regioisomeric products can be expected. In fact, the stereochemistry of the anomeric proton H1′, identified by its characteristic chemical shift at 5.15 ppm, was found by analysis of the coupling constant between H1′ and H2′ (7.2 Hz) to be, as expected, in the β-configuration. Although purely chemical syntheses of HMMA-Gluc have been low yielding, this enzyme-assisted synthesis was found to stereoselectively produce HMMA-Gluc in very high yield (71%).

Kinetic Analysis of Glucuronidation in Microsomes UGT activities in microsomes are expected to differ with respect to species and microsomal enzyme inducer. To study the biocatalytic efficiency of various microsomes toward glucuronidation, kinetic parameters for HMMA glucuronidation were determined using Aroclor 1254-induced and non-induced rat liver and human liver microsomes. All three types of microsomes catalyzed HMMA glucuronidation and the apparent kinetic parameters are shown in Table 1. The apparent V_{max} of Aroclor 1254-induced rat liver microsomes was 64.7 nmol/min/mg protein, two fold higher than from non-induced rat liver microsomes. Human liver microsomes showed a lower apparent V_{max} as compared with both rat liver microsomes. K_m values of 1.9—7.6 mM were obtained for all three microsomes which, while low, are sufficient for the purpose of enzymatic synthesis. These results suggest that Aroclor 1254-induced rat liver microsomes are a suitable biocatalyst for an enzyme-assisted synthesis of HMMA-Gluc.

Human UGT Isozyme Catalysis of HMMA Glucuronidation It is known that the metabolism of MDMA involves *N*-demethylation by CYP2D6 to DHMA. DHMA can undergo subsequent *O*-methylation mediated by catechol *O*-methyltransferase (COMT) to HMMA as shown in Chart 1. The hydroxyl group of HMMA can be further metabolized by phase II enzymes to produce conjugates, the glucuronide/sulfate, which are excreted into urine. Although the majority of MDMA metabolites are conjugates, there have been no reports on the UGT isoforms involved in HMMA glucuronidation. Therefore, enzyme analyses were performed on the 12 kinds of human microsomes that express UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17). Initially, an HPLC method to quantitate the amount of HMMA-Gluc in the enzyme reaction mixture was tried; however, it was difficult to detect the UV absorbance from HMMA-Gluc because of overlap from impurity peaks derived from insect cell microsomes (data not shown). Therefore, an LC-MS method, in selected ion monitoring mode set for HMMA-Gluc at *m/z* 372, was used. Results from the LC-MS study are shown in Fig. 2. In the case of the UGT1A series, HMMA-Gluc was hardly detectable, even for the 1A1, 1A6 and 1A9 isozymes which are known to catalyze the glucuronidation of hydroxyl groups in phenolic compounds.[15] In contrast, production of HMMA-Gluc was observed in the UGT2B series, with UGT2B15 being especially productive. The V_{max} and K_m values for human UGT2B15 were determined to be 1.6 nmol/min/mg protein and 3.8 mM, respectively, showing that human UGT2B15 exhibits high glucuronidation efficiency with HMMA.

Table 1. Kinetic Parameters for HMMA Glucuronidation by Liver Microsomes

	Aro[a]	Rat[b]	Human[c]
V_{max} (nmol/min/mg protein)	64.7	30.6	16.7
K_m (mM)	6.7	1.9	7.6

a) Aroclor 1254-induced rat liver microsomes. *b*) Non-induced rat liver microsomes. *c*) Pooled human liver microsomes.

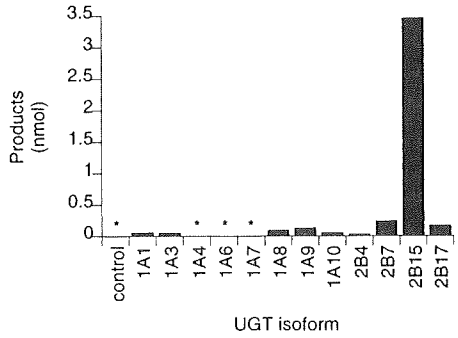

Fig. 2. Glucuronidation of HMMA by Microsomes from Insect Cells Expressing Human UGT Isoforms

* Not detected.

Discussion

MDMA metabolism involves demethylation to DHMA catalyzed by CYP2D6, followed by *O*-methylation to HMMA, catalyzed by COMT. Although some HMMA is detected in urine samples as a metabolite of MDMA, most of it is conjugated by phase II enzymes to give a glucuronide or sulfate which is excreted into urine.[9,10] If metabolites of MDMA were readily available as authentic standards, the detection of these metabolites along with HMMA or MDMA in urine samples would constitute a powerful analytical method for unequivocal proof of MDMA use. Unfortunately, the conjugate metabolites are not commercially available and are difficult to synthesize. In this report, we focused on HMMA-Gluc as a major urinary metabolite of MDMA and developed a useful method based on enzyme-assisted synthesis using rat liver microsomes for its production. Liver microsomes from Aroclor 1254-induced rats were used as a highly active source of mammalian UGT. After purification by protein precipitation and preparative HPLC, the structure of HMMA-Gluc was characterized by ^{1}H-NMR and mass spectrometry. This enzymatic method was highly stereoselective, producing the β-anomer. Pure HMMA-Gluc was isolated in very high yield (71%, 13.2 mg), in amounts sufficient for the analysis of MDMA consumption and for enzyme kinetics studies.

An LC-MS method was employed for identification of glucuronidation activities of human UGT isoforms toward HMMA. High levels of enzymatic activity responsible for the formation of HMMA-Gluc from HMMA were only observed in UGT2B15 which is an isoform that catalyzes the glucuronidation of flavonoids and androgenic and estrogenic steroids.[16] A number of endogenous phenolic compounds, drugs, and hydroxylated metabolites of drugs are substrates for the UGT1A series, but interestingly, HMMA is not glucuronidated by any of its members.

Oxazepam, a commonly used 1,4-benzodiazepine anxiolytic drug, is known to be cleared primarily by glucuronidation catalyzed by UGT2B15.[17] The glucuronidation of oxazepam by human subjects is gender dimorphic, arising from a genetic polymorphism for UGT2B15. Such an effect of UGT2B15 polymorphism on gender differences may be con-

sidered in the context of MDMA activity, *i.e.*, it is known that increasing doses of MDMA produce more hallucinogen like perceptual alterations, particularly in women. As in the case of oxazepam, this gender difference might be attributable to the involvement of UGT2B15 in the glucuronidation of HMMA.

In conclusion, we have demonstrated a large scale preparation of HMMA-Gluc by enzyme-assisted synthesis. Kinetic parameters indicated that using Aroclor 1254-induced rat liver microsomes was superior to using non-induced rat liver microsomes or human liver microsomes. In addition, we showed HMMA is glucuronidated to HMMA-Gluc by human UGT2B15. Further studies, utilizing enzyme-assisted synthesis of other illegal drug metabolites that are difficult to prepare by chemical synthesis, for example, psilocin, are now in progress.

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—Regular Articles—

植物系違法ドラッグ製品及び法規制植物試料の Direct Analysis in Real Time (DART)-TOFMS を用いた迅速スクリーニング法の検討

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Simple and Rapid Screening for Psychotropic Natural Products Using Direct Analysis in Real Time (DART)-TOFMS

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Direct Analysis in Real Time (DART) is a novel ionization technique that provides for the rapid ionization of small molecules under ambient conditions. To investigate the trend of non-controlled psychotropic plants of abuse in Japan, a rapid screening method, without sample preparation, was developed using DART-time of flight mass spectrometer (TOFMS) for plant products. The major psychotropic constituents of these products were determined using liquid chromatography-mass spectrometry (LC/MS). As a result of the DART-TOFMS analyses of 36 products, the protonated molecular ions $[M+H]^+$, corresponding to 6 kinds of major hallucinogenic constituents (mescaline, salvinorin A, *N,N*-dimethyltryptamine, harmine, harmaline and lysergamide), were detected in 21 products. It was possible to estimate their accurate elemental compositions through exact mass measurements. These results were consistent with those of the LC/MS analyses and the contents of the 6 psychotropic constituents were in the range from 0.05 to 45 μg/mg. Typical controlled narcotic drugs, tetrahydrocannabinol, opioid alkaloids and psilocin were also directly detected in marijuana cigarette, opium gum and magic mushroom respectively. Although it is difficult to estimate the matrix effects caused by other plant ingredients, the DART-TOFMS could be useful as a simple and rapid screening method for the targeted psychotropic natural products, because it provides the molecular information of the target compounds without time-consuming extraction and pre-treatment steps.

Key words——Direct Analysis in Real Time (DART); TOFMS; LC/MS; psychotropic plants

緒　　言

平成18年度の薬事法改正により，平成19年4月より指定薬物制度が施行され，違法ドラッグに対する規制が強化されてきている．しかし一方で，規制が厳しくなった化学合成化合物の代替品として，植物系違法ドラッグ製品の流通が深刻な問題となっている．植物系違法ドラッグ製品に関しては，製品が同一名称であっても異なった植物が流通していたり，特定の活性成分を含有する植物が多数存在するために同定が困難であったり，観賞用として流通している植物の近縁種であったりと，一律に規制することが困難である．このような製品の流通実態を調査するためには，まずは，個々の植物成分等についての研究を行うとともに，実際の製品を分析するための，効率のよいスクリーニング手法の検討が重要な課題である．

近年開発されたイオン化法 Direct Analysis in Real Time (DART™) の動作原理は，導入されたHeガスをニードル電極の放電によりプラズマ化し，励起状態の中性気体分子として大気ガス中に放出すると，測定対象物に直接作用しイオン化することによる．大気圧下で非接触に試料をイオン化でき，さらに質量検出器に time of flight mass spectrometer (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] また，DART は試料表面からイオン化が可能な他手法 fast atom bombardment (FAB) 法及び matrix-assisted laser desorption ionization (MALDI) 法と比較して，マトリックスが不要であり，イオン源と質量検出器間が開放されているため，液体，固体等の試料形態を問わず，間にかざすだけで試料表面がイオン化される利

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点がある．既にDARTを使用した薬品,[2] 香料,[3] 食品添加物,[4] 薬物,[5] 培養植物[6,7]等への分析適用例が報告されている．

植物系違法ドラッグ製品は，乾燥植物の葉，樹皮，種子，樹脂，粉末など多様な形態で流通している．これらの製品について，抽出操作等の前処理を省いて直接分析が可能であれば，成分スクリーニングの簡便化，迅速化が期待できる．本研究では様々な形態の植物系違法ドラッグ36製品を対象として，DART-TOFMSを用いて，代表的な植物由来幻覚成分である*N*,*N*-dimethyltryptamine (DMT), mescaline, harmine, harmaline, salvinorin A及びlysergamide (LSA) の検出を試みた (Fig. 1)．また，同製品について，過去に行ったLC/MS分析の結果[8]と比較し，植物系違法ドラッグ製品のスクリーニング分析におけるDART-TOFMSの有用性について論じた．さらに，法律で規制されている植物試料として，大麻，psilocin, psilocybin含有キノコ(いわゆるマジックマッシュルーム) 及びあへんに着目し，これら試料中の主活性成分分析に適用して，本法の有用性を検討した．

実 験 方 法

1. 試料 2004–2007年に市場で流通していた，向精神様活性を標榜する様々な形態の植物系違法ドラッグ36製品［乾燥植物（葉・花）細片11，粉末8，樹脂状5，種子4，樹皮3，タバコ状2，その他3試料］を測定試料とした．これらの36製品は，LC/MS分析により，植物由来活性成分及び故意に添加された合成化合物について，既報[8]により同定済みのものである．また，これら製品中に含まれる代表的な植物由来幻覚成分DMT， mescaline, harmine, harmaline, salvinorin A, LSAの含有量は，LC/MS分析による既報[8]のものを参照した．大麻取締法，麻薬及び向精神薬取締法及びあへん法等の法律で規制されている植物試料は，大麻2種，マジックマッシュルーム1種及びあへん2種を使用した．

2. 試薬 DMT, mescaline HCl, psilocin, psilocybinは過去に報告した方法[9]で合成したものを使用した．Salvinorin Aは徳島文理大学香川薬学部代田 修博士より，LSAは星薬科大学の細江智夫博士より供された．Harmine及びharmalineはAldrich社 (MO, USA) より，Δ9-tetrahydrocannabinol (THC) はCerilliant社 (TX, USA) より購入した．Morphine HCl, codeine H_3PO_4, thebaine, papaverine HCl及びnoscapine HClは塩野義製薬から購入又は供されたものを使用した．各化合物は過去にHPLC, TLCを用いて純度確認を行い，標品として使用しているものを用いた．それぞれの化合物は1 mg/mlのメタノール溶液を作成し，定性用の標準化合物溶液とした．その他の試薬はHPLC用又は試薬特級に準じたものを使用した．また，LC/MS測定時の抽出溶液の膜ろ過フィルターとしてUltraFree-MC (孔径 0.45 μm) Millipore社製

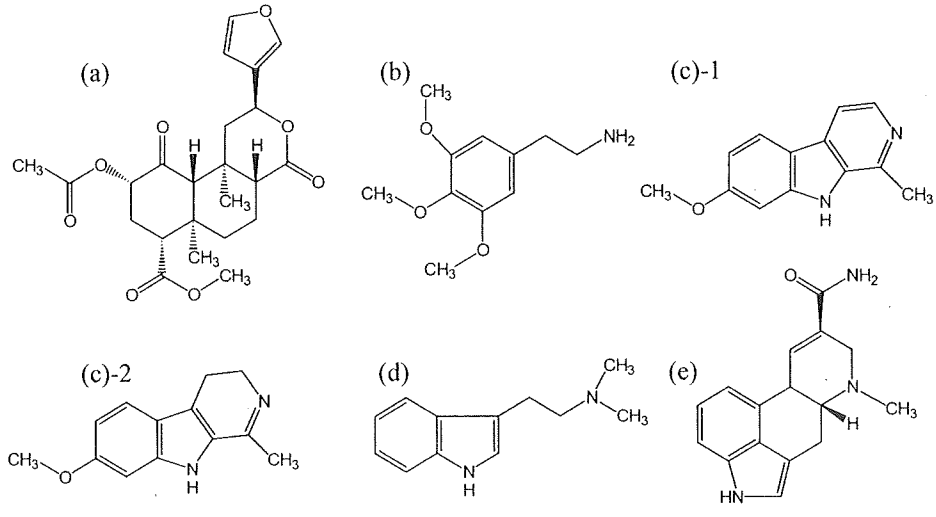

Fig. 1. Chemical Structures of Six Kinds of Major Hallucinogenic Plant Constituents Studied in This Study
(a): salvinorin A, (b): mescaline, (c)-1: harmine, (c)-2: harmaline, (d): *N*,*N*-dimethyltryptamine (DMT), (e): lysergamide (LSA).

(MA, USA) を使用した.

3. DART-TOFMS 測定条件 DART-TOFMS 測定装置として, イオン源 Direct Analysis in Real Time (DART) (日本電子社製) に質量分析計 AccuTOF JMS-T100 (日本電子社製) を連結したものを使用した. 測定条件は以下の通りである. 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 diphenhydramine ($C_{17}H_{21}NO$) 溶液を用いた.

DART 条件

Positive mode; gas flow: He, 2.0 l/min, gas temp.: 200–250℃, 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: 10–1000 (Da)

4. DART-TOFMS 測定方法 測定試料を DART イオン源と TOFMS の間にかざすことで測定した. 乾燥した植物試料 (葉, 樹皮, 根) 等は試料片をピンセットでつまみ, そのまま先端をイオン源にかざした. また粉末, 樹脂は薬包紙に包むか, 微量をガラス棒の先端に付着させ測定を行った. 種子は乳棒を用いて粗く砕き, 内容物をガラス棒の先端に微量付着させた. また, 溶液はガラス棒に液体を付着させたのちに先端をかざして測定した. 同一試料を 1 回の分析中で数回繰り返し測定し, 得られたスペクトルの精密質量値から化合物の組成推定を行った. 試料から分析対象化合物の $[M+H]^+$ に相当するイオンピークが検出された場合には, さらに定性用標準化合物メタノール溶液を使用して分析を行い, 両者の測定値を比較した.

5. LC/MS 測定方法 粉末, 樹脂状の製品は 10 mg, その他の乾燥植物等は乳鉢で粉砕し 20 mg を量り取り, メタノール 2 ml を加えて 10 分間超音波抽出を行った. 2000 rpm で 5 分間遠心後, 上清を膜ろ過して LC/MS 測定用試料とした. 測定機器に LC/MSD 1100 (Agilent, CA, USA), カラムに Atlantis dC18 (2.1×150 mm, 5 μm) (Waters, MA, USA) を使用し positive mode で測定を行った. その他の詳細条件は過去の報告に記載した通りである.[8]

結果及び考察

1. 植物系違法ドラッグ製品の分析 DART-TOFMS で試料を測定するに当たり, 2004–2007 年に市場で流通していた様々な形態の植物系違法ドラッグ製品 36 試料を測定試料とした. 乾燥した葉, 樹皮, サボテンの皮などからは, 試料の一部をイオン源にかざすと, そのままの状態で容易に成分ピークが検出できた. 種子は表面からでは検出できず, 殻を砕いて中身をガラス棒に微量付着させることで検出可能となった.

DART イオン源を用いた質量分析では, カラムによる分離を行わないので各成分はイオン化されると同時に検出され, スペクトル強度はイオン化の容易さに大きく左右される.[1] また, DART-TOFMS を用いた分析では前処理が不要で, 測定時間も 1 分程度と非常に迅速であった. さらに DART 測定に必要な試料は微量で, 葉 1 枚, 種子 1 粒から目的成分を検出できるため, 同一試料を LC/MS 等で測定可能であり, スクリーニングの信頼性を高めることができる. したがって, 1 次スクリーニングを簡便な DART-TOFMS で行ったのち, LC/MS 等を用いて確認及び定量を行う手法が有効である.

DART-TOFMS の positive mode で植物系違法ドラッグ製品の測定を行った結果, 測定した 36 製品のうち, 分析対象とした植物由来幻覚成分 6 化合物 (DMT, mescaline, harmine, harmaline, salvinorin A, LSA) に相当する $[M+H]^+$ イオンが主なピークとして 21 製品から検出された (Table 1). 本測定結果と過去に報告した LC/MS 分析による成分分析結果[8] を比較すると, 検出化合物は両測定方法で一致した. さらに, 表中にこれら化合物について LC/MS 定量分析の結果を参考値として記載した. DART-TOFMS を用いた定量分析は, TLC を装着した分析についての報告があるものの, その再現性, 定量性は LC/MS に比べて低い.[10] また, DART-TOFMS 分析の検出限界について LC/MS 分析と単純に比較することはできないが, 今回使用した植物試料の対象成分量の範囲ではすべて検出可能であった.

分析対象化合物を検出した代表的な製品について, Fig. 2 に DART-TOFMS スペクトル及び同製品メタノール抽出液の LC/MS スペクトル及びクロマト

Table 1. DART-TOFMS and LC/MS Analyses of the 36 Plant Products Advertized Psychotropic Effects

Compounds	Plant products (Indicated name)	Form	DART-TOFMS			LC/MS[b]	
			Detected[a] (samples)	Accurate mass measurements	Estimated elemental compositions	Detected (samples)	Amount (μg/mg)
Salvinorin A	Salvia	Dried leaves, Cigarette	9	433.1887/ 373.1666	$C_{23}H_{29}O_8$/ $C_{21}H_{25}O_6[M+H\text{-}60]^+$	9	3.0–23.0
Mescaline	San pedro *etc.*	Cactus, Powder	3	212.1268	$C_{11}H_{18}NO_3$	3	2.0–17.0
Harmine Harmaline	Harmala *etc.*	Bark, Seeds, Powder, Resin	5	213.1037 215.1276	$C_{13}H_{13}N_2O$ $C_{13}H_{15}N_2O$	5	0.8–35.0 0.1–45.0
DMT	Ayahuasca *etc.*	Bark	2	189.1397	$C_{12}H_{17}N_2$	2	11.0, 12.0
LSA	Woodrose *etc.*	Seeds	2	268.1448	$C_{16}H_{18}N_3O$	2	0.05, 2.0

a) Detected the ion corresponding to the protonated molecular ion of the targeted compound. *b*) Data are from ref. 8).

グラムを示した.

「アヤワスカ」を標榜する乾燥植物片2製品から, DMTの$[M+H]^+$に相当するイオンをメインピークとして検出し, 精密質量の測定値189.1397からDMTのプロトン付加体の組成式$C_{12}H_{17}N_2$(理論値189.1392)が推測された[Fig. 2(a)].

Salvinorin Aに関しては, DART-TOFMS測定において$[M+H]^+$に相当するイオンと主ピークとして$[M+H\text{-}60]^+$に相当するイオンが9製品から検出された. 香料が添加されているものや, 他の植物と混合している製品(タバコ状)等についても分析したが, いずれの試料からもm/z 433及び373の精密質量値が測定可能であり, プロトン付加体の組成式が推定可能であった. LC/MSによる定量分析では9製品中のsalvinorin Aの含有量は3–23 μg/mgであった. Figure 2(b)にsalvinorin Aを含有する代表的な製品(乾燥葉)から得られたDART-TOFMSスペクトル及びLC/MSの測定結果を示した.

LSAの$[M+H]^+$に相当するイオンが検出された2製品は外見の異なる種子でwoodroseとRivea corymbosaの表記があり, LC/MS測定によるLSA含有量はそれぞれ2 μg/mg, 0.05 μg/mgと大きく異なった. LSAは分解し易く, LC/MS測定においては, 迅速な抽出操作, 測定が求められるが, DART-TOFMSを用いた分析は前処理が不要であり, 含有量0.05 μg/mgの試料からも検出可能であった[Fig. 2(c)]. また, LSA含有植物にはiso LSAが存在することが報告されている.[11] LC/MS分析では分離可能であっても, DART-TOFMSでは異性体を区別できないため, 同一ピークとして検出されると考えられた.

Mescalineでは, DART-TOFMS測定において3製品から$[M+H]^+$に相当するピークが検出された. また, 精密質量の測定値212.1285から組成式$C_{11}H_{18}NO_3$(理論値212.1287)が推測された[Fig. 2(d)]. LC/MS分析においては乾燥したサボテン試料を粉砕する必要があるが, DART-TOFMSによる分析では試料片から容易に測定が可能であった.

Harmine, harmalineは含有が確認された5製品のすべてで同時に検出され, LC/MS測定における含有量に相関なくharmine由来の$[M+H]^+$ピークに相当するm/z 213の強度が, harmaline由来の$[M+H]^+$に相当するm/z 215より強く観察された[Fig. 2(e)]. Harmine, harmaline含有植物製品は, 種子や樹皮等の植物体及び樹脂や粉末状の製品が存在したが, 加工された製品より植物体において, 両化合物のピーク強度が高い傾向にあった.

以上の結果から, DART-TOFMSを用いた分析は, 今回分析対象とした植物由来幻覚成分を製品中から検出するのに優れた方法と考えられた. 特に, Salvinorin A含有植物(*Salvia divinorum*)は, Salvinorin Aとともに, 平成19年4月より指定薬物として規制されているが, 呈色反応やイムノアッセイなどの簡易スクリーニング手法は現在のところ報告されていない. また, Salvinorin Aは, 植物製品のメタノール抽出物を用いたGC/MSやHPLC等の分析において, configurationの異なる化合物や分解物が一部検出されることが報告されており,[12] 様々な形態の製品から前処理を行わず迅速に測定することが可能なDART-TOFMSは, 有用な分析手法であると考えられた. しかし, 高濃度に添加される

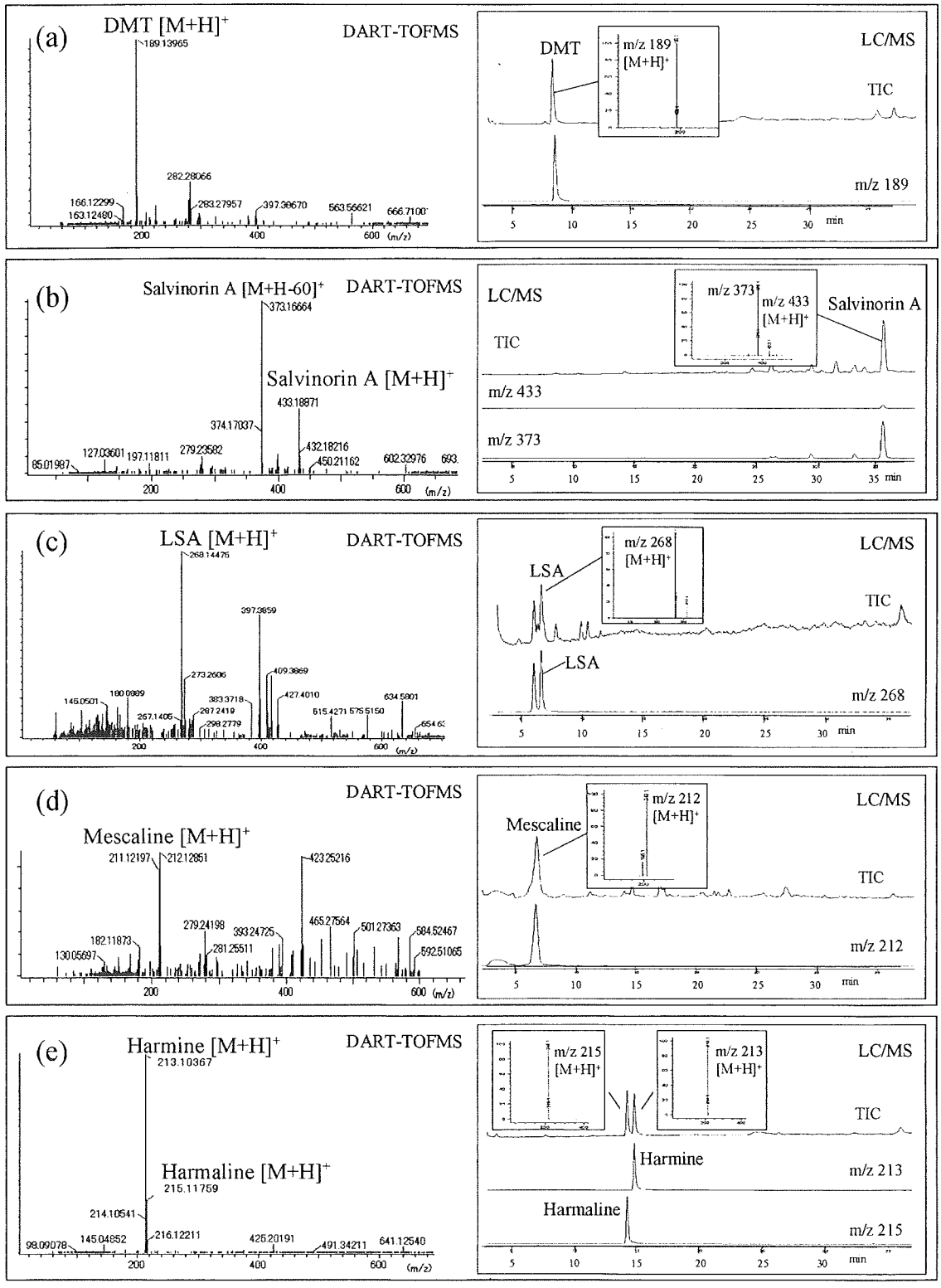

Fig. 2. DART-TOFMS Mass Spectra and LC/MS Ion Chromatograms of the Products

(a): "Ayawaska" (bark sample contained DMT), (b): "Salvia" (dried leaf sample contained salvinorin A), (c): "Rivea corymbosa" (seeds sample contained LSA), (d): "San pedro" (dried cactus sample contained mescaline), (e): "Harmala" (seeds sample contained harmine and harmaline).

可能性のある合成化合物や常在成分によるイオン化の妨害も否定できないため，強度の大きい他ピークが検出された場合には注意が必要である．また，構造類似体や異性体の多い合成違法ドラッグ成分においては，組成式が同一な化合物が多く DART-TOFMS のみでは判別できないことに留意しなけれ

ばならない.

2. 法律で規制されている植物試料の分析 法律で規制されている植物試料として，大麻（*Cannbis sativa* L.），psilocin，psilocybin 含有キノコ（いわゆるマジックマッシュルーム）及び，アヘン試料の DART-TOFMS による測定を行い Fig. 3 に結果を示した.

大麻試料では，乾燥葉及びタバコ状の試料から，代表的な大麻成分である THC, cannnabidiol (CBD) 及び cannabinol (CBN) の $[M+H]^+$ に相当するイオンを主なピークとして検出することが可能であった．また，精密質量の測定値(315.2330 及び 311.2018)から，THC/CBD 及び CBN のプロトン付加体の組成式 $C_{21}H_{31}O_2$ 及び $C_{21}H_{27}O_2$（理論値 315.2324 及び 311.2011）が推定可能であった [Fig. 3(a)].

乾燥キノコ試料は試料片を直接イオン源にかざしたところ，psilocin の $[M+H]^+$ に相当するイオンを主ピークとして検出することが可能であった．また，精密質量の測定値 205.1324 から psilocin のプロトン付加体の組成式 $C_{12}H_{17}N_2O$（理論値 205.1341）が推定可能であった [Fig. 3(b)]．しかし，主活性成分の 1 つである psilocybin に相当する質量のイオンピークは検出されなかった．Psilocybin は熱等により容易に脱リン酸化され psilocin に分解されるため，GC/MS 分析では psilocin として検出されることが報告されている.[13] DART-TOFMS による測定でも同様に，標準品の psilocybin 溶液を測定したところ，イオン化時の熱により psilocin として検出されることを確認しており，そのために今回乾燥キノコ試料から psilocybin が検出されなかったと考えられる.

あへん試料については樹脂状と粉末状の試料を，薬包紙に少量取りイオン源にかざして測定したところ，主要なあへんアルカロイドの $[M+H]^+$ に相当するイオンが検出された．Morphine ($C_{17}H_{19}NO_3$, 理論値 286.1443，測定値 286.1431)，codeine ($C_{18}H_{21}NO_3$, 理論値 300.1559，測定値 300.1579)，thebaine ($C_{19}H_{21}NO_3$, 理論値 312.1599，測定値 312.1595)，papaverine ($C_{20}H_{21}NO_4$, 理論値 340.1549，測定値 340.1535）及び noscapine ($C_{22}H_{23}NO_7$, 理論値 414.1553，測定値 414.1542）が，測定値よりプロトン付加体の組成式が推定可能であった 5 化合物であ

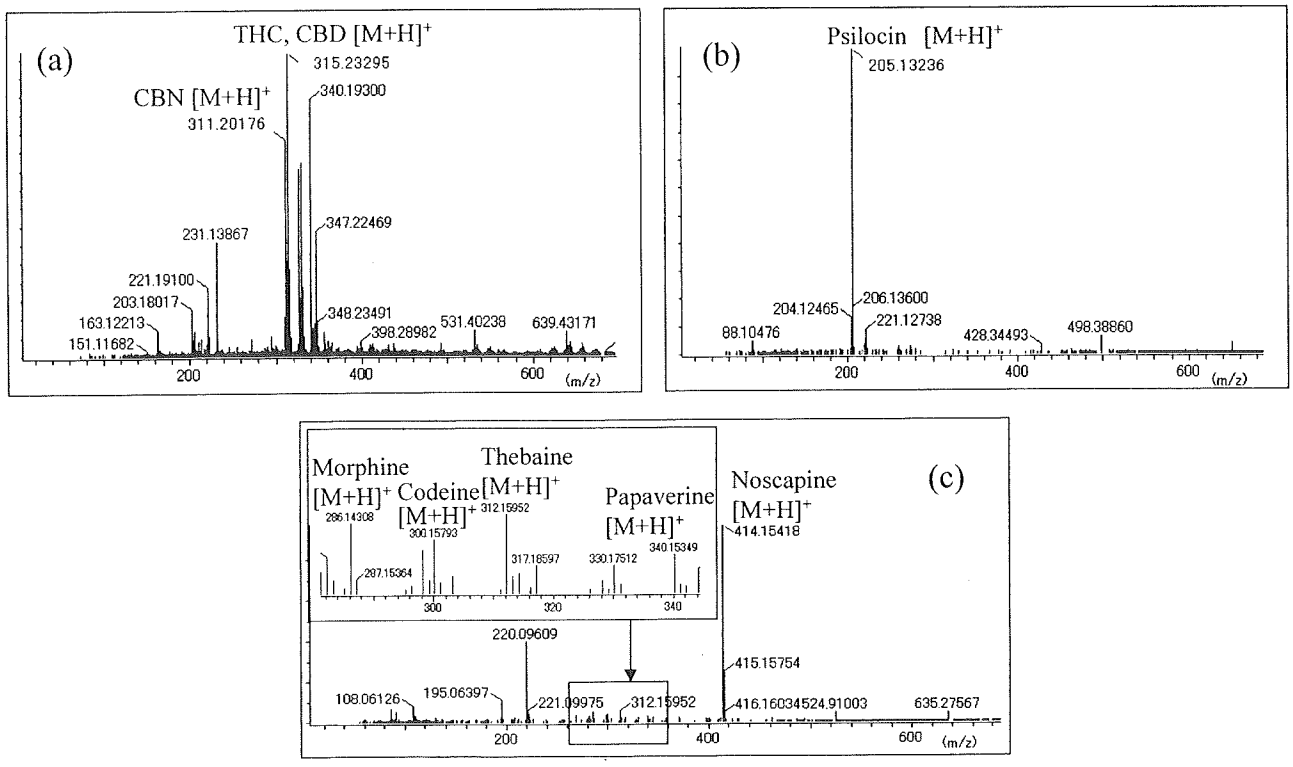

Fig. 3. DART-TOFMS Mass Spectra

(a): marijuana cigarette sample contained CBN, THC and CBD, (b): dried mushroom sample contained psilocin, (c): opium resin contained morphine, codeine, thebaine, papaverin and noscapin.

る［Fig. 3(c)］．樹脂試料からは noscapine が，粉末試料からは papaverine が主ピークとして検出されたが，アルカロイド中最も含量が高い morphine のイオン強度は低かった．そこで，同濃度に調整したあへんアルカロイド5化合物のメタノール溶液を，ガラス棒に付着させて DART-TOFMS で測定したところ，morphine のイオン強度が最も低く，他化合物が検出された 10 μg/ml において検出ができなかった．したがって，あへん試料を DART で確認する際は，morphine より noscapine 等の成分を指標とするのが望ましいと考えられた．

結　　論

DART-TOFMS を用いて，乾燥植物，樹脂，粉末等の形態を有する植物系違法ドラッグ製品及び法規制植物について，抽出操作等の前処理を行わずに測定を行った．その結果，今回測定を行った植物に含有される代表的な幻覚成分や活性成分に相当するピークが検出でき，さらに精密質量値より化合物の組成推定を行うことで，含有成分の推定が可能であった．LC/MS や GC/MS 等他の分析手法と比較すると，DART-TOFMS では前処理が不要で測定時間が短く簡便であり，キャリーオーバーが起き難い等の利点がある．したがって，一次スクリーニングを DART-TOFMS で行ったのち，LC/MS や GC/MS 等の分離分析手段を用いて成分の同定，定量を行うという一連の手法が植物違法ドラッグの分析法として有用であると考える．

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Note

Effects of *Psilocybe argentipes* on Marble-Burying Behavior in Mice

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***Psilocybe argentipes* is a hallucinogenic mushroom. The present study examined the effects of *P. argentipes* on marble-burying behavior, which is considered an animal model of obsessive-compulsive disorder. *P. argentipes* significantly inhibited marble-burying behavior without affecting locomotor activity as compared with the same dose of authentic psilocybin. These findings suggest that *P. argentipes* would be efficient in clinical obsessive-compulsive disorder therapy.**

Key words: *Psilocybe argentipes*; hallucinogenic mushrooms; psilocybin; marble-burying behavior; obsessive-compulsive disorder

Obsessive-compulsive disorder (OCD) is a mental illness that is widespread (2–3% of the population worldwide) and chronically disabling.[1)] This psychiatric condition is characterized by recurring obsessions and compulsions that significantly interrupt the daily functioning of the patient. Currently, selective serotonin (5-HT)-reuptake inhibitors (SSRIs) represent first-line pharmacotherapy for OCD. Nevertheless, the onset of action is slow, and 30–50% of patients do not respond at all to these agents.[2,3)]

Rodents use bedding material to bury noxious materials like glass marbles. No habituation to these novel objects occurs on serial testing or by prehousing with marbles.[4)] Moreover, SSRIs that have been used to treat human OCD inhibit marble-burying behavior without affecting locomotor activity. Hence marble-burying behavior has generally been considered as an animal model of OCD.[5)]

Francisco *et al.*[3)] recently found that psilocybin is associated with acute reductions in OCD symptoms in several subjects without significant adverse effects. Psilocybin is the main psychoactive substance in many hallucinogenic mushrooms. The structural similarities of psilocybin and psilocin to serotonin, a neurotransmitter also derived from tryptophane, is believed to be the cause of their action on the serotonin receptor in the brain and of hallucination (Fig. 1). Psilocybin is a potent 5-HT_{1A} and 5-$HT_{2A/2C}$ receptor agonist. The mechanisms of action of OCD are largely unclear, but are probably diverse for various subjects, since OCD presents a broad range of symptoms. Hallucinogenic mushrooms produce a variety of psychoactive agents aside from psilocybin, such as psilocin, baeocystin, norbaeocystin, bufotenin, and aeruginascin. Hence they show therapeutic action in a huge variety of subjects beyond the authentic materials. Hallucinogenic mushrooms have potential value as a novel form of pharmacotherapy, but no previous investigations have investigated the medicinal use of hallucinogenic mushrooms without psychotherapy. In addition, few animal model studies have examined the use of psilocybin in OCD, and the effects on the marble-burying behavior have yet to be determined.

The present study investigated the effects of hallucinogenic mushrooms on marble-burying behavior as compared with the effects of antipsychotic agents such as fluvoxamine. Mycelia of *Psilocybe argentipes*, one of the most popular hallucinogenic mushrooms in Japan, were used as the microbiological material. The amount of psilocybin in *P. argentipes* was determined by high-performance liquid chromatography (HPLC). We also examined the effects of authentic psilocybin on marble-burying behavior in comparison with *P. argentipes*.

Five-week-old male ICR mice (Charles River Laboratories Japan, Kanagawa, Japan) were used in each experiment. For $\geq$7 d before behavioral tests, all the animals were maintained in an air-conditioned room under controlled temperature (22 $\pm$ 2 °C) and relative humidity (55 $\pm$ 5%). The mice were housed in plastic cages with sawdust and kept under a light-dark cycle (lights on 07:00–19:00). They were allowed ad-libitum access to food (MF; Oriental Yeast, Tokyo) and water, except during the experiments. All procedures involving animals were conducted in accordance with the "Guidelines for Animal Experiments" of Takasaki University of Health and Welfare. Marble-burying behavior tests were conducted between 09:00 and 17:00. The mice were placed individually in plastic cages (22.5 × 38.5 × 18.5 cm) containing 25 glass marbles (diameter, 15 mm) evenly spaced on sawdust (depth, 5 cm) without food or water. At the same time, the locomotor activity of the mice was measured using an MDC system (MDC-W01,

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Abbreviations: OCD, obsessive compulsive disorder; 5-HT, 5-hydroxytryptamine; SSRIs, selective serotonin reuptake inhibitors

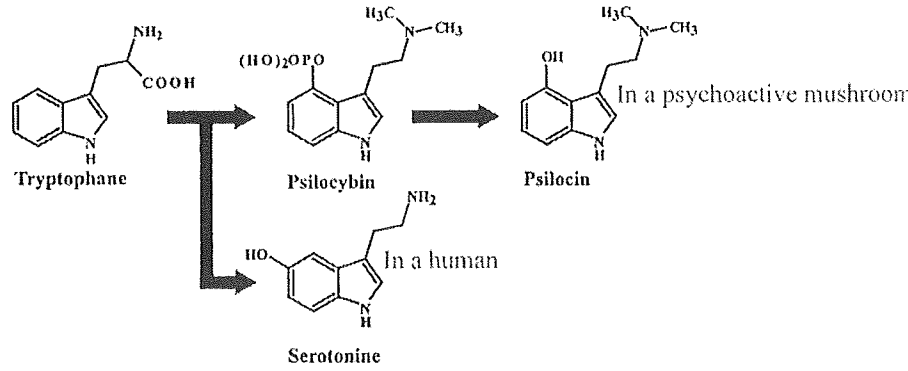

Fig. 1. Chemical Structures of Tryptophane Derivatives in Metabolic Biotransformation.

MDC-WR1; Brain Science Idea, Osaka, Japan) comprising a sensor monitor mounted above the cage to detect changes in heat across multiple zones of the cage. Body heat radiated by the animal was detected with the sensor head of the monitor. Thus, the system allowed monitoring and counting of all spontaneous movements. The results for marble-burying behavior were expressed as the number of marbles buried at least two-thirds deep within 30 min and average total counts of locomotor activity for 30 min.

Fluvoxamine maleate (Tocris Cookson, Ellisville, MO), psilocybin, and psilocin were dissolved in saline for the marble-burying behavior test. Authentic psilocybin and psilocin were prepared as reported previously (psilocybin, psilocin, and hallucinogenic mushrooms such as *P. argentipes* are controlled as narcotic drugs by law in Japan).[6] The fungus *P. argentipes* from our laboratory collection was cultivated in potato-dextrose liquid medium, consisting of 20% potato extract and 2% dextrose, at 28 ± 2 °C for 14 d. Cultivated mycelia were freeze-dried and powdered using an electric blender (WB-1; Osaka Chemical, Osaka, Japan) for 30 s. Powdered mycelia were dissolved in saline for the marble-burying behavior test. Fluvoxamine was administered intraperitoneally, while the other agents were administered orally at a volume of 0.1 ml/10 g body weight 30 min before the marble-burying behavior test.

Powdered mycelia from *P. argentipes* (0.5 g) were extracted with 50 ml of methanol for 24 h at room temperature. The crude extract was filtered through a 0.45-μm polyvinylidene fluoride (PVDF) filter (Whatman, Florham Park, NJ) before injection into the HPLC apparatus. The liquid chromatograph consisted of a HTEC-500 electrochemical detection system and EPC-500 chromatographic data system (Eicom, Kyoto, Japan). The chromatographic column used contained Partisil-10 octadecyl silane, 10 μm (250 mm × 4.6 mm inside diameter; GL Science, Tokyo). The mobile phase comprised 90% 0.1 mol/l aqueous citrate-phosphate buffer (pH 3.1) and 10% ethanol. The flow-rate was 1 ml/min, and the impressed voltage was 750 mV. All measurements were performed at room temperature.

Values were expressed as means ± standard error of the mean. Data was assessed by multivariate analysis of variance, followed by Dunnett's test. Values of $p < 0.05$ were regarded as statistically significant.

The present study investigated the effects of the hallucinogenic mushroom *P. argentipes* on marble-burying behavior, which is considered an animal model

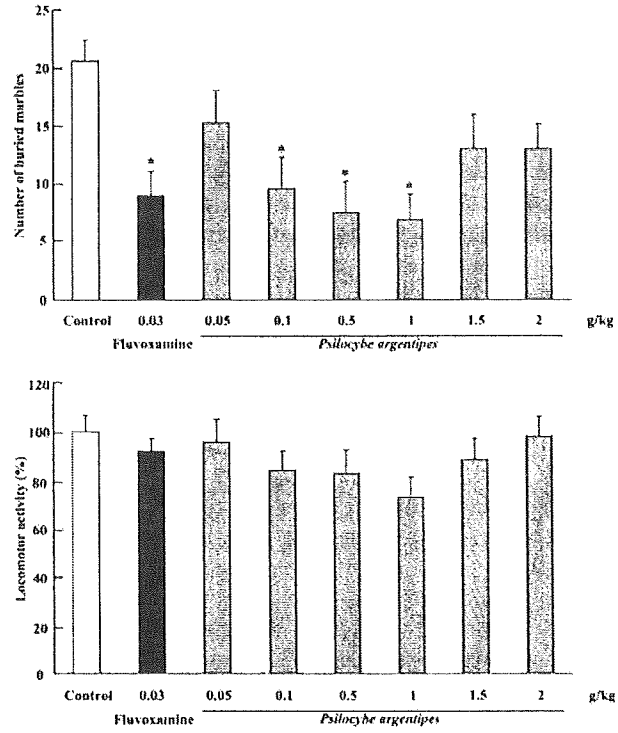

Fig. 2. Effects of *Psilocybe argentipes* on Marble-Burying Behavior and Locomotor Activity in Mice.
Values are expressed as mean ± S.E.M. for 10 mice. $^*p < 0.05$, compared to the control group.

of OCD. SSRIs used in clinical treatment inhibit marble-burying behavior without affecting locomotor activity.[7] *P. argentipes* at a dose of 0.05–2 g/kg showed a trend toward inhibited marble-burying behavior, while a dose of 0.1–1 g/kg significantly reduced the number of buried marbles (Fig. 2). Moreover, *P. argentipes* did not significantly affect locomotor activity at any dose (Fig. 2). The relationship between dose and number of buried marbles showed an inverted bell curve. This represents a very unusual result for the marble-burying behavior test, since many chemicals show a proportional relationship, high doses reducing both the number of buried marbles and locomotor activity.[7] Generally, *P. argentipes* shows a dose-proportional hallucinogenic effect.[8] The reduction in marble-burying behavior with *P. argentipes* was hence probably not associated with the hallucinogenic effect. In addition, hallucinogenic effects strongly involve agonistic effects on 5-HT_{2A} receptors.[9] The reduction in marble-burying behavior with *P. argentipes*

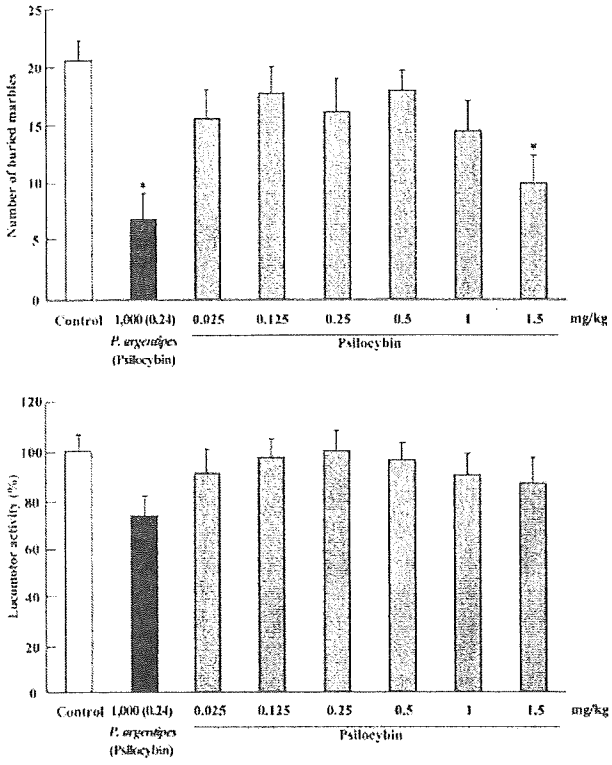

Fig. 3. Effects of Psilocybin on Marble-Burying Behavior and Locomotor Activity in Mice.

Values are expressed as mean ± S.E.M. for 10 mice. $^{*}p < 0.05$, compared to the control group.

thus appears unrelated to agonistic effects on 5-HT_{2A} receptors. The number of buried marbles was comparable between *P. argentipes* and fluvoxamine, which is used in anti-OCD pharmacotherapy. The dose of fluvoxamine was set based on the findings of Ichimaru *et al.*,[10] since fluvoxamine significantly inhibits marble-burying behavior without affecting locomotor activity. In addition, no negative effect was observed, for example, the weight gain or loss or loose stools. Some case reports have described reduced OCD symptoms with ingestion of hallucinogenic mushrooms.[11,12] In one case, hallucinogenic mushroom therapy was maintained as a treatment for OCD symptoms for 4 years.[11] The results show that hallucinogenic mushrooms such as *P. argentipes* can exhibit anti-OCD effects in clinical use.

Quantitative analysis by HPLC showed that mycelia from *P. argentipes* contained 0.024% psilocybin and 0.0008% psilocin. *P. argentipes* at a dose of 0.1–1 g/kg thus contained about 23.8–238 μg/kg psilocybin and 0.8–8 μg/kg, psilocin. This psilocybin content was comparable to other hallucinogenic mushrooms containing high concentrations of psilocybin.[13]

Psilocybin at a dose of 0.025–1.5 mg/kg showed a slight trend toward inhibiting marble-burying behavior, while a dose of 1.5 mg/kg resulted in significantly reduced marble-burying behavior (Fig. 3). In addition, psilocybin did not have any significant effect on locomotor activity during the marble-burying behavior test (Fig. 3). No results of the marble-burying behavior test with psilocybin have previously been described. However, Moreno *et al.* showed that psilocybin markedly decreased human OCD symptoms to varying degrees under controlled conditions.[3] The amount of psilocybin required to exert a significant effect was larger than with *P. argentipes*. These results suggest that the anti-OCD effects of hallucinogenic mushrooms such as *P. argentipes* are probably not the same as those of authentic psilocybin. Hallucinogenic mushrooms produce a wide variety of tryptamine derivatives other than psilocybin.[13] Some of these, such as psilocin, baeocystin, norbaeocystin, bufotenin, and aerginascin, have psychoactive effects, although many hallucinogenic mushrooms produce smaller amounts of them than psilocybin.[13,14] These findings suggest that inhibition of marble-burying behavior by *P. argentipes* is due to the involvement of a variety of psychoactive substances.

In conclusion, *P. argentipes* significantly reduced marble-burying behavior in mice, animal model of OCD, without adversely effecting locomotor activity. Moreover, it inhibited marble-burying behavior more effectively than authentic psilocybin, and at lower doses. These findings suggest that *P. argentipes* exhibits anti-OCD activity in clinical use. In addition, materials other than psilocybin are probably involved in reductions in marble-burying behavior following *P. argentipes* administration.

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

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ABSTRACT

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

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Chemicals and reagents

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

2.2. Instrumentation

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

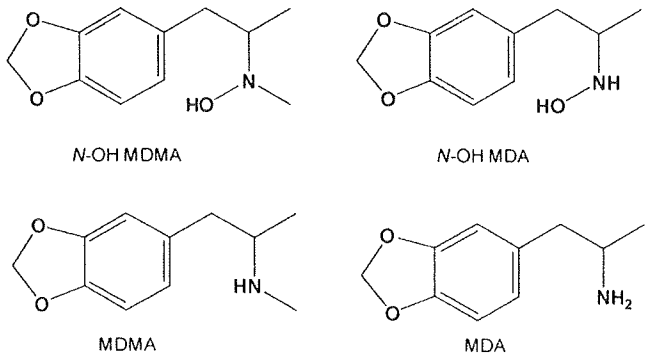

Fig. 1. Structures of *N*-OH MDMA and its metabolites.

2.3. Animal experiments

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

2.4. Sample preparation

2.4.1. Stock solution

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

2.4.2. Stability of N-OH MDMA

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

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

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

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

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

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

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

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

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

3. Results and discussion

3.1. Stability of N-OH MDMA

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

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

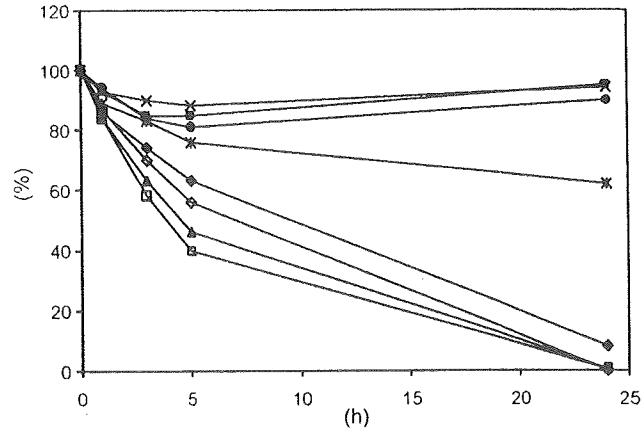

Fig. 2. Stabilities of *N*-OH MDMA oxalate in phosphate buffers at different pHs. ■, pH 3.0; ×, pH 4.0; ●, pH 5.0; *, pH 6.0; ◆, pH 7.0; ▲, pH 8.0; □, pH 9.0; ◇, pH 10.0.

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

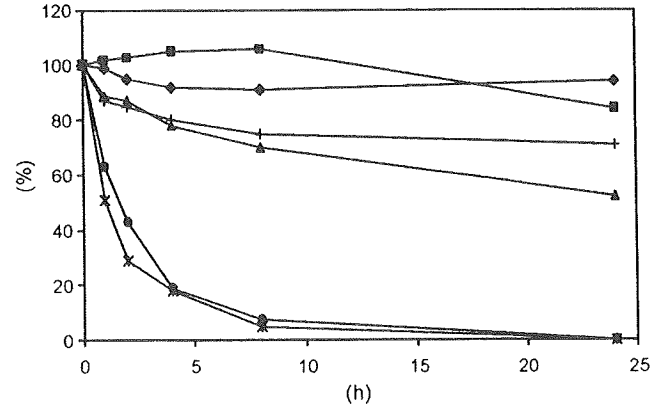

Fig. 3. Stabilities of *N*-OH MDMA oxalate in urine samples kept under different conditions. ●, kept at room temperature; ■, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept at room temperature; ▲, kept on ice; ◆, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept on ice; *, the control urine was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

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

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

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

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

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

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

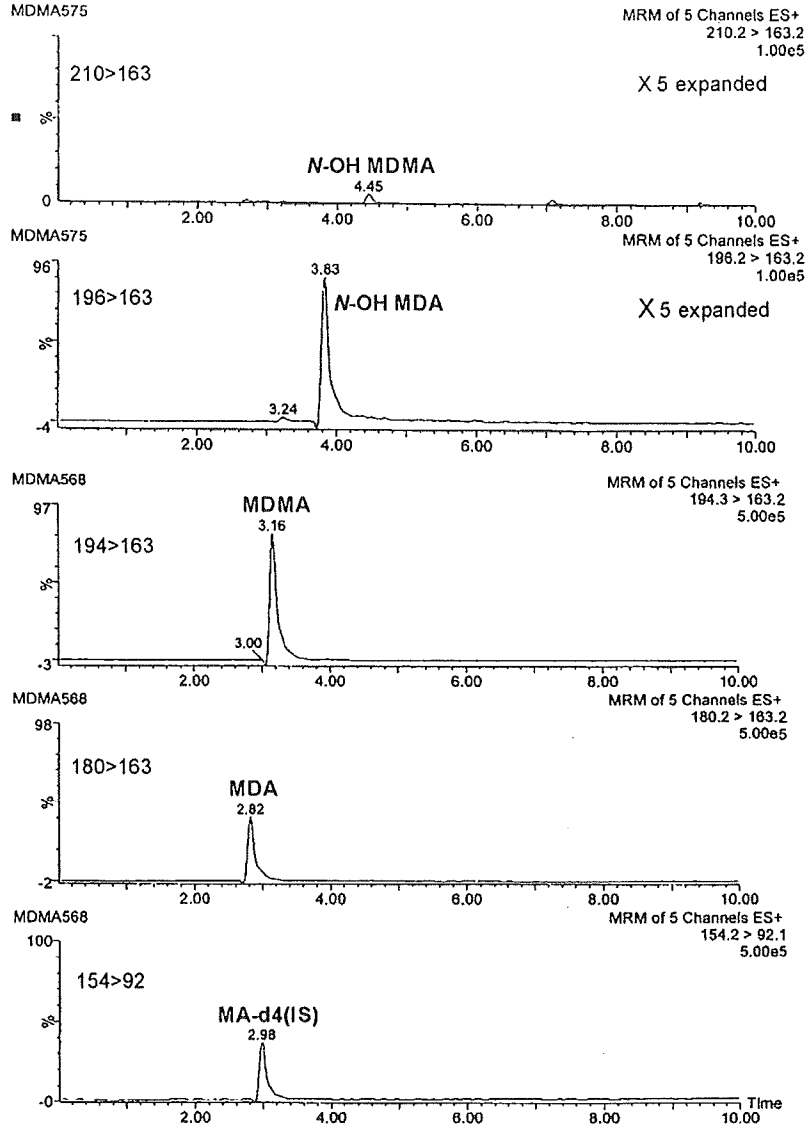

Fig. 4. UPLC–MS/MS MRM chromatograms of the extract from the rat urine 0–10 h after the last administration of *N*-OH MDMA (5 mg/kg, *i.p.*, rat 1).

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

3.2. Pre-treatment steps

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

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

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

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

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

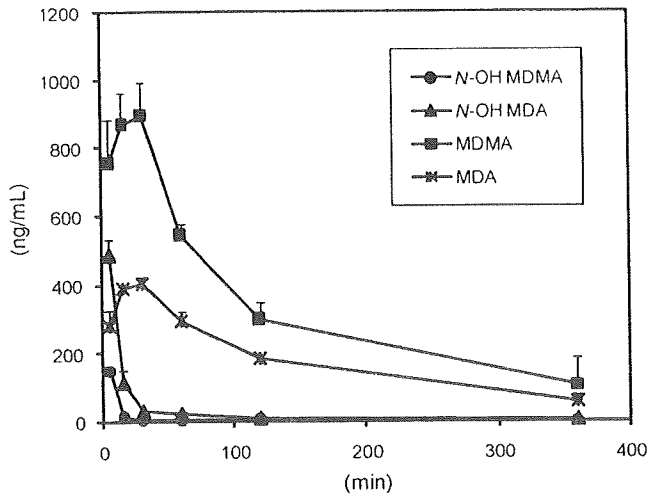

Fig. 5. Time courses of rat plasma drug concentrations after the first administration of *N*-OH MDMA (5 mg/kg, *i.p.*, $n = 3$). The error bar indicates each standard deviation.

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

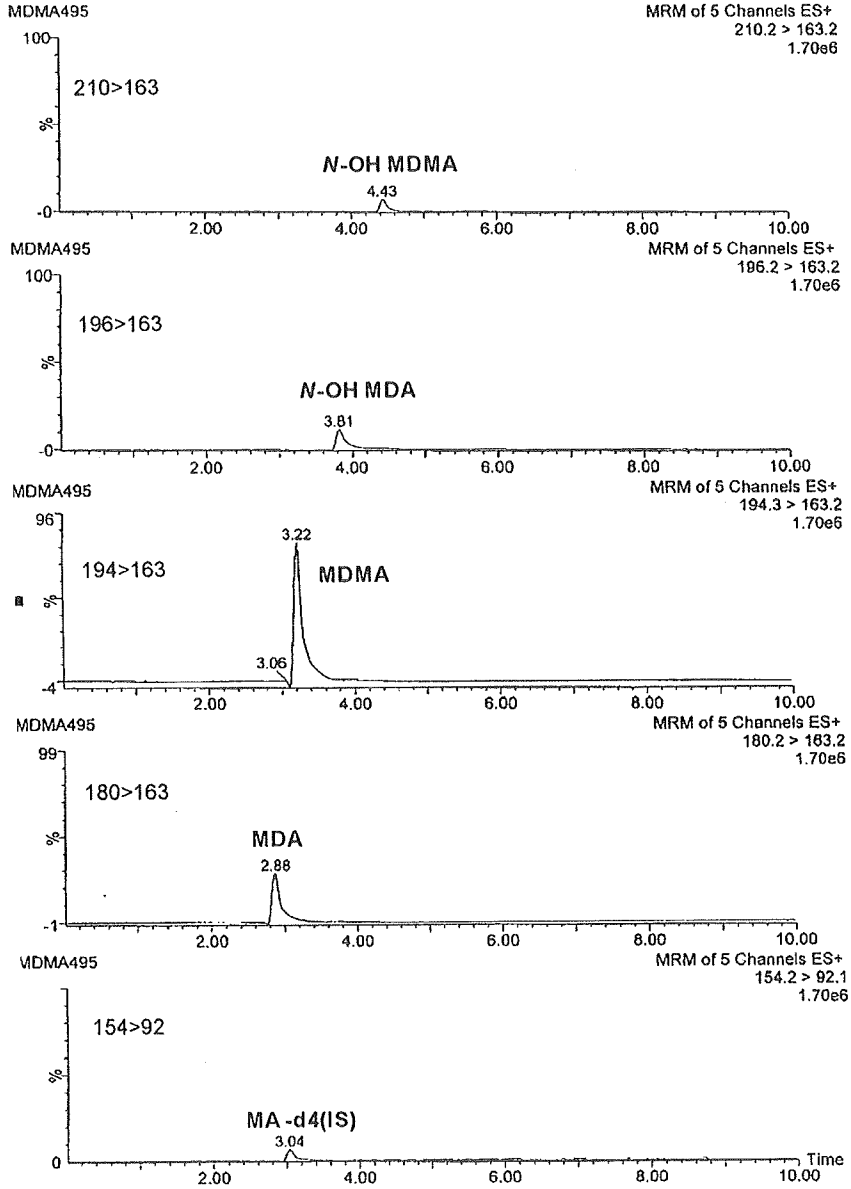

Fig. 6. UPLC–MS/MS MRM chromatograms of the extract from the rat plasma 5 min after the first administration of *N*-OH MDMA (5 mg/kg, *i.p.*, rat 1).

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

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

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

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

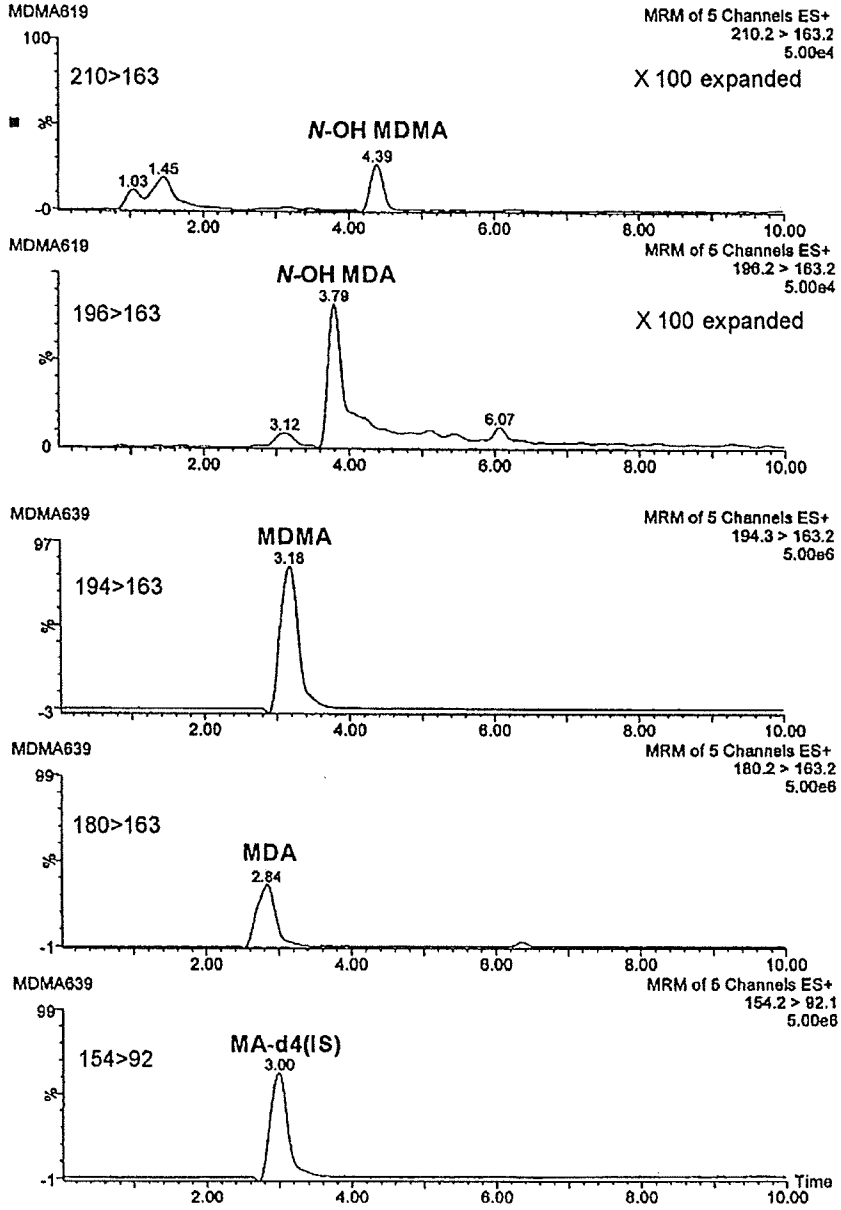

Fig. 7. UPLC–MS/MS MRM chromatograms of the extract from the rat hair collected 4 weeks after the first administration of *N*-OH MDMA (5 mg/kg × 10 days, *i.p.*, rat 1).

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

3.6. Drug concentrations in rat hair

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

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

4. Conclusions

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

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