, the physico-chemical properties of dextromethorphan/levomethorphan and their metabolites could be significantly related to their concentrations in the hair samples. Additionally, the drug concentrations in the rat hair (collected 4 weeks after the first administration) reflected the total amounts of drugs in the plasma of rats administered with dextromethorphan/levomethorphan for ten successive days, and the differences might become more distinct. The detection of the parent compounds from hair samples would provide useful information regarding the monitoring of their use over a long period.

In the DA rat samples, obvious differences in the ratios of the metabolites were found between the dextro and levo forms. These differences were most clearly detected in the hair samples. 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 investigate the differences of their metabolic properties between dextromethorphan and levomethorphan, DA rat and human liver microsomes were studied. As a result, we have shown the enantioselective metabolism of levomethorphan, not only in DA rats but also in human liver microsomes, especially with regards to the *O*-demethylation. Because it is well-known that CYP2D6 (mainly related to *O*-demethylation of dextromethorphan) is polymorphically expressed in humans, it may be difficult to discuss the enantioselective metabolism in humans who can be classified as poor, intermediate and extensive metabolizers of dextromethorphan. In future studies, the metabolic properties of these drugs using CYP2D6 enzymes (having a variety of phenotypes) should be examined to clarify the effects of their genotypes on the enantioselective *O*-demethylation of levomethorphan observed in this study.

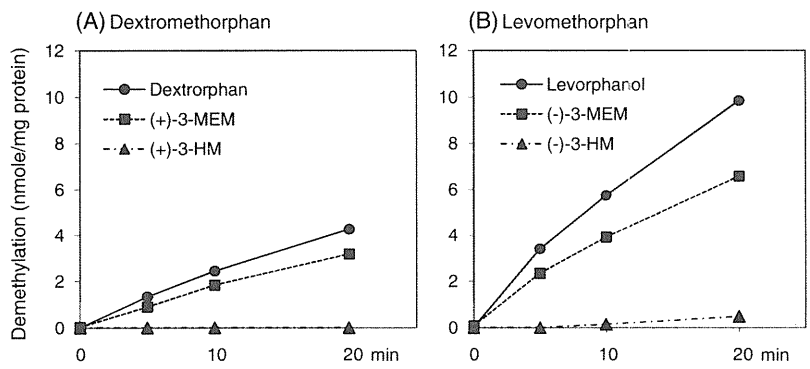


Fig. 6 Demethylation of (**A**) dextromethorphan and (**B**) levomethorphan in human liver microsomes

Conclusions

In this present study, we have established procedures for chiral analyses of dextromethorphan, levomethorphan, and their *O*-demethyl and/or *N*-demethyl metabolites in rat plasma, urine, and hair using LC-MS/MS. These analytical methods might be applied to human samples and could be useful for discriminating dextromethorphan use from levomethorphan use although further studies should be carried out using authentic human samples for forensic toxicological purposes. In addition, we have found the enantioselective metabolism of levomethorphan, not only in DA rats but also in human liver microsomes, especially with regards to the *O*-demethylation. This is the first report describing the differences in metabolic properties between dextromethorphan and levomethorphan in rats and humans.

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

References

1. Mutschler J, Koopmann A, Grosshans M, Hermann D, Mann K, Kiefer F (2010) Dtsch Arztebl Int 107:537–540
2. Chyka PA, Erdman AR, Manoguerra AS, Christianson G, Booze LL, Nelson LS, Woolf AD, Cobaugh DJ, Caravati EM, Scharman EJ, Troutman WG (2007) Clin Toxicol 45:662–677
3. Banken JA, Foster H (2008) Ann NY Acad Sci 1139:402–411
4. Shin EJ, Lee PH, Kim HJ, Nabeshima T, Kim HC (2008) J Pharmacol Sci 106:22–27
5. Bryner JK, Wang UK, Hui JW, Bedodo M, MacDougall C, Anderson IB (2006) Arch Pediatr Adolesc Med 160:1217–1222
6. Miller SC (2005) Addict Biol 10:325–327
7. Logan BK, Goldfogel G, Hamilton R, Kuhlman J (2009) J Anal Toxicol 33:99–103
8. Rammer L, Holmgren P, Sandler H (1988) Forensic Sci Int 37:233–236
9. Woods JW, Carney J (1978) NIDA Res Monogr 18:54–66
10. Trescot AM, Datta S, Lee M, Hansen H (2008) Pain Physician 11: S133–S153
11. Jacqz-Aigrain E, Funck-Brentano C, Cresteil T (1993) Pharmacogenetics 3:197–204
12. Schmid B, Bircher J, Preisig R, Küpfer A (1985) Clin Pharmacol Ther 38:618–624
13. Gorski JC, Jones DR, Wrighton SA, Hall SD (1994) Biochem Pharmacol 48:173–182
14. Köppel C, Tenczer J, Arndt I, Ibe K (1987) Arzneimittelforschung 37:1304–1306
15. Lutz U, Bittner N, Lutz RW, Lutz WK (2008) J Chromatogr B 871:349–356
16. Kristensen HT (1998) J Pharm Biomed Anal 18:827–838
17. Aumatell A, Wells RJ (1993) J Chromatogr Sci 31:502–508
18. Lin SY, Chen CH, Ho HO, Chen HH, Sheu MT (2007) J Chromatogr B 859:141–146
19. Bendriss EK, Markoglou N, Wainer IW (2001) J Chromatogr B 754:209–215
20. Afshar M, Rouini MR, Amini M (2004) J Chromatogr B 802:317–322
21. Hendrickson HP, Gurley BJ, Wessinger WD (2003) J Chromatogr B. B 788:261–268
22. Kim SC, Chung H, Lee SK, Park YH, Yoo YC, Yun YP (2006) Forensic Sci Int 161:185–188
23. Spanakis M, Vizirianakis IS, Mironidou-Tzouveleki M, Niopas I (2009) Biomed Chromatogr 23:1131–1137
24. Rodrigues WC, Wang G, Moore C, Agrawal A, Vincent MJ, Soares JR (2008) J Anal Toxicol 32:220–226
25. Kim EM, Lee JS, Park MJ, Choi SK, Lim MA, Chung HS (2006) Forensic Sci Int 161:198–201
26. Bagheri H, Es-haghi A, Rouini MR (2005) J Chromatogr B 818:147–157
27. Eichhold TH, McCauley-Myers DL, Khambe DA, Thompson GA, Hoke SH 2nd (2007) J Pharm Biomed Anal 43:586–600
28. Kuhlenbeck DL, Eichold TH, Hoke SH 2nd, Baker TR, Mensen R, Wehmeyer KR (2005) Eur J Mass Spectrom 11:199–208
29. Lutz U, Völkel W, Lutz RW, Lutz WK (2004) J Chromatogr B 813:217–225
30. Vengurlekar SS, Heitkamp J, McCush F, Velagaleti PR, Brisson JH, Bramer SL (2002) J Pharm Biomed Anal 30:113–124
31. Sunouchi M, Fukuhara M, Ohno Y, Takanaka A (1988) J Toxicol Sci 13:193–204
32. Ozawa S, Ohta K, Miyajima A, Kurebayashi H, Sunouchi M, Shimizu M, Murayama N, Matsumoto Y, Fukuoka M, Ohno Y (2000) Xenobiotica 10:1005–1017
33. Kikura-Hanajiri R, Kawamura M, Saisho K, Kodama Y, Goda Y (2007) J Chromatogr B 855:121–126
34. Kikura-Hanajiri R, Kawamura M, Miyajima A, Sunouchi M, Goda Y (2010) Forensic Sci Int 198:62–69
35. Bochner F, Somogyi AA, Chen ZR (1994) Xenobiotica 24:543–552
36. Kerry NL, Somogyi AA, Mikus G, Bochner F (1993) Biochem Pharmacol 45:833–839
37. Zysset T, Zeugin T, Küpfer A (1988) Biochem Pharmacol 37:3155–3160
38. Nakahara Y, Takahashi K, Kikura R (1995) Biol Pharm Bull 18:1223–1227
39. Nakahara Y, Ochiai T, Kikura R (1992) Arch Toxicol 66:446–449