CP-47,497 might be due to slower metabolisms than Δ^9-THC caused by the absence of allylic methyl hydroxylation, although the metabolites of CCH, CP-47,497 and JWH-018 have not been completely elucidated.

Atwood et al. [32] reported that JWH-018 had profound CB_1 receptor-mediated effects on cellular signaling and neurotransmission, although the impact of CCH and CP-47,497 on CB1 receptor-mediated activities remains unclear. The diversity in specificity of the synthetic cannabinoids and their metabolites for CB_1, CB_2 and other receptors may also account for their differences on the duration of changes in EEG power densities and suppression of the locomotor activities.

Here, we demonstrated that the synthetic cannabinoids CCH, CP-47,497 and JWH-018 changed EEG power spectra and suppressed the locomotor activity of rats more significantly and for a longer duration than Δ^9-THC, indicating that these synthetic cannabinoids are associated with potent pharmacological actions in the central nervous system. Therefore, the psychotropic effects of the synthetic cannabinoids need to be clarified by further detailed study.

At present, it is difficult to assume health risks associated with the use of these synthetic cannabinoids, because only limited information about their pharmacology, toxicology and metabolism is currently available. However, these compounds may have large potential to cause harm by acting as CB receptor agonists. Because the composition of synthetic cannabinoids varies considerably in herbal products [7,8], accidental overdosing of those compounds may cause serious psychotropic symptoms. Therefore, further studies are essential to reveal the risks and to prevent serious health problems caused by the abuse of these compounds.

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—Article—

デザイナードラッグとして検出された合成カンナビノイドの異性体分析について

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Isomeric Analysis of Synthetic Cannabinoids Detected as Designer Drugs

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Recently, many psychotropic herbal products, named such as "Spice", were distributed worldwide *via* the Internet. In our previous study, several synthetic cannabinoids were identified as adulterants in herbal products being available in Japan due to their expected narcotic effects. Among those, two derivatives of Δ^9-tetrahydrocannabinol (Δ^9-THC), which is major psychotropic cannabinoid of marijuana, cannabicyclohexanol (CCH, 3-[2-hydroxy-4-(2-methylnonan-2-yl) phenyl] cyclohexan-1-ol) and CP-47,497 (3-[2-hydroxy-4-(2-methyloctan-2-yl) phenyl] cyclohexan-1-ol), have been controlled as designated substances (Shitei-Yakubutsu) under the Pharmaceutical Affairs Law since November 2009. CCH was detected together with its trans-form (1-epimer) in many herbal products, and CCH and CP-47,497 have two chiral centers in the structures. However, the pharmaceutical activities of the isomers of CCH have not been reported. This study presents chiral separations of CCH, its trans-form and CP-47,497 in the products using LC-circular dichroism (CD) and LC-MS analyses. The enantiomeric pairs of CCH, its trans-form and CP-47,497 were separated, respectively. Subsequently, the analyses of the herbal products showed that CCH and its trans-form existed as mixtures of enantiomers and the relative ratios of CCH and the trans-form enantiomers ranged from 42/58% to 53/47% and from 33/67% to 52/48%, respectively.

Key words——synthetic cannabinoids; chiral separation; cannabicyclohexanol; CP-47,497; designer drug; LC-MS

緒　　言

近年，違法ドラッグ市場では，ハーブやお香と称し，植物系違法ドラッグ製品が多数販売される傾向がみられる．その中でも特に，カンナビノイド様の作用を標榜する植物系製品の流通が増加している．われわれはこれまでに，これら植物系違法ドラッグ製品の成分分析を行い，製品中から数種類の合成カンナビノイドを単離・同定しているが，[1-4] これら化合物はすべてカンナビノイド様の薬理作用を有する化合物として合成されたものであった．そのうち，大麻の主活性成分 Δ^9-tetrahydrocannabinol (Δ^9-THC) の誘導体である cannabicyclohexanol (CCH, 3-[2-hydroxy-4-(2-methylnonan-2-yl) phenyl] cyclohexan-1-ol), CP-47,497 (3-[2-hydroxy-4-(2-methyloctan-2-yl) phenyl] cyclohexan-1-ol) は，平成 21 年 11 月に 1*RS*, 3*SR* のラセミ体(**1**, **2**), (**5**, **6**)として，指定薬物に指定された (Fig. 1)．CCH (**1** or **2**) 及び CP-47,497 (**5** or **6**) は，それぞれ 1, 3 位に不斉炭素を有する化合物であるが，CCH の trans 体 (1-epimer, **3** or **4**) が CCH とともに多くの製品から検出されている．[3,5] また，(−)-CP-47,497 (**5**) は，(+)-CP-47,497 (**6**) よりも若干強いカンナビノイド活性が報告されているが，[6,7] 製品中に含有されるこれら化合物の立体異性体分析は報告されていない．さらに，CP-47,497 の 4-hydroxypropyl 体であり，代表的なカンナビノイド受容体 (cannabinoid receptor: CB-R) アゴニストである CP-55,940 (Fig. 1) は，(−)-体が (+)-体よりも 88 倍強い CB_1-R 親和性を持つことが知られている．[6] また，現在日本での検出事例はないが，ヨーロッパやアメリカで流通が報告されている合成カンナビノイド HU-210 (アメリカでは麻薬に指定) は強力な CB-R アゴニストであるが，[8] その enantiomer である HU-211 はカンナビノイド様の向精神活性がないことが知られている (Fig. 1)．[9,10] このように，これ

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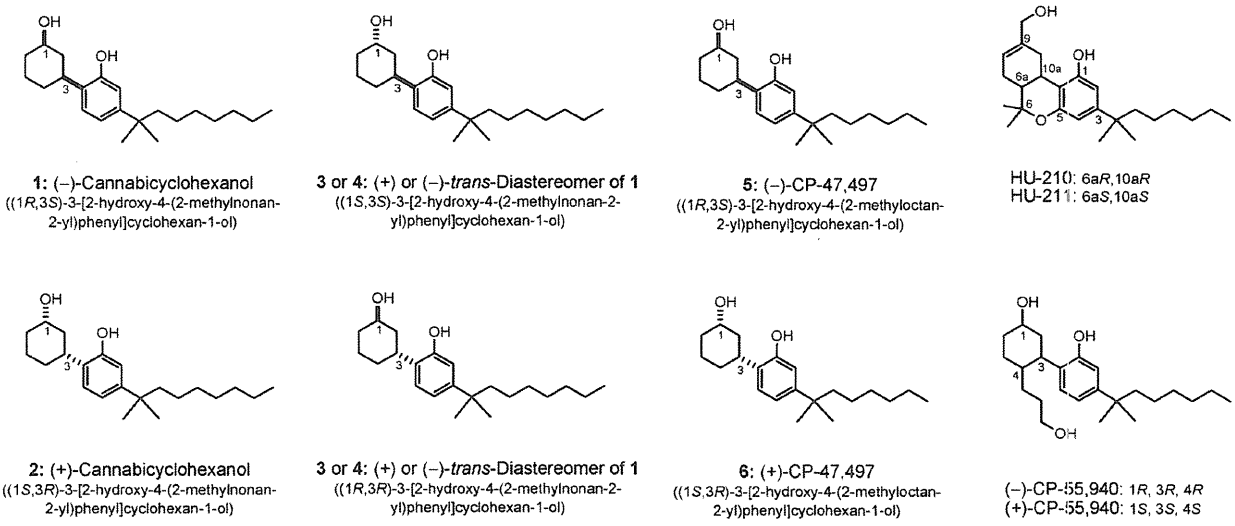

Fig. 1. Structures of Synthetic Cannabinoid Enantiomers

ら合成カンナビノイドの光学異性体別の活性確認は，薬理学的にみて興味深い課題であり，そのためにはまず，光学異性体分析法の確立が重要となる．また，レギュラトリーサイエンスの観点からみると，違法薬物中の合成カンナビノイドがどのような異性体組成を持つか実態を調査することは，今後の規制と取り締まりのあり方を考える面で重要である．そこで本研究では，LC-circular dichroism (CD) を用いて，CCH, trans 体及び CP-47,497 をキラル HPLC カラムにより光学分割を行うと同時に，各異性体の CD スペクトルを測定することにより，光学異性体分析を行った．さらに，違法ドラッグ製品についてはキラル HPLC カラムを用いた LC-MS 分析を行い，各化合物の enantiomer の相対比を調べたので報告する．

実 験 方 法

1. 分析試料及び試薬 平成 20–21 年度にインターネットを介して購入したカンナビノイド様作用を標榜した植物系違法ドラッグ製品中で，あらかじめ GC-MS 及び LC-MS 分析により，CCH 及び trans 体，CP-47,497 のいずれかが検出された 37 製品を分析に用いた．[3] なお，既報[3]で分析した 34 製品に加え，新たに合成カンナビノイドが検出された 3 製品についても，分析に用いた．CCH 及び trans 体は Melvin らの方法[11]に従って筆者らが合成し，精製したものを用いた．CAY-10596 (CCH の製品名)，(±)-CP-47,497, (−)-CP-47,497 及び (+)-CP-47,497 は Cayman Chemical 社より購入した．HPLC, LC-MS の移動相に用いたアセトニトリルは HPLC グレードを使用した．その他の試薬は市販特級品を使用した．抽出溶液の膜ろ過には，Ultrafree-MC (0.45 μm filter unit, MILLIPORE 社製) を用いた．

2. 測定用試料の調製法 植物細片は乳鉢で粉末化して 10 mg を使用し，MeOH 1 ml を加え，超音波下 10 分間抽出を行った．さらに膜ろ過を行い，不溶物を取り除いて測定試料とした．

3. LC-CD 分析条件 装置：[HPLC] JASCO Gulliver Series, カラム：Chiralpak-IA3 (2.1 mm i.d. ×150 mm, 3 μm, Daicel) 又は Chiralpak-IA (4.6 mm i.d.×250 mm, 5 μm, Daicel)，移動相：0.1%ギ酸水溶液/0.1%ギ酸アセトニトリル (30/70)，測定波長：210–420 nm，流速：0.2 ml/min 又は 1 ml/min，カラム温度：30℃，注入量：1 μl，検出：UV 検出器 (UV-970) 及び円二色性 (CD) 検出器 (CD-2095)．

4. LC-MS キラル分析条件 装置：[LC] Waters ACQUITY UPLC/[MS] Waters Single Quadrupole Detector (SQD)，カラム：Chiralpak-IA3 (2.1 mm i.d.×150 mm, 3 μm, Daicel)，移動相：0.1%ギ酸水溶液/0.1%ギ酸アセトニトリル (35/65)，測定波長：190–500 nm，流速：0.2 ml/min，カラム温度：30℃，注入量：1–3 μl，検出：

フォトダイオードアレイ検出器（PDA）及び質量検出器.

5. 質量分析条件 イオン化：エレクトロスプレーイオン化 (ESI) 法, Positive and negative mode, Desolvation gas flow: N_2 650 l/h, Desolvation gas temp.: 350°C, Cone voltage: 30 V, Capillary voltage: 3000 V, scan range: m/z 50–500.

各化合物の検出は，LC-MS のネガティブモードにおける各製品中の CCH (**1** or **2**) (Mw 332)，trans 体 (**3** or **4**) (Mw 332) の脱プロトン化分子イオン ($[M-H]^-$ m/z 331) 及び CP-47,497 (**5** or **6**) (Mw 318) の脱プロトン化分子イオン ($[M-H]^-$ m/z 317) をモニタリングすることにより行った．また，製品中の各化合物の enantiomer の相対比は，脱プロトン化分子イオンピークの面積比から算出した.

結　　果

1. 合成カンナビノイドの LC-CD 及び LC-MS による光学異性体分析 LC-CD 分析の結果，合成品である CCH (**1** and **2**)，trans 体 (**3** and **4**) 及び市販品 (±)-CP-47,497 (**5** and **6**) の各 enantiomer はそれぞれ良好に分離した [Figs. 2(A)–(C)]．また，違法ドラッグ製品中から単離した CCH[1] も合成品と同様に enantiomer として完全に分離した (Data not shown)．また，市販品の (−)-CP-47,497 (**5**) 及び (+)-CP-47,497 (**6**) はそれぞれ 1 ピークとして検出され，CD においてもそれぞれ正と負に検出された [Figs. 2(D) and (E)]．さらに，LC 分析中に stop-flow mode として，CCH (**1** and **2**) 及び (±)-CP-47,497 (**5** and **6**) それぞれの CD スペクトルを測定したところ，両化合物のスペクトルは酷似していた [Figs. 2(F) and (G)]．CCH と CP-47,497 の立体構造が類似していることから (Fig. 1)，各 enantiomer の絶対構造には相関があると考えられるため，(−)-CCH は化合物 **1**，(+)-CCH は化合物 **2** と強く推定された.

さらに，LC-MS により標品及び違法ドラッグ製品の MeOH 抽出物について分析を行った (Figs. 3 and 4)．標品の結果は先述と同様であるが，Cayman Chemical 社において (1*R*,3*S*)-3-[2-hydroxy-4-(2-methylnonan-2-yl) phenyl] cyclohexan-1-ol (**1**) として販売されていた CAY-10596 についても分析したところ，表記とは異なり enantiomer の混合物 (**1** and **2**) であることが明らかとなった [Fig. 4(D)]．また，製品 No. 6 を分析した結果，CCH (**1** and **2**) 及び trans 体 (**3** and **4**) の enantiomer は良好に分離した [Fig. 4(E)]．また，標品である CCH, trans 体及び (±)-CP-47,497 の各 enantiomer のピーク面積比は Table 1 に示した.

2. 違法ドラッグ製品中の各合成カンナビノイド enantiomer の相対比の傾向 次に，違法ドラッグ 37 製品について光学異性体分析を行った (Table 1)．その結果，全製品において，含まれていた CCH 及び trans 体は enantiomer として完全分離した．CCH の enantiomer のピーク面積比 (**1**/**2**) は 42/58(%)–53/47(%) であり，trans 体のピーク面積比 (**3**/**4**) は 33/67(%)–52/48(%) であった (Table 1)．したがって，CCH 及び trans 体は各 enantiomer の混合物として製品中に混入されていることが明らかとなった．また，CP-47,497 は製品 No. 1 からのみ極少量検出され，enantiomer のピーク面積比 (**5**/**6**) は 52/48(%) であった (Table 1)．なお，標品として用いた CCH, trans 体及び (±)-CP-47,497 もピーク面積上，若干の enantio excess が観察されるが，これら化合物の光学が完全にラセミ体であるという証明はなく，その原因解明は行っていない.

考　　察

今回分析を行った CCH, CP-47,497 は，大麻の主活性成分 Δ^9-THC の誘導体として 1980 年代に Pfizer により CB-R アゴニストとして合成された化合物であり，その構造に不斉炭素を有している．これまでに，合成カンナビノイドの光学分割についてはいくつか報告があり，前述の HU-210 及びその enantiomer である HU-211 の光学分割については，Abu-Lafi らが報告している.[12] また，(±)-CP-47,497 (**5** and **6**) については，trans 体と比較して，より強いカンナビノイド様の鎮痛作用を有すること，さらに，(−)-CP-47,497 (**5**) は，(+)-CP-47,497 (**6**) よりも 2 倍強い CB_1-R 親和性を持つことが報告されているが,[6,11] CCH については立体異性体の活性報告はない．今回分析を行った違法ドラッグ 37 製品については，Table 1 にも示したが，製品中の CCH と trans 体の比率については，CCH (cis 体) の割合が 65–>90% とばらつきがあること

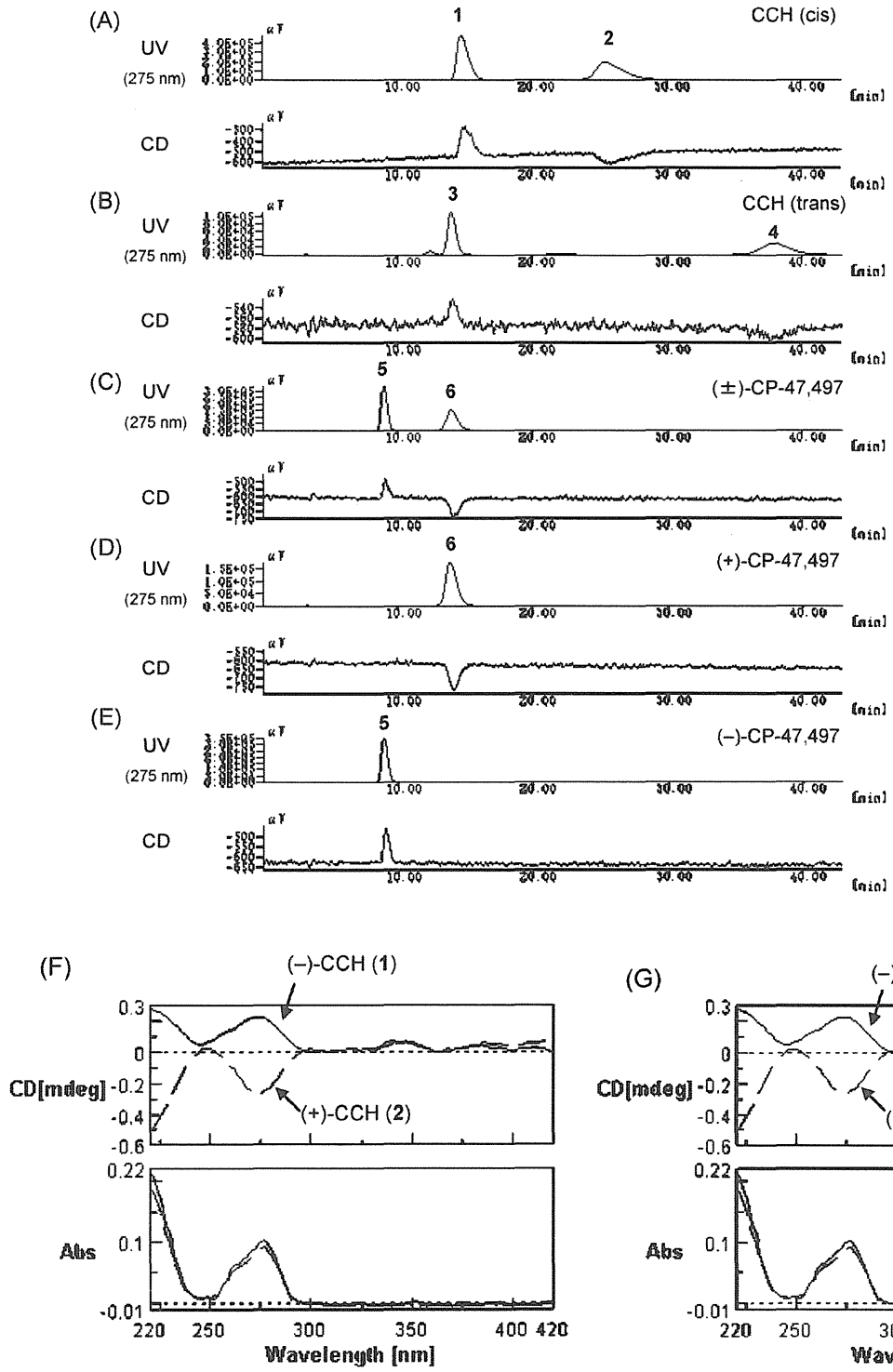

Fig. 2. LC-UV and CD Chromatograms of the Standard of Synthetic Cannabinoid Enantiomers

The compounds CCH (A), CCH trans-form (B), (±)-CP-47,497 (C), (+)-CP-47,497 (D) and (−)-CP-47,497 (E) were analyzed. Upper and lower traces: UV and CD signals at 275 nm. CD and UV spectra of the each peak of CCH (F) and (±)-CP-47,497 (G) at the stopped-flow mode.

を既に報告している.[3] この点については，以下のように考えると合理的に説明可能となる．すなわち，既報[11]の方法により CP-47,497 と同様に合成されたと推測すると，合成ルートには次の 2 ルートが考えられる．(1) 前駆体であるケトン体のカルボニル基を sodium borohydride ($NaBH_4$) により還

Table 1. Relative Ratios (Peak Areas of MS Chromatograms) of Synthetic Cannabinoid Enantiomers (**1–6**) in the Herbal Products

No.	Product form	CCH **1** (%) / **2** (%)	CCH-trans **3** (%) / **4** (%)	CP47,497 **5** (%) / **6** (%)	Ratio of the cis-form (**1**+**2**/**1**+**2**+**3**+**4**)[a]
	Cannabicyclohexanol (CCH)	52 / 48	− / −	− / −	−
	Cayman CAY10596	43 / 57	− / −	− / −	−
	CCH-trans	− / −	46 / 54	− / −	−
	(±)-CP-47,497	− / −	− / −	44 / 56	−
1	Dried leaf (cutting)	48 / 52	47 / 53	52 / 48	##
2	Dried leaf (cutting)	42 / 58	38 / 62	n.d.[b] / n.d.	##
3	Dried leaf (cutting)	45 / 56	44 / 56	n.d. / n.d.	###
4	Dried leaf (cigarette type of No. 2)	45 / 55	48 / 52	n.d. / n.d.	##
5	Dried leaf (cigarette type of No. 3)	45 / 55	44 / 56	n.d. / n.d.	+
6	Dried leaf (cutting)	46 / 54	49 / 51	n.d. / n.d.	##
7	Dried leaf (cutting)	45 / 55	48 / 52	n.d. / n.d.	##
8	Dried leaf (cutting)	49 / 51	52 / 48	n.d. / n.d.	##
9	Dried leaf (cutting)	46 / 54	48 / 52	n.d. / n.d.	##
10	Dried leaf (cutting)	47 / 53	49 / 51	n.d. / n.d.	##
11	Dried leaf (cutting)	47 / 53	48 / 52	n.d. / n.d.	###
12	Dried leaf (cigarette type of No. 11)	47 / 53	48 / 52	n.d. / n.d.	##
13	Dried leaf (cutting)	47 / 53	51 / 49	n.d. / n.d.	###
14	Dried leaf (cutting)	51 / 49	47 / 53	n.d. / n.d.	+
15	Dried leaf (cutting)	50 / 50	44 / 56	n.d. / n.d.	+
16	Dried leaf (cutting)	46 / 54	33 / 67	n.d. / n.d.	##
17	Dried leaf (cigarette type of No. 16)	44 / 56	39 / 61	n.d. / n.d.	##
18	Dried leaf (cigarette type)	51 / 49	50 / 50	n.d. / n.d.	##
19	Dried leaf (cutting)	47 / 53	50 / 50	n.d. / n.d.	##
20	Dried leaf (cutting)	45 / 55	45 / 55	n.d. / n.d.	###
21	Dried leaf (cigarette type)	45 / 55	49 / 51	n.d. / n.d.	##
22	Dried leaf (cutting)	47 / 53	47 / 53	n.d. / n.d.	+
23	Dried leaf (cutting)	47 / 53	46 / 54	n.d. / n.d.	+
24	Dried leaf (cutting)	45 / 55	44 / 56	n.d. / n.d.	###
25	Dried leaf (cutting)	46 / 55	n.d. / n.d.	n.d. / n.d.	###
26	Dried leaf (cutting)	47 / 53	42 / 58	n.d. / n.d.	###
27	Dried leaf (cutting)	48 / 52	43 / 57	n.d. / n.d.	###
28	Dried leaf (cutting)	43 / 57	43 / 57	n.d. / n.d.	+
29	Dried leaf (cutting)	47 / 53	45 / 55	n.d. / n.d.	+
30	Dried leaf (cutting)	45 / 55	42 / 58	n.d. / n.d.	+
31	Dried leaf (cutting)	43 / 57	42 / 58	n.d. / n.d.	+
32	Dried leaf (cutting)	53 / 47	52 / 48	n.d. / n.d.	###
33	Dried leaf (cutting)	49 / 51	44 / 56	n.d. / n.d.	+
34	Dried leaf (cigarette type)	50 / 50	48 / 52	n.d. / n.d.	+
35	Dried leaf (cutting)	49 / 51	46 / 54	n.d. / n.d.	+
36	Dried leaf (cutting)	44 / 56	47 / 53	n.d. / n.d.	+
37	Dried leaf (cutting)	47 / 53	45 / 55	n.d. / n.d.	+

[a] The number of '+' indicates a ratio of the cis-form (**1**+**2**) as estimated from signal intensities in the corresponding GC-MS chromatograms. ### : >90%, ## : <90%–80%, +: <80%–65%.[3] [b] Not detected (S/N<3).

元し，cyclohexanol とした後，palladium on carbon (Pd-C) でフェノール基の保護基を外す方法，(2) 前駆体であるケトン体を先に Pd-C でフェノール基の保護基を外した後，$NaBH_4$ によりカルボニル基を還元する方法である．どちらも蓋然性のある合成ルートであり，標品を合成する際の予備的検討では，両法とも合成可能なことを確認している．それぞれの製品が異なったルートで合成されたとする

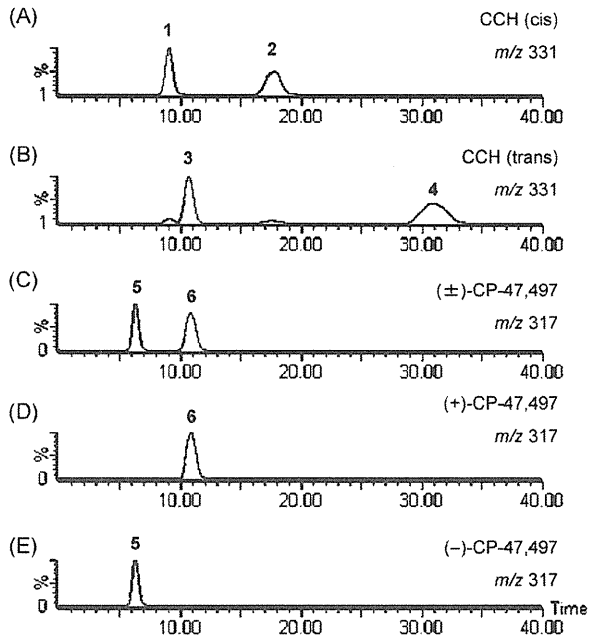

Fig. 3. LC-MS Chromatograms of the Standard of Synthetic Cannabinoid Enantiomers at m/z 331 (A, B) and m/z 317 (C-E).

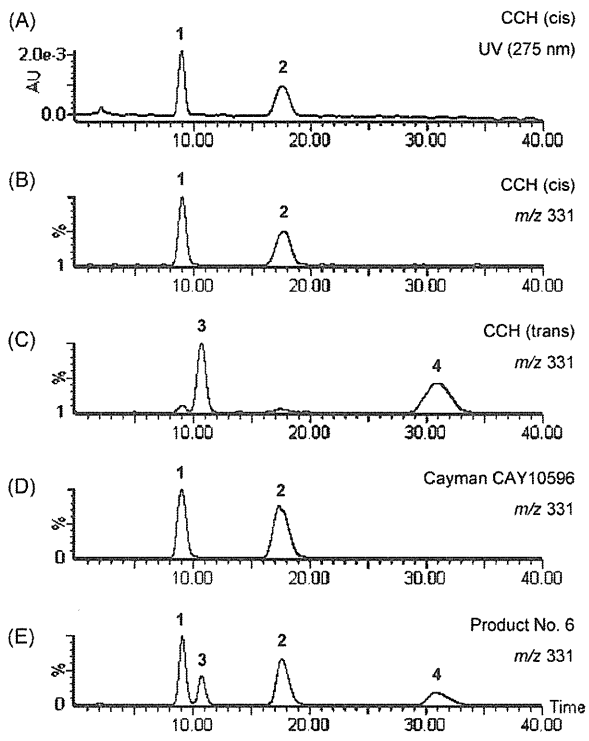

Fig. 4. LC-UV (A) and m/z 331 MS Chromatograms (B–E) of the Standard of Synthetic Cannabinoid Enantiomers and the Product No. 6

と，当然 CCH と trans 体の比率が異なる可能性が高く，ばらつきが大きくなる．さらに，本分析により，CCH 及び trans 体は enantiomer の混合物として製品中に混入されていることを示した（Table 1）．このように，製品毎に立体異性体の含量が異なっていることから，今後，合成カンナビノイドの製造地域や製造元等の情報が入手できた場合，情報を入手できた製品と各製品の diastereo excess (d.e.) 及び enantio excess (e.e.) 等を比較することにより，流通経路を推定できる可能性がある．また，本研究を基礎として，CCH と trans 体の各光学異性体の薬理活性が明らかとなれば，今後，より適切な規制行うことが可能であるものと考えられる．

結　　論

本研究では，LC-CD 及び LC-MS を用いて，指定薬物である cannabicyclohexanol (CCH)，CP-47,497，及び CCH の trans 体の光学分割を行ったところ，各 enantiomer はそれぞれ良好に分離した．さらに，違法ドラッグ 37 製品について分析を行った結果，製品中に含まれていた CCH, trans 体及び CP-47,497 は enantiomer としてそれぞれ完全分離した．したがって，CCH, trans 体及び CP-47,497 は enantiomer の混合物として製品中に混入されていることが明らかとなった．したがって，合成カンナビノイドを含め様々な違法ドラッグ成分の流通実態調査を行う場合，各成分の光学異性体分析及び同定も重要であるものと考えられた．

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—Note—

Direct Analysis in Real Time (DART)-TOFMS を用いた尿中覚せい剤及び 3,4-methylenedioxymethamphetamine (MDMA) 迅速スクリーニング法の検討

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Simple and Rapid Screening for Methamphetamine and 3,4-Methylenedioxymethamphetamine (MDMA) and Their Metabolites in Urine Using Direct Analysis in Real Time (DART)-TOFMS

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An ionization technique, direct analysis in real time (DART) has recently been developed for the ambient ionization of a variety samples. The DART coupled with time-of-flight mass spectrometry (TOFMS) would be useful as a simple and rapid screening for the targeted compounds in various samples, because it provides the molecular information of these compounds without time-consuming extraction. In this study, we investigated rapid screening methods of illicit drugs and their metabolites, such as methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA), amphetamine (AP) and 3,4-methylenedioxyamphetamine (MDA) in human urine using DART-TOFMS. As serious matrix effects caused by urea in urine samples and ionizations of the targeted compounds were greatly suppressed in the DART-TOFMS analyses, simple pretreatment methods to remove the urea from the samples were investigated. When a pipette tip-type solid-phase extraction with a dichloromethane and isopropanol mixed solution as an eluent was used for the pretreatment, the limits of detection (LODs) of 4 compounds added to control urine samples were 0.25 μg/ml. On the other hand, the LODs of these compounds were 0.5 μg/ml by a liquid-liquid extraction using a dichloromethane and hexane mixed solution. In both extractions, the recoveries of 4 compounds from urine samples were over 70% and these extraction methods showed good linearity in the range of 0.5–5 μg/ml by GC-MS analyses. In conclusion, our proposed method using DART-TOFMS could simultaneously detect MA, MDMA and their metabolites in urine at 0.5 μg/ml without time-consuming pretreatment steps. Therefore it would be useful for screening drugs in urine with the molecular information.

Key words——direct analysis in real time; methamphetamine; 3,4-methylenedioxymethamphetamine; urine; time-of-flight mass spectrometry

緒　　言

近年開発された Direct Analysis in Real Time (DART™) イオン化法は，大気圧下で非接触的に試料をイオン化でき，さらに質量検出器に time-of-flight mass spectrometry (TOFMS) を用いることで，精密質量測定に基づく元素組成推定が可能となる.[1] DART では液体，固体等の試料形態を問わず，イオン源にかざすだけで物質の表面がイオン化され，分子量の測定が可能であるため，成分分析の簡便化及び迅速化が期待できる．既に DART を使用した食品中の異物[2-5]や成分，[6-9] 食品容器包装中の添加物，[10,11] 医薬品，[12,13] 違法薬物，[14-16] 生体試料中の代謝物，[17-19] その他様々な分野における分析適応例が報告されている．また，われわれは過去に DART-TOFMS を用いた植物系違法ドラッグ製品及び法規制植物の簡便なスクリーニング法について報告している.[20]

尿中乱用薬物の 1 次スクリーニング法として，イムノクロマトグラフィーを用いた簡易検査キットによる検査法が，簡便な方法として多くの場面で使用されている．これら簡易検査キットは，尿を反応パネルの上にのせ，試薬を滴下する等の単純な操作で判定できるが，日本における代表的な乱用薬物である覚せい剤 methamphetamine (MA) 及び合成麻薬 3,4-methylenedioxymethamphetamine (MDMA) を

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識別して同時に検出可能なイムノクロマトグラフィーキットは現在のところ販売されていない．そこで，本研究ではMA及びMDMA，それら化合物の代謝物amphetamine (AP)及び3,4-methylenedioxyamphetamine (MDA)を同時に判別可能な，DART-TOFMSを用いた尿中乱用薬物のスクリーニング法の検討を行った．

実験方法

1. 試薬　分析対象薬物としてAP硫酸塩，MA塩酸塩，MDMA塩酸塩，MDA塩酸塩，またGC-MS分析時の内標準物質としてMA重水素標識体(2-methylamino-1-phenylpropane-2,3,3,3-d4, MA-d4)の各化合物水溶液を使用した．MA塩酸塩は大日本住友製薬から購入し，その他の化合物は過去に合成し，論文で報告したものを使用した．[21,22] 前処理用の尿素分解酵素としてurease Type C3 from Jack beans, 1490000 U/g (Sigma Aldrich社，MO, USA)を，マイクロ固相抽出用ピペットチップはOmix pipette tips C18及びC4(Varian社，CA, USA)を，タンパク沈殿時の溶液膜ろ過フィルターとしてUltraFree-MC（孔径0.45 μm）(Millipore社，MA, USA）を使用した．その他の試薬は試薬特級を用い，尿試料はボランティアから得た尿を薬物フリーのヒトコントロール尿として使用した．ヒト尿試料採取及びその取り扱いは，国立医薬品食品衛生研究所研究倫理委員会による倫理審査の承認を経て，倫理委員会の定める規定に則り，遵守すべき基準に従って実施した．

2. 前処理法　尿中の尿素による影響を低減し，DART-TOFMS測定における薬物の検出感度を向上させるために以下に示した簡易前処理法を検討した．

2-1. 固相抽出法　マイクロ固相抽出ピペットチップ（Omix pipette tips C18又はC4）を用いて各薬物標準溶液を加え濃度0.5–5 μg/mlに調製した尿試料中の化合物を，以下に示した操作で抽出を行った．①固相の活性化：メタノール100 μl 2回，純水100 μl 2回ピペット操作を行う．②試料の保持：各薬物を添加したヒトコントロール尿100 μlに0.1 M水酸化ナトリウム水溶液20 μlを加え，液中でピペット操作を5回繰り返す．③洗浄：純水100 μl 2回，5%メタノール水溶液100 μl 2回ピペット操作を行う．④溶出：溶出溶媒50 μl中でピペット操作を5回繰り返す．得られた溶出液をDART-TOFMS分析に使用した．

2-2. 液–液抽出法　ヒトコントロール尿1 mlに各薬物標準溶液を0.5–5 μg/mlになるよう添加し，少量の25%アンモニア水を加えた．さらに500 μlの有機溶媒を加えて振とうし，静置後有機溶媒層を測定に使用した．

2-3. 酵素処理法　尿素除去のために文献[23]に従い，各薬物を添加したヒトコントロール尿100 μlにurease（1000 U/mlリン酸緩衝溶液）10 μlを加え，37℃で10分間振とうし酵素反応を行った．

3. DART-TOFMS分析　DART-TOFMS測定装置として，イオン源Direct Analysis in Real Time (DART)に質量分析計AccuTOF JMS-T100（ともに日本電子社製）を連結したものを使用した．測定は各試料溶液をガラス棒の先端に付着させ，同一試料を1回1–2分の分析中で複数回DARTイオン源にかざし，スペクトルの確認を行った．なお，質量校正にはPEG600を使用し，各測定の内標準物質としてcaffeine（$C_8H_{10}N_4O_2$）溶液を用いた．その他の測定条件は以下の通りである．

DART条件：Positive mode; gas flow: He, 2.0 l/min; gas temp.: 200℃; needle: 3200 kV; electrode 1: 100 V; electrode 2: 250 V

TOFMS条件：Postive mode; orifice 1: 15 V, 80℃; orifice 2: 5 V; ring lens: 5 V; ion guide: 500 V; reflectron: 950 V; mass range: 100–500 (Da)

4. GC-MS分析　各前処理法における尿中からのMA, AP, MDMA, MDAの回収率及び，濃度範囲の直線性を確認するために，各処理を行った後GC-MSを用いて定量分析を行った．マイクロ固相抽出用ピペットチップ若しくは液–液抽出による抽出液に，内標準物質MA-d4水溶液（最終濃度2 μg/ml）及び酸性メタノールをMA, AP遊離塩基の揮発防止のために微量添加した後，窒素気流下で蒸発乾固させた．Trifluoroacetic anhydride (TFAA, Sigma Aldrich社）100 μl及び酢酸エチル100 μlを加え，60℃で20分間反応させ，過剰の試薬を窒素気流下で留去し，酢酸エチル100 μlに溶解して，各化合物のTFA体としてGC-MS測定を行った．また，酵素処理溶液については，反応液に内標準物質MA-d4水溶液（最終濃度2 μg/ml）及びエタノー

ル 0.9 ml を加えてタンパクを沈殿させた後，溶液をフィルターろ過し，窒素気流下で蒸発乾固させ，TFAA を用いて同様に誘導体化して GC-MS 測定試料とした．

GC-MS は Selected ion monitoring（SIM）モードで測定を行い，各化合物と内標準物質とのピーク面積比を算出し，0.5–5.0 μg/ml の濃度範囲における各化合物の直線性を検討した．回収率は，各濃度における標準溶液と前処理溶液のピーク面積比（各化合物/内標準物質）を比較することにより算出した．

GC-MS 測定装置として，6890N GC-5975MSD (Agilent 社製) を用いた．

GC-MS 条件：Column: HP-1MS (30 m×0.25 mm i.d., 0.25 μm, Agilent); Gas: He; Flow: 1.0 ml/min; injection volume: 1 μl; splitless mode; Injection temp.: 200℃; Column temp.: 60℃ (1 min hold) −20 ℃/min–280℃ (5 min hold); ionization: EI; transfer temp.: 280℃; monitoring ions: *m/z* 140 (TFA-AP), 154 (TFA-MA, TFA-MDMA), 135 (TFA-MDA), 158 (TFA-MA-d4)

結果及び考察

1. 標準溶液及び尿中薬物の直接分析　対象薬物の標準水溶液に，25%アンモニア水を微量加え遊離塩基として測定を行った．DART イオン源に同一試料を繰り返しガラス棒の先端に付着させてかざし，TOFMS により複数回の化合物スペクトル確認を行った．その結果 MA, AP, MDMA, MDA 水溶液のプロトン付加分子イオン $[M+H]^+$ の検出は，各化合物 0.5 μg/ml まで確認可能であった（S/N>3）．なお，理論値と測定値の質量差が 10 mmu 以内の精度で組成推定可能な濃度は各化合物 1 μg/ml 以上であった．Table 1 に測定化合物及び尿素 dimer の組成式（プロトン付加体）及びモノアイソトピック質量値を示した．

次に尿試料に MA 水溶液を添加して測定を行ったところ，尿素（CH_4N_2O）の dimer（$[2M+H]^+$: 121.0725）によるイオン化抑制が認められ，MA のプロトン付加分子イオン $[M+H]^+$ が確認できる濃度（S/N>3）は大幅に低下し，20 μg/ml 程度であった（Fig. 1）．そこで，DART-TOFMS による尿中薬物分析において，尿素の影響を低減し検出感度を向上させるための簡易的な前処理法を検討した．

2. マイクロ固相抽出用ピペットチップを用いた分析　マイクロ固相抽出用ピペットチップは，チップ先端に固定相を充填しており，マイクロピペットに装着して通常のピペット操作で溶液を吸引，吐出することで一連の固相抽出操作を行うことが可能であり，尿中薬物分析の前処理にも用いられている．[24]

Table 1. Elemental Compositions of Targeted Compounds, Caffeine and Urea (Dimer) and Their Exact Mass (Calculated)

Compound	Elemental compositions (protonated)	Exact mass (calculated)
AP	$C_9H_{14}N$	136.11262
MA	$C_{10}H_{16}N$	150.12827
MDA	$C_{10}H_{14}NO_2$	180.10245
MDMA	$C_{11}H_{16}NO_2$	194.11810
caffeine	$C_8H_{11}N_4O_2$	195.08820
urea (dimer)	$C_2H_9N_4O_2$	121.07255

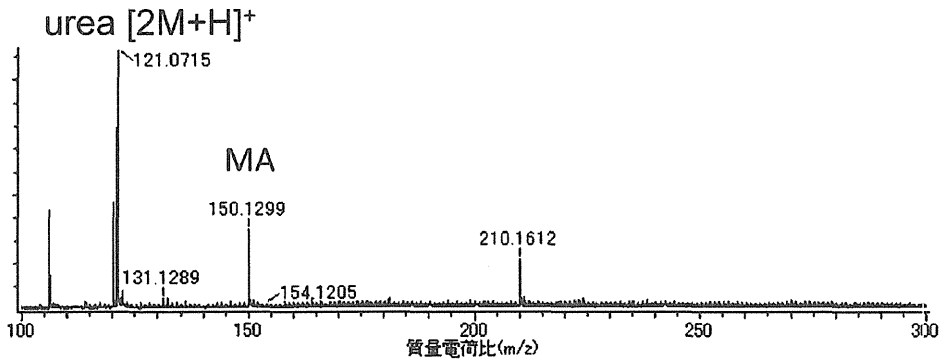

Fig. 1. DART-TOFMS Mass Spectrum of the Urine Sample Spiked with MA (20 μg/ml) without Any Pretreatments

DART-TOFMS では溶出液をそのままガラス棒に付着させて測定を行うため，溶出溶媒を尿試料の半量とすることで，各薬物を約2倍に濃縮することが可能であった．溶出溶媒としてメタノールのみ，メタノール/アセトニトリル混液，ジクロロメタン/イソプロパノール混液を用いて，DART-TOFMS 測定を行ったところ，いずれの溶出溶媒においても，4化合物のプロトン付加分子イオンピーク $[M+H]^+$ が確認できた．最も感度が良好であったジクロロメタン/イソプロパノール混液（3：1）と固相チップ C18 の組み合わせでは，尿中薬物濃度 0.25 μg/ml で4化合物の $[M+H]^+$ が確認可能であった（S/N >3）[Fig. 2(A)]．

3. 液-液抽出を用いた分析　液-液抽出は尿中薬物の簡便な抽出法であり，様々な抽出溶媒が用いられている．本研究では，DART-TOFMS 分析において4化合物の検出感度がよく，他成分による妨害が少ない抽出溶媒を検討した．抽出溶媒をそのままガラス棒に付着させ DART-TOFMS で測定した結果，酢酸エチルを用いた場合，dimer である $[2M+H]^+$ 177 が強く検出され，4化合物の検出感度が低下した．ジエチルエーテルを用いると，

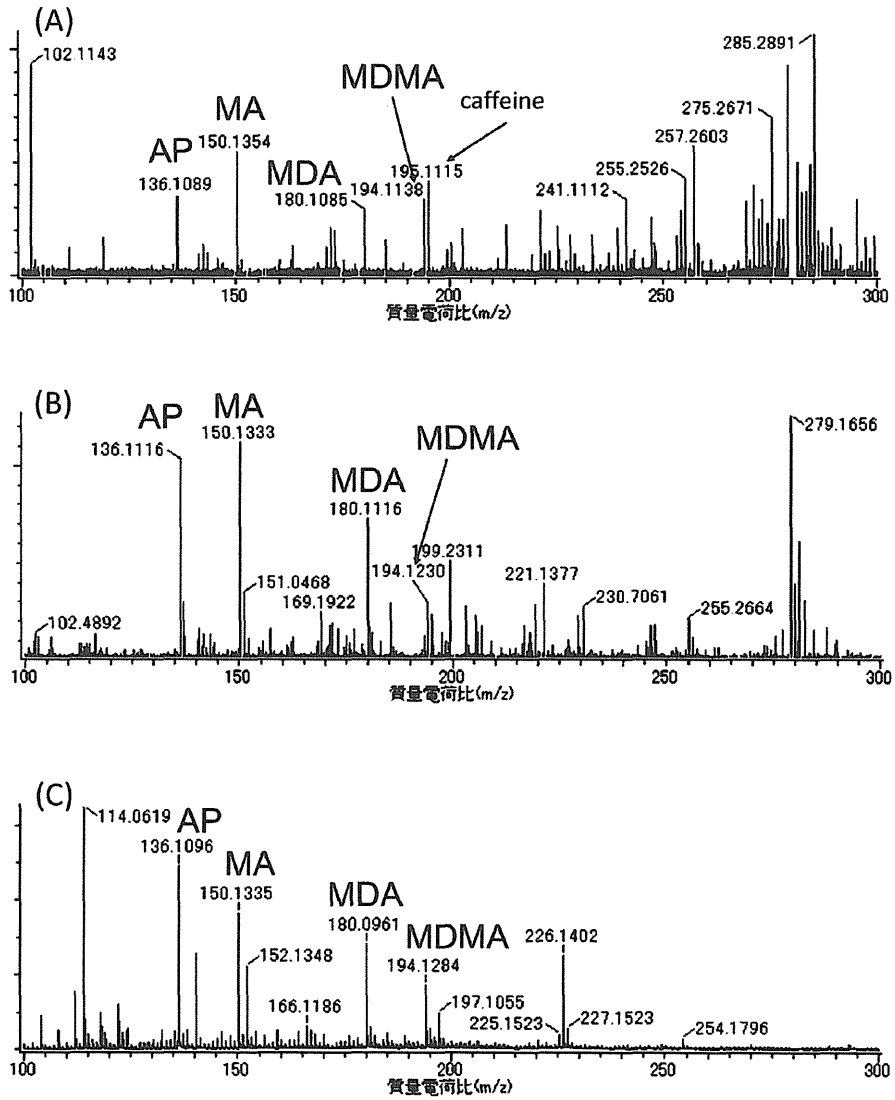

Fig. 2. DART-TOFMS Mass Spectra of the Extracts from the Urine

(A) at 0.25 μg/ml by C18 pipette tip-type solid-phase extraction (B) at 0.5 μg/ml by liquid-liquid extraction with a mixed solution of dichloromethane and hexane and (C) at 5 μg/ml treated with urease.

DART 測定中に揮発してしまうため，安定した測定が困難であった．ヘキサンを用いると，尿中常在成分の低減が確認できたが，AP 及び MDA の検出感度が他 2 化合物に比べ低下した．また，ジクロロメタンを使用した場合は，有機溶媒が下層なため，DART 測定時においてガラス棒に有機溶媒層のみを付着させることが困難であった．そこで，ヘキサンにイソプロパノール，エタノール，アセトン，ジクロロメタンを加えた混合液について検討を行った結果，ヘキサン/ジクロロメタンを 2：1 で混合すると，有機層が上層となり 4 化合物の検出感度が改善された．Figure 2(B)に示したように，0.5 μg/ml の尿中薬物濃度で 4 化合物のイオンピーク $[M+H]^+$ が確認可能であり (S/N>3)，他の溶媒抽出液と比較して最もよい結果が得られた．

4. 酵素処理による分析　酵素反応溶液をガラス棒に付着させて，DART-TOFMS により測定を行ったところ，尿素の分解は確認できたが他成分の影響により，4 化合物とも 1 μg/ml 以下の濃度でイオンピーク $[M+H]^+$ の検出が困難であった．Figure 2(C)に 4 化合物を 5 μg/ml 添加し，urease 処理を行ったヒトコントロール尿試料の DART-TOFMS スペクトルを示した．

5. GC-MS 分析による各前処理液の定量結果

以上の DART-TOFMS による測定結果に基づき，各前処理法の抽出効率を確認するため GC-MS を用いて処理液について定量分析を行った．

固相抽出法では，溶出溶媒としてジクロロメタン/イソプロパノール混液 (3：1)，固相に C18 を用いた操作法で，各化合物の尿中からの回収率が，0.25 μg/ml (最終濃度 0.5 μg/ml) では AP，MDA において 70%以下であったが，0.5–5 μg/ml (最終濃度 1.0–10 μg/ml) の濃度範囲では 4 化合物ともに 70%以上の回収率を示し [Fig. 3(A)]，直線性も R^2>0.990 であった．

液–液抽出法においては，抽出液にヘキサンのみを使用した場合，AP 及び MDA の回収率が 40–50 %と低い値を示した．一方，ジクロロメタンを使用した場合は，回収率が 90%以上であった．そこで，ヘキサン/ジクロロメタンを 2：1 で混合したところ，4 化合物すべての回収率が各濃度で 70%以上に向上し [Fig. 3(B)]，直線性も R^2>0.990 であった．

また，酵素処理法においては，タンパク沈殿及び膜ろ過後，誘導体化を行った試料を GC-MS で分析した結果，1.0–5.0 μg/ml の濃度範囲では回収率が 60–80%であったが，GC-MS クロマトグラム上で尿中常在成分による妨害が大きく，0.5 μg/ml では

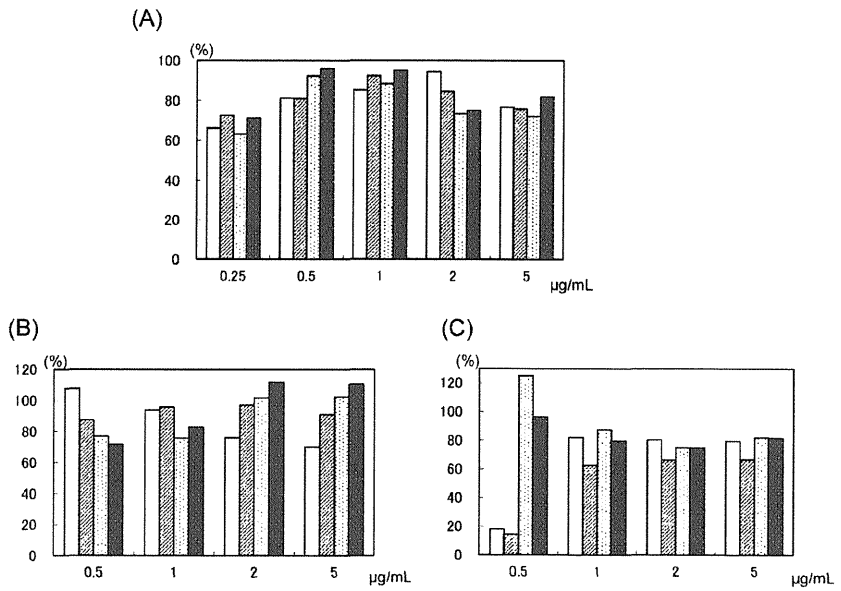

Fig. 3. Recoveries of Targeted Compounds

AP (□), MA (▨), MDA (⬚) and MDMA (■). (A) from urine samples extracted by C18 pipette tip-type solid-phase extraction, (B) a liquid-liquid extraction with a mixed solution of dichloromethane and hexane and (C) treated with urease.

各化合物の回収率が大きくばらついた [Fig. 3(C)].

結　　論

MA, AP, MDMA, MDA を添加したヒトコントロール尿試料を DART-TOFMS により測定したところ，尿素等の常在成分による目的化合物のイオン化抑制が認められ，検出感度が大幅に低下した．そこで，簡易抽出法を検討した結果，溶出溶媒にジクロロメタン/イソプロパノール混液を用いたマイクロ固相抽出用ピペットチップにより，各薬物 0.25 μg/ml の濃度まで，プロトン付加分子イオンピーク $[M+H]^+$ が検出可能であった (S/N>3)．また，ヘキサン/ジクロロメタン混液を用いた液-液抽出では，0.5 μg/ml まで検出が可能であった (S/N>3)．一方，urease を用いて尿中尿素を分解する手法では，尿中成分による妨害が大きく，1 μg/ml 以下の濃度では検出が困難であった．本研究に用いた簡易前処理法の抽出効率を確認するために，上記の前処理溶液中薬物を GC-MS により測定した結果，固相抽出法及び液-液抽出法で 4 化合物の回収率は 70 %以上となり，0.5-5 μg/ml の尿中薬物濃度範囲で良好な直線性を示した．

本研究において，簡単な前処理を行うことにより，常在成分によるイオン化抑制が低減し，尿試料中の薬物分子イオンピークが DART-TOFMS により検出可能であった．さらに十分なスペクトル強度が得られる高濃度試料においては，精密質量値から，組成推定を行うことが可能であった．本法は，米国乱用薬物・精神衛生サービス管理局 (SAMHSA) 等の推奨するカットオフ値濃度 0.5 μg/ml 以上の尿中 MA 及び MDMA について，固相抽出又は液-液抽出による前処理に 1 分程度，DART-TOFMS による測定で 1-2 分と，合計 3 分以内の分析時間で同時に検出することが可能であり，また，高濃度の場合は組成推定による判別が可能である．さらに，代謝物を同時検出することにより，尿への MA，MDMA の混入を否定でき，スクリーニングの信頼性が高まる．既存の尿検査簡易キットでは MA と MDMA 及び代謝物の同時検出が困難であることを考慮すると，DART-TOFMS は尿中のこれら薬物の 1 次スクリーニング法として有用であると考えられた．しかし，組成式が同一な化合物については，DART-TOFMS のみで判別できないことに留意する必要がある．また本研究では，尿中に存在する可能性がある他の成分（薬物や添加物を含む）が及ぼす影響は検討しておらず，今後，実際の薬物使用者を含む様々な尿試料について検討を加える必要がある．

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Identification of a Novel Cannabimimetic Phenylacetylindole, Cannabipiperidiethanone, as a Designer Drug in a Herbal Product and Its Affinity for Cannabinoid CB_1 and CB_2 Receptors

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A new cannabimimetic phenylacetylindole (cannabipiperidiethanone, 1) has been found as an adulterant in a herbal product which contains two other known synthetic cannabinoids, JWH-122 and JWH-081, and which is distributed illegally in Japan. The identification was based on analyses using GC-MS, LC-MS, high-resolution MS and NMR. Accurate mass spectrum measurement showed the protonated molecular ion peak of 1 at *m/z* 377.2233 $[M+H]^+$ and the molecular formula of 1 was $C_{24}H_{29}N_2O_2$. Both mass and NMR spectrometric data revealed that 1 was 2-(2-methoxyphenyl)-1-{1-[(1-methylpiperidin-2-yl)methyl]-1*H*-indol-3-yl}ethanone. Compound 1 has a mixed structure of known cannabimimetic compounds: JWH-250 and AM-2233. Namely, the moiety of phenylacetyl indole and *N*-methylpiperidin-2-yl-methyl correspond to the structure of JWH-250 and AM-2233, respectively. However, no synthetic, chemical or biological information about 1 has been reported. A binding assay of compound 1 to cannabinoid receptors revealed that 1 has affinity for the CB_1 and CB_2 (IC_{50}=591, 968 nM, respectively) receptors, and shows 2.3- and 9.4-fold lower affinities than those of JWH-250. This is the first report to identify cannabimimetic compound (1) as a designer drug and to show its binding affinity to cannabinoid receptors.

Key words synthetic cannabinoid; JWH-081; JWH-122; 2-(2-methoxyphenyl)-1-{1-[(1-methylpiperidin-2-yl)methyl]-1*H*-indol-3-yl}ethanone; JWH-250; designer drug

Numerous psychotropic products have been made readily available *via* the Internet. In Japan, various herbal products with brand names such as "Spice" and "herbal incense," hinting at cannabis-like effects, began to appear in 2008, following their advent in several European countries in 2006. In early 2009, we reported that these herbal products contained synthetic cannabinoids such as cannabicyclohexanol (CCH) and JWH-018 as psychoactive adulterants.[1,2] German groups have also found these compounds in some herbal products.[3] More than 20 synthetic cannabinoids have been detected as psychoactive ingredients in herbal products around the world since 2009,[4—10] and ten of those cannabinoids—CCH, CP-47,497, JWH-018, JWH-073, JWH-250, JWH-015, JWH-122 (**2**), JWH-081 (**3**), JWH-200 and JWH-251—were controlled as designated substances (Shitei-Yakubutsu) under the Pharmaceutical Affairs Law in Japan as of May 2011. Most of these compounds were synthesized as cannabimimetic substances having affinities to cannabinoid CB_1 and/or CB_2 receptors in the course of drug development.[11] However, some of these synthetic cannabinoids have been abused as psychoactive drugs in place of *Cannabis sativa* L. (cannabis, marijuana, hemp), which naturally contains psychoactive cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC). During our successive survey of designer drugs distributed in Japan, we found a new compound (**1**) contained in a herbal product together with two known cannabimimetic substances, JWH-122 (**2**) and JWH-081 (**3**) (Fig. 1). In the present study, we describe the identification of the novel phenylacetylindole (**1**) and its affinity to cannabinoid CB_1 and CB_2 receptors.

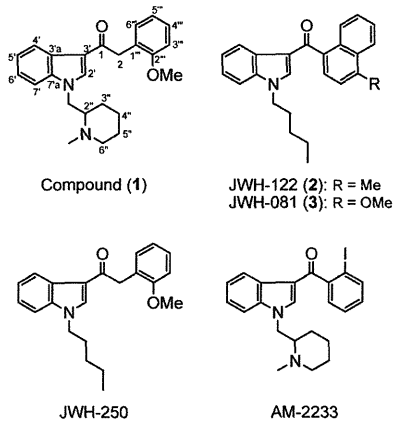

Fig. 1. Structures of the Detected Compounds (**1**—**3**) and Related Cannabimimetic Indoles

Experimental

Chemicals and Reagents JWH-122 (**2**), JWH-081 (**3**) and JWH-250 were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). (*R*)-(+)-WIN-55,212-2 was purchased from Sigma (St. Louis, MO, U.S.A.). All other common chemicals and solvents were of analytical reagent grade or HPLC grade.

Sample for Analysis The analysis sample was purchased *via* the internet in January 2011 as a herbal product being sold in Japan. The product contained 2 g of mixed dried plants.

Preparation of Sample Solution For qualitative analyses, 10 mg of the herbal product was crushed into powder and extracted with 1 ml of MeOH under ultrasonication for 10 min. After centrifugation (5 min, 3000 rpm), the supernatant solution was passed through a centrifugal filter (Ultrafree-MC, 0.45 μm filter unit; Millipore). If necessary, the solution was diluted with MeOH to a suitable concentration before instrumental analyses.

Analytical Conditions The sample solution was analyzed by GC-MS (electron impact (EI)) and LC-MS (electrospray ionization (ESI)) analyses according to our previous report.[5] The accurate mass spectrum of the target compound was measured using a direct analysis in real time (DART) ion source coupled to a time-of-flight (TOF) mass spectrometer (AccuTOF JMS-100LC; JEOL, Tokyo, Japan) in a positive mode.[12] The measurement conditions were as previously reported.[5]

For NMR analysis, pyridine-d_5 (99.96%) was purchased from the ISOTEC division of Sigma-Aldrich (St. Louis, MO, U.S.A.). The NMR spectra were obtained on ECA-600 spectrometers (JEOL). Assignments were made *via* ^{1}H-NMR, ^{13}C-NMR, heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), double quantum filtered correlation spectroscopy (DQF-COSY), one-dimensional total correlation spectroscopy (1D-TOCSY), and rotating frame nuclear Overhauser effect (ROE) spectra. For isolation of the compound, recycling preparative HPLC (Japan Analytical Industry, Tokyo, Japan) was used with a JAIGEL-GS310 column (500 mm×20 mm i.d.; Japan Analytical Industry) and monitored by UV absorbance and refractive index (RI) detectors.

Isolation of Compound 1 A 2-g sample of the herbal product was extracted with 150 ml of $CHCl_3$/MeOH (1 : 1) by ultrasonication for 1 h. The extractions were repeated three times, and the supernatant fractions were combined and evaporated to dryness. The extract was placed on a preparative TLC plate (Silica Gel 60, 20×20 cm, 2 mm; Merck, Darmdstadt, Ger-

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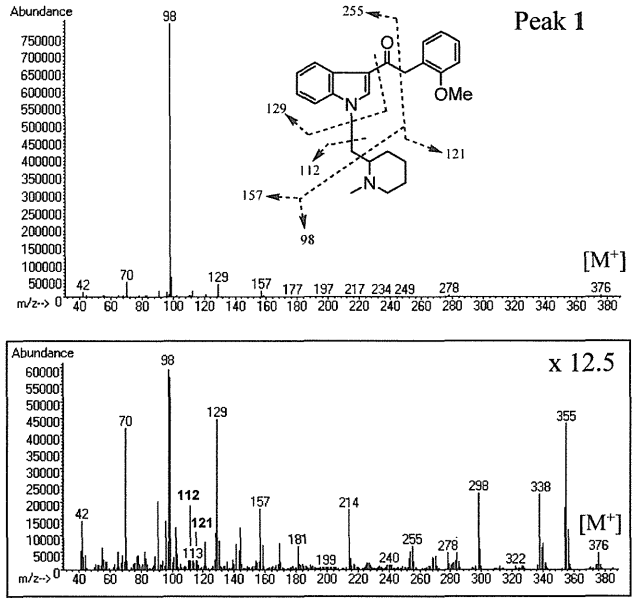

Fig. 2. GC-EI Mass Spectrum of the Detected Peak at 52.67 min (**1**)

many), which was then developed using $CHCl_3$/MeOH (20 : 1). A portion of the silica gel in the TLC plate that contained the target compound was detected by UV 254 nm and DART-TOF-MS, and scraped from the plate. The target compound was then eluted with $CHCl_3$/MeOH (1 : 1) to obtain fraction 1, and fraction 1 was further purified by recycling preparative HPLC with $CHCl_3$/MeOH (1 : 1) to obtain compound **1** (43 mg).

Compound **1**: A pale yellow oil; UV (MeOH) λ_{max} nm: 242, 300; ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz): see Table 1; EI-MS *m/z* (% relative intensity): 376 (0.6, $[M^+]$), 355 (5), 338 (3), 298 (3), 255 (0.8), 214 (2), 157 (2), 129 (6), 121 (1), 112 (2), 98 (100) and 70 (5), as shown in Fig. 2; DART-TOF-MS *m/z*: 377.2233 $[M+H]^+$ (Calcd for $C_{24}H_{29}N_2O_2$: 377.2229).

Binding Assay for Cannabinoid CB_1 and CB_2 Receptors The binding affinities of **1** and JWH-250 for the CB_1/CB_2 receptors were determined by the competition of agonist [^{3}H]-CP-55,940 (PerkinElmer Inc., MA, U.S.A.) binding to human recombinant cannabinoid CB_1/CB_2 receptors. To determine the IC_{50} values of the tested compounds, eight different concentrations of each compound in the range of 3 nM to 10 μM were investigated. (*R*)-(+)-WIN-55212-2, which is a cannabinoid receptor agonist, was used as a positive control.

Results and Discussion

Identification of Compound 1 An unknown peak **1** was detected along with two major peaks **2** (JWH-122) and **3** (JWH-081) in the GC-MS and LC-MS chromatograms of the herbal product (data not shown). The compounds for the peaks **2** and **3** were completely identical to JWH-122 and JWH-081, respectively, by direct comparison with the authentic samples.[5,9] The unknown peak **1** at 52.67 min in the GC-MS chromatogram showed a mass spectrum having 12 major ion peaks, as shown in Fig. 2. The LC-MS analysis determined that the peak **1** at 4.4 min showed a major ion peak at *m/z* 377 $[M+H]^+$ and absorbance maxima at 242 and 300 nm of the UV spectrum (data not shown). In the accurate mass spectrum obtained by DART-TOF-MS with direct exposure of the sample extract to the ion source, the major ion peak showed a protonated molecular ion peak ($[M+H]^+$) at *m/z* 377.2233 in the positive mode, suggesting that the molecular formula of **1** was $C_{24}H_{29}N_2O_2$.

The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited 28 protons and 24 carbons as shown in Table 1. The NMR spectra of **1**

Table 1. NMR Data[a] of JWH-250 and Compound **1**

Position	JWH-250[b]	Compound **1**[c]	
	^{13}C	^{13}C	^{1}H
1	193.1	192.2	—
2	40.9	40.7	4.43, 1H, d, *J*=15.1 Hz, overlapped
			4.40, 1H, d, *J*=15.1 Hz, overlapped
2′	135.0	136.4	8.37, 1H, s
3′	116.1	116.1	—
3′a	126.8	126.6	—
4′	122.8	122.3	8.88, 1H, d-like, *J*=7.5 Hz
5′	123.1	123.0	7.36, 1H, m, overlapped
6′	122.4	122.0	7.38, 1H, m, overlapped
7′	109.7	110.3	7.55, 1H, d, *J*=6.8 Hz
7′a	136.6	137.3	—
N-$\underline{CH_2}$	—	48.5	4.48, 1H, dd, *J*=14.1, 4.1 Hz
			4.00, 1H, dd, *J*=14.1, 8.3 Hz
1″	47.1	—	—
2″	29.5	62.3	2.31, 1H, m
3″	29.0	28.6	1.17, 1H, m
			1.10, 1H, m
4″	22.3	23.0	1.40, 1H, m, overlapped
			0.93, 1H, m
5″	13.9	25.0	1.38, 2H, m, overlapped
6″	—	56.2	2.74, 1H, d-like, *J*=11.3 Hz
			1.95, 1H, m
1‴	124.7	124.9	—
2‴	156.9	157.2	—
3‴	110.5	110.4	6.91, 1H, d, *J*=8.3 Hz
4‴	128.0	127.7	7.25, 1H, ddd, *J*=8.3, 7.6, 1.4 Hz
5‴	120.7	120.2	6.97, 1H, ddd, *J*=7.2, 7.6, 1.1 Hz
6‴	131.0	131.0	7.50, 1H, d-like, *J*=7.2 Hz
N-Me	—	42.7	2.34, 3H, s
OMe	55.4	54.7	3.65, 3H, s

a) Recorded at 600 MHz (^{1}H) and 150 MHz (^{13}C), respectively; data in δ ppm (*J* in Hz). *b*) Recorded in $CDCl_3$. *c*) Recorded in pyridine-d_5.

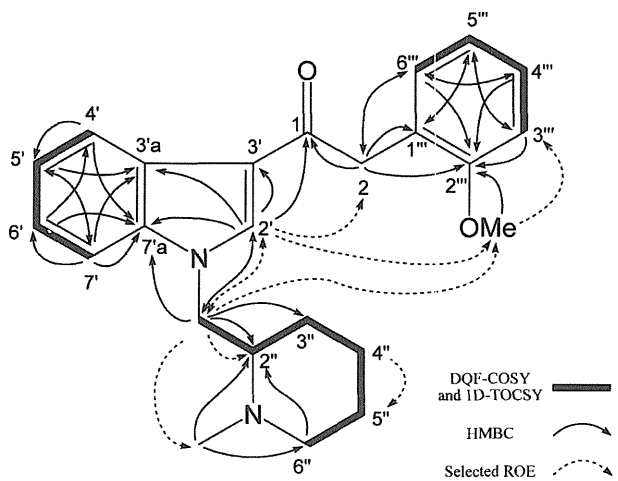

Fig. 3. DQF-COSY, 1D-TOCSY, HMBC and Selected ROE Correlations of **1**

(Table 1, Fig. 3) showed the presence of a methoxy group, a carbonyl carbon (C-1) with a methylene group which was adjacent to the carbonyl group (position-2), an indole group (positions-2′, 3′, 3′a, 4′, 5′, 6′, 7′, 7′a) and a phenyl group (positions-1‴ to 6‴). These spectra were very similar to those of the *o*-methoxy phenylacetyl indole, JWH-250 (Fig. 1, Table 1), except for the remaining data indicating a $C_7H_{14}N_1$

Table 2. Effect of Synthetic Cannabinoids on [^{3}H]-CP-55,940 Binding to Human Cannabinoid Receptors

Compound	IC_{50} (nM)		
	CB_1	CB_2	Ratio CB_1/CB_2
1	591	968	0.61
JWH-250	260	103	2.52
(*R*)-(+)-WIN-55,212-2[a]	45.6	13.8	3.30

a) Positive control, cannabinoid receptor agonist.

unit in place of the *n*-pentyl group. The HMBC and ROE spectra of **1** confirmed that the indole, methoxy, phenyl and acetyl groups were in the same arrangement as in JWH-250 (Figs. 1, 3). The ^{13}C-carbon, the HMQC, the DQF-COSY and the 1D-TOCSY spectra of the remaining unit suggested the existence of a 1,2,6-substiuted hexane moiety and one independent methyl group. The chemical shifts at the three carbons of C-2″, C-6″ and the independent methyl suggested that these carbons were connected to the nitrogen atom, and the HMBC correlations between the methylene protons (H-6″) and the methine carbon (C-2″) and between the *N*-methyl protons at δ_H 2.34 and the C-2″ and C-6″ carbons confirmed that the remaining unit of **1** was a *N*-methylpiperidin-2-ylmethyl group (Fig. 3). The connection of the remaining unit to the indole nitrogen was revealed by the HMBC correlations from the bridging methylene protons (*N*-C<u>H</u>$_2$) to the two carbons (C-2′, C-7′a) and from the methine proton at the 2′-position to the bridging methylene carbon (*N*-<u>C</u>H$_2$) (Fig. 3). The observed ROE correlations also supported the structure, as shown in Fig. 3. On the basis of these mass and NMR spectral data (Figs. 2, 3, Table 1), the structure of compound **1** was finally deduced as 2-(2-methoxyphenyl)-1-{1-[(1-methylpiperidin-2-yl)methyl]-1*H*-indol-3-yl}ethanone. This is the first report of this compound, and it was revealed that **1** has a mixed structure of known cannabimimetic compounds: JWH-250 and AM-2233 (Fig. 1). Considering its structure, compound **1** has been named cannabipiperidiethanone. By using chiral HPLC analysis, compound **1** has been revealed to exist as a racemic mixture (data not shown).

Binding Activity of Compound 1 to Cannabinoid CB_1 and CB_2 Receptors No chemical or biological information about compound **1** has yet been reported. However, **1** has a mixed structure of known cannabimimetic compounds, JWH-250 and AM-2233 (a racemic compound), and both compounds have been reported to possess affinity to cannabinoid CB_1 and CB_2 receptors (JWH-250: K_i=11, 33 nM, respectively; AM-2233: K_i=2.8, 2.9 nM, respectively).[13,14] Therefore, we thought that **1** might have some cannabinoid receptor-binding activity. Subsequently, the binding affinity of **1** to cannabinoid CB_1 and CB_2 receptors was determined in competition with agonist [^{3}H]-CP-55,940 binding, as shown in Table 2. As a result, **1** was shown to have affinity for the CB_1 and CB_2 receptors (IC_{50}=591, 968 nM, respectively), and to have 1.6-fold selectivity for the CB_1 receptor (Table 2). The affinities of **1** for the CB_1 and CB_2 receptors were 2.3- and 9.4-fold lower than those of JWH-250, and 13- and 70-fold lower than those of (*R*)-(+)-WIN-55,212-2, as shown in Table 2. Since the chiral resolution of AM-2233 has been reported and the (*R*)-(+)-enantiomer has very high affinities for the CB_1 and CB_2 receptors, 300- and 260-fold greater than those of the (*S*)-(−)-enantiomer,[14] it might be of additional interest to determine the affinities of each enantiomer of **1** from the view point of medicinal chemistry.

In this study, we first identified a novel cannabimimetic compound (**1**) in an illegal product and revealed its affinity for cannabinoid CB_1/CB_2 receptors. When certain synthetic cannabinoids became controlled substances under Japanese law, new analogs of the controlled substances replaced them as adulterants. Since the pharmacological and toxicological data for most of these cannabimimetic compounds have not been reported, there are serious health risks involved in their use. Therefore, we are continuously monitoring such compounds in illegal products to prevent their abuse.

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Rapid enantiomeric separation and simultaneous determination of phenethylamines by ultra high performance liquid chromatography with fluorescence and mass spectrometric detection: application to the analysis of illicit drugs distributed in the Japanese market and biological samples

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A rapid enantiomeric separation and simultaneous determination method based on ultra high performance liquid chromatography (UHPLC) was developed for phenethylamine-type abused drugs using (*R*)-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((*R*)-(−)-DBD-Py-NCS) as the chiral fluorescent derivatization reagent. The derivatives were rapidly enantiomerically separated by reversed-phase UHPLC using a column of 2.3-μm octadecylsilica (ODS) particles by isocratic elution with water–methanol or water–acetonitrile systems as the mobile phase. The proposed method was applied to the analysis of products containing illicit drugs distributed in the Japanese market. Among the products, 1-(3,4-methylenedioxyphenyl)butan-2-amine (BDB) and 1-(2-methoxy4,5-methylenedioxyphenyl)propan-2-amine (MMDA-2) were detected in racemic form. Furthermore, the method was successfully applied to the analysis of rat-hair specimens from rats that were continuously dosed with diphenyl(pyrrolidin-2-yl)methanol (D2PM). Using UHPLC-fluorescence (FL) detection, (*R*)- and (*S*)-D2PM from hair specimens were enantiomerically separated and detected with high sensitivity. The detection limits of (*R*)- and (*S*)-D2PM were 0.12 and 0.21 ng/mg hair, respectively (signal-to-noise ratio (S/N) = 3). Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: phenethylamines; diphenyl(pyrrolidin-2-yl)methanol (D2PM); (*R*)-(−)-DBD-Py-NCS; chiral derivatization method; ultra high performance liquid chromatography (UHPLC)

Introduction

Health hazards caused by the abuse of illicit drugs occur frequently among young people and have become a serious concern. Such drugs are easily obtainable via the Internet, adult shops, street markets, and so on. The use of illicit drugs is also the gateway to narcotic and psychostimulant drugs abuse. In Japan, the Pharmaceutical Affairs Law was revised, and the regulation was tightened by introducing a system of controlled substances, designated as Shitei-Yakubutsu, in April 2007 (31 compounds and 1 plant).[1,2] Under this Act, compounds that have potential harmful health effects are designated Shitei-Yakubutsu, and rapid response to such compounds is facilitated. This system temporarily decreased the distribution of designated substances in Japan. However, due to synthetic modification, structural analogs of designated compounds may slip past regulations. As of August 2011, 60 substances (classified as 26 phenethylamines, 12 tryptamines, 6 alkyl nitrites, 4 piperazines, 10 cannabinoids, 1 diterpene, and 1 plant) are listed as designated substances; the list is continually revised and improved as necessary. Diphenyl(pyrrolidin-2-yl)methanol (D2PM), and 1-(2-fluorophenyl)-*N*-methylpropan-2-amine (*N*-methyl-2FMP), were recently added to the designated substances list; there is concern that analogs of these substances may be distributed as new illicit drugs. In particular, D2PM and its analogs are organocatalysts used for various asymmetric syntheses; however, such chemical reagents are

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likely abused.[3–7] To prevent distribution of these substances, it is crucial to establish an analytical method of detection before they enter the Japanese market. Therefore, development of simple and rapid screening methods of illicit drugs and their structurally related compounds is required.

Most phenethylamine compounds are chiral, and their enantiomers can possess different pharmacological activities and pharmacokinetic/pharmacodynamic properties. For example, it is well known that the enantiomers of methamphetamine and amphetamine differ in their biological and metabolic activities. The *d*-isomer has the greatest biological activity, whereas the *l*-isomer is far less active.[8,9] Therefore, it is important to ensure enantiomeric purity by chiral separation. Furthermore, relevant information can be gathered by indentifying the manufacturing method, the producer countries, and their sources by analyzing impurities and determining of the ratio of optical isomers in the distributed illicit drugs.

Numerous strategies for enantiomeric separation of chiral compounds are available using various separation techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), capillary electrophoresis (CE), and capillary electrochromatography (CEC).[10–18] Among these methods, HPLC is one of the most effective tools for chiral separation. The methods are divided into three broad classes: chiral stationary phase (CSP) methods, chiral mobile phase methods, and chiral derivatization methods.[15–17] CSP methods use packing materials combined with chiral molecules at the carrier surface as the stationary phase. A number of CSP methods have already been developed and are widely used. However, to perform chiral separations of target enantiomers by HPLC, the CSP must be selected through a trial-and-error process based solely on prior experience. Chiral mobile phase methods form a diastereomer complex by passing the sample through a column using a mobile phase containing chiral molecules. This method does not require the column to be packed with chiral molecules or complicated handling; however, the kinds of enantiomers that can be separated using this method are limited. Indeed, direct chromatographic separation using hydroxypropyl-β-cyclodextrin (HP-β-CD) as a chiral mobile phase additive has been investigated for the chiral separation of amphetamine and its derivatives; baseline separations could not be achieved because of the peak broadening.[19] These basic compounds tend to broaden peaks in consequence of residual silanol in the column. On the other hand, the chiral derivatization method does not require the comparatively expensive analytical column containing a CSP, and analysis can be performed using a conventional HPLC column such as an ODS column. Guillame *et al.* reported that *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC) would be effective for enantiomeric separation of amphetamine, its derivatives, and several β-blockers (atenorol, propranorol, and so on) using HPLC–UV.[19] Furthermore, using a fluorescence derivatization method such as HPLC-fluorescence (HPLC-FL) detection has the advantage of highly sensitive detection.

In our previous study, we reported an HPLC-FL method for the enantiomeric separation of D2PM and psychotropic methylphenidate (MPH) using a chiral fluorescent derivatization reagent, (*R*)-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((*R*)-(−)-DBD-Py-NCS).[20] However, to the best of our knowledge, the method and the reagent have not been applied for the analysis of other drugs.

In this study, we conducted to develop a method for rapid enantiomeric separation and simultaneous determination of phenethylamine-type abused drugs and established a detection method using UHPLC-FL and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). UHPLC is an analytical technique developed in the last half decade that is an extension of conventional HPLC techniques; it uses small particles in the separation column and pumping of the mobile phase under ultra-high pressure conditions.[21–23] Application to the analysis of phenethylamine drugs distributed on the Japanese market and the analysis of rat-hair specimens after oral dose of racemic, (*R*)-, and (*S*)-D2PM are also discussed.

Experimental

Materials and reagents

The hydrochloric acid salts of racemic phenethylamines, i.e. 1-(3,4-methylenedioxyphenyl)butan-2-amine (BDB), 1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine (DOI), 2-ethylamino-1-phenylpropan-1-one (*N*-ethylcatinone), 1-(4-fluorophenyl)propan-2-amine (4-FMP), 2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one (bk-MBDB), 2-ethylamino-1-(3,4-methylenedioxyphenyl)propan-1-one (bk-MDEA), 1-(2-fluorophenyl)-*N*-methylpropan-2-amine (*N*-methyl-2FMP), 2-(methylamino)-1-(4-methylphenyl)propan-1-one (4-methylmethocatinone), 1-(2-methoxy4, 5-methylenedioxyphenyl) propan-2-amine (MMDA-2), and 1-(4-methoxyphenyl)-*N*-methylpropan-2-amine (PMMA) were obtained from the National Institutes of Health Sciences (NIHS, Tokyo, Japan). Products sold in the past as legal substances on the Japanese market were used for the determination of the phenethylamine-type abused drugs. Racemic methylphenidate hydrochloride (MPH), (2R,2′R)-(+)-*threo*-methyl α-phenyl-α-(2-piperidyl)acetate hydrochloride (D-MPH), (2S,2′S)-(−)-*threo*-methyl α-phenyl-α-(2-piperidyl)acetate hydrochloride (L-MPH), and leucine enkephalin were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). (*R*)-Diphenyl(pyrrolidin-2-yl)methanol ((*R*)-(+)-α,α-diphenyl-2-pyrrolidinemethanol, (*R*)-D2PM), (*S*)-diphenyl(pyrrolidin-2-yl)methanol ((*S*)-(−)-α,α-diphenyl-2-pyrrolidinemethanol, (*S*)-D2PM), α-(4-piperidyl)benzhydrol (PBH as internal standard), (*R*)-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((*R*)-(−)-DBD-Py-NCS), (*R*)-(+)-4-nitro-7-(2-chloroformylpyrrolidin-1-yl) -2,1,3-benzoxadiazole ((*R*)-(+)-NBD-Pro-COCl), 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), and triethylamine were obtained from Tokyo Kasei Co. (Tokyo, Japan). Diethyl ether, dimethyl sulfoxide (DMSO), and trifluoroacetic acid (TFA) were obtained from Kanto Kagaku Co. (Tokyo, Japan). Sodium dodecyl sulfate (SDS) and hydrochloric acid (HCl) were purchased from Wako Pure Chemicals (Osaka, Japan). Acetonitrile (CH_3CN), methanol (CH_3OH), and formic acid (FA) were of LC–MS grade (Wako Pure Chemicals, Osaka, Japan). Saline was purchased from Otsuka Pharmaceutical Factory, Inc. (Naruto, Japan). All other reagents were of analytical-reagent grade and were used without further purification.

UHPLC-FL and ESI-TOF-MS conditions

A Shimadzu (Kyoto, Japan) ultra-fast liquid chromatograph system consisting of two LC-20AD pumps, a degasser (DGU-20A_3) and an auto-injector (SIL-20AC_{HT}) was used. Reversed-phase liquid chromatography was performed using TSK-gel ODS-140HTP column (2.1 mm i.d. × 100 mm, 2.3 μm, Tosoh, Tokyo). The column

