ことでSal Aを短時間（分析時間10分）で分析できることを確認した．続いて，各種シソ科植物メタノールエキスを分析した結果，*Salvia divinorum* のみが陽性となる結果を得た．本法を用いることで簡便に幻覚性サルビアを鑑別できることを明らかにした．

さらに，抗-SalA MAb産生ハイブリドーマを材料として，より安価に調製可能で，高機能化が可能な組換え抗体についても作製を試み，組み換えタンパク質を得，精製することが出来た．得られたFabについてSalA-HSA（ヒト血清アルブミン）を固相化抗原としたELISAを行ったが，固相化抗原に対する反応性を確認することができなかった．通常，不溶性画分に発現したタンパクは不活性な状態で発現するため，巻き戻しが必要となる．本研究では，様々な巻き戻し法の中から効率的な再生が期待できる透析法を採用したが，この結果より，巻き戻しがうまくいかなかった可能性が高いものと考えられた．今後，特に，正しいジスルフィド結合形成を促すために，巻き戻しの際に添加する還元剤，酸化剤の選定，濃度の調整が，重要な検討課題と考えられる．

C.17. 抗カチノン抗体の取得と競合的 ELISA の開発

作製したCA-Suc-BSAコンジュゲートをマウスに 4 度免疫感作した段階で血中抗体化を測定した結果，十分な血中抗体価を得ることができた．続いて，常法により細胞融合を実施し，融合後のハイブリドーマの選抜を行うことで，最終的にCA-Suc-BSAを認識するMAbを産生するハイブリドーマ 1 種を樹立した．続いて，樹立したハイブリドーマを大量培養の後，培地中に分泌されたMAbの精製を行った．精製した抗CA MAbのアイソタイプはIgGタイプの抗体で，サブタイプは 2a，軽鎖はκ鎖であった．

抗CA MAbを活用した競合的ELISAを確立するために，まず最適一次抗体濃度の検討を行った．3種の固相化抗原をイムノプレートに固相化した後，ブロッキングを行い，続いて種々の濃度に調製した抗CA MAbをプレートに分注した．二次抗体反応，基質溶液の添加の後，得られた吸光度を測定したところ，一次抗体の濃度に依存して吸光度の上昇が認められ，十分な発色が得られた条件を，競合的 ELISA に用いる最適一次抗体濃度と決定した．次に，先に決定した最適濃度の一次抗体を用いて間接競合法によるCAの検出を試みた．その結果，CA を直接キャリアータンパクに結合した固相化抗原を用いた場合に，CA を検出可能な最も感度の高い競合的ELISAの開発に成功した．

本研究成果により，簡便高感度なチャット（*Catha edulis*）鑑別法の構築が可能と考えられる．作製した抗CA MAbは遊離のCAに対する反応性を示さないことから，今後，抗体が反応性を有する CA 誘導体を見出し，誘導体をターゲットとした間接競合法による ELISA を構築する予定である．

D. 結論

本研究は，厚生労働省の乱用薬物行政と乱用薬物取締りに直接貢献することを目的に遂行されている．麻薬指定される化合物は，本研究で事前に分析法が確立されることで，指定後の迅速な取締りが行われることになる．また，代謝物の合成と生体試料からの分析が遂行されることで，使用罪に対し始めて対応することが可能となる．さらに，今後対応が必要とされる植物系乱用薬物について，本研究で事前にその規制の範囲が検討され，分析，鑑定法が確立されることで，適切な規制を行うことが出来る．

また，大麻や麻薬含有植物では，栽培事犯が増加しているが，本研究の結果，簡便正確な鑑定法が確立することで，迅速な取締りが行われることになり，国民の危機リスクを低減させることになる．

特に，本研究では，デキストロメトルファンとその光学異性体レボメトルファン（麻薬）の代謝経路が示されたことにより，レボメトルファン使用事犯への対応がより容易になったものと考える．また，これらの化合物の内因性代謝物の尿内変動をNMRで直接観測し，多変量解析することで，両者が識別可能であるばかりでなく，その依存度についても推定できる可能性が示唆された．

また各種押収品に関する調査・分析結果は，捜査，取締現場での，要求に直接対応するものである

植物系の研究では，大麻種子1粒により，その大麻の産地を予測できる方法が示され，今後の大麻のプロファイリング分析に有効な手段となるものと考えられる．さらに，本研究で行った不正大麻に関する実態調査は，日本初のものであり，日本でも，THC高含有大麻が流通していることを示した．

ケシについても，近縁な植物種と考えられ，形態による識別が難しい未規制ケシ属植物，*Papaver pseudo-orientale* (PPO)について，一般栽培が可能なオニゲシ（PO），法規制植物であるハカマオニゲシ(PB)と，PCRにより容易に区別できることが示された．また，同様ケシ，アツミゲシ，ヒナゲシ間においても識別が可能なプライマーが設計され，形態や含有成分からの識別に依存していたケシ属植物の識別が，遺伝子情報を用いることにより，より客観的かつ迅速，簡便に可能となることが明らかになった．従って，今後，PPOについて，現実的に本種を規制していく可能性を考えることが出来るようになったものと言える．また，開発された分析法は，種子1粒-数粒で鑑別を可能とする手法であり，実際に発芽させ植物の形態を確認することが困難なケシ属植物の，植物種鑑別に威力を発揮するものと期待される．さらに，FTAカードで採集した核酸試料を検体とした規制植物種鑑別法について成功したが，本手法は，フィールドでの試料採取から保管まで，冷蔵施設等を必要とせず，実用性が非常に高いシステムと考えられる．さらに，*Salvia divinorum*やカートといった乱用の可能性のある植物体の迅速確認法が確立されつつある等，本研究は，今後の乱用薬物取り締まりに直接的に貢献できるものと言える．

E. 健康危機情報

特になし

F. 研究発表等

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新聞報道

読売新聞朝刊 平成24年12月31日「危険性を増す大麻」

研究成果の刊行に関する一覧表

原著論文

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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

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

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

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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

* Corresponding author. Tel.: +81 3 3700 8764; fax: +81 3 3707 6950.
E-mail address: kikura@nihs.go.jp (R. Kikura-Hanajiri).

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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).

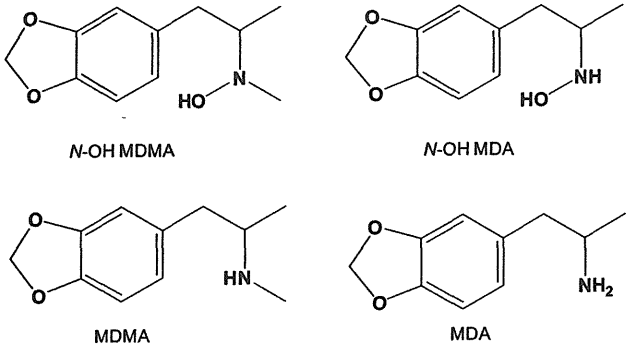

Fig. 1. Structures of *N*-OH MDMA and its metabolites.

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.

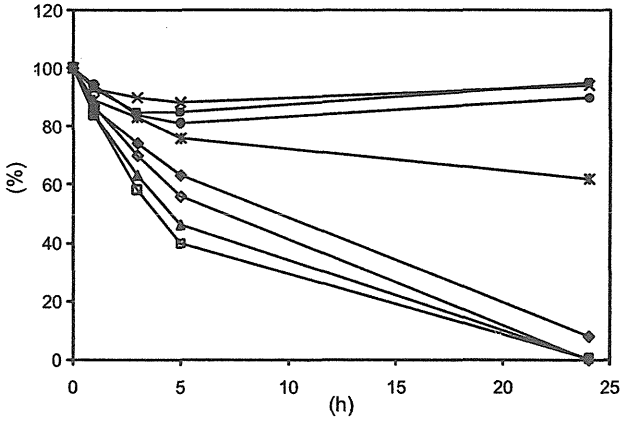

Fig. 2. Stabilities of *N*-OH MDMA oxalate in phosphate buffers at different pHs. ■, pH 3.0; ×, pH 4.0; ●, pH 5.0; *, pH 6.0; ◆, pH 7.0; ▲, pH 8.0; □, pH 9.0; ◇, pH 10.0.

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

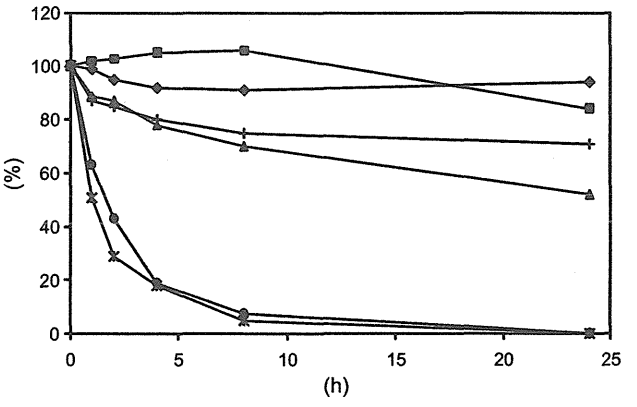

Fig. 3. Stabilities of *N*-OH MDMA oxalate in urine samples kept under different conditions. ●, kept at room temperature; ■, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept at room temperature; ▲, kept on ice; ◆, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept on ice; *, the control urine was 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.

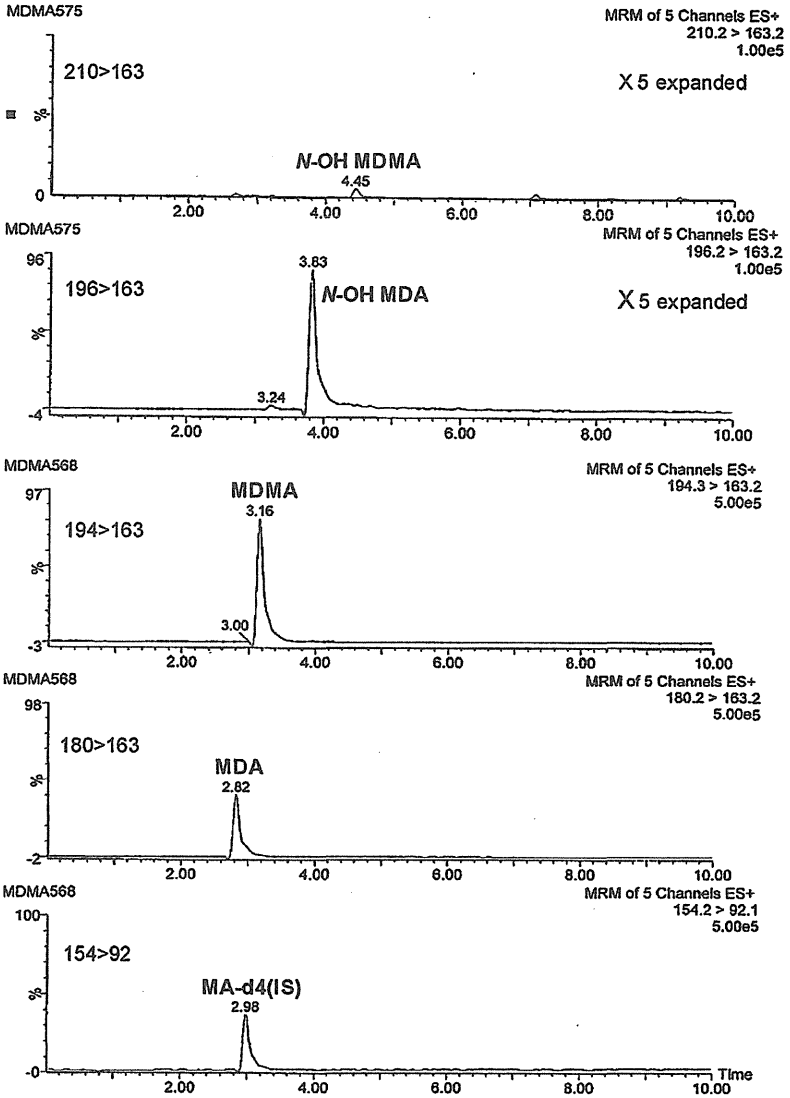

Fig. 4. UPLC–MS/MS MRM chromatograms of the extract from the rat urine 0–10 h after the last administration of *N*-OH MDMA (5 mg/kg, *i.p.*, rat 1).

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^{TM}) 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.

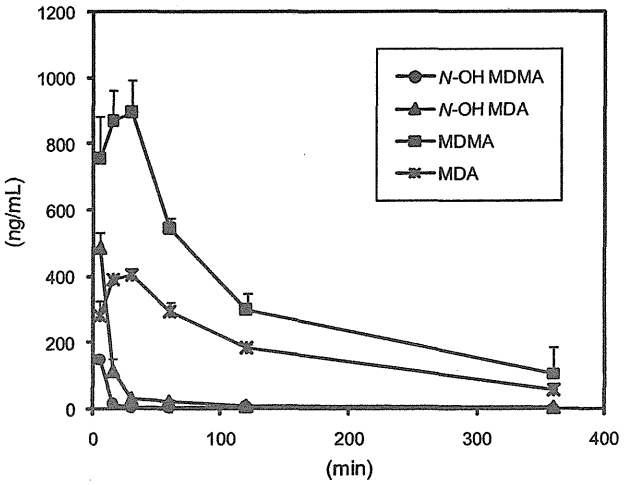

Fig. 5. Time courses of rat plasma drug concentrations after the first administration of *N*-OH MDMA (5 mg/kg, *i.p.*, $n = 3$). The error bar indicates each standard deviation.

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.

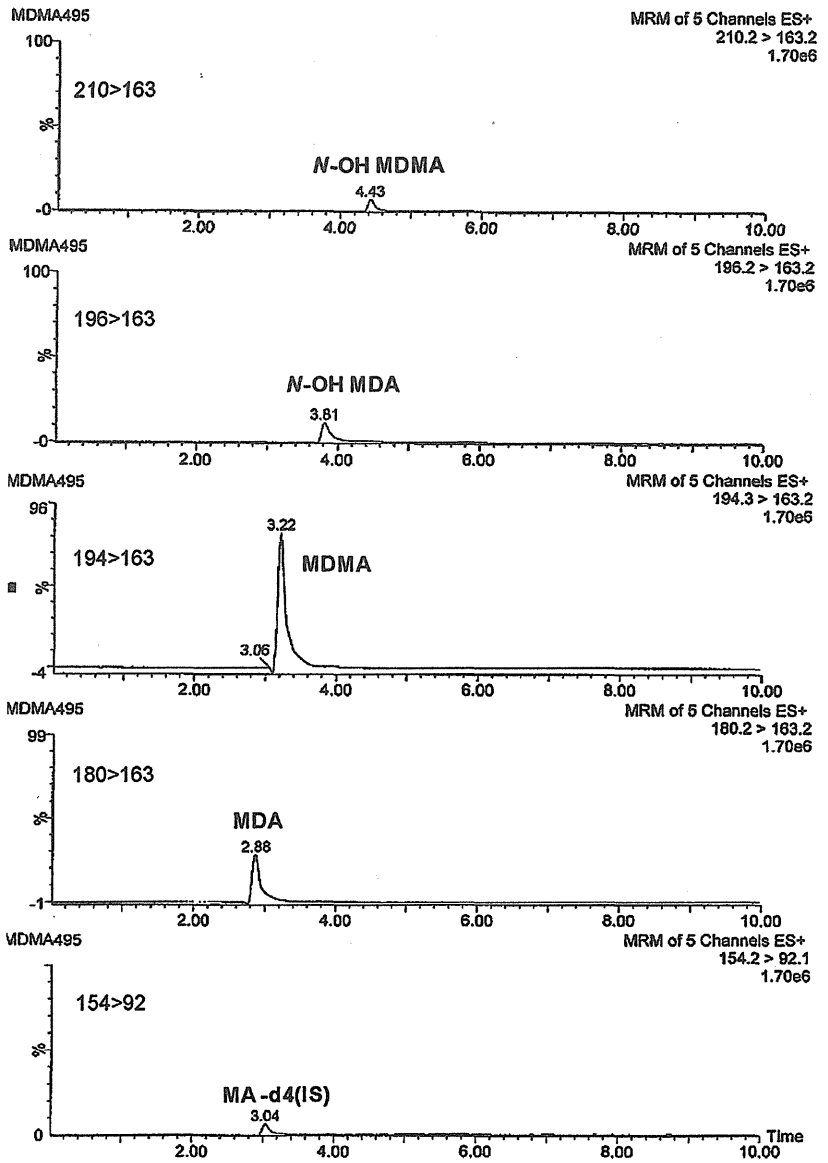

Fig. 6. UPLC–MS/MS MRM chromatograms of the extract from the rat plasma 5 min after the first administration of *N*-OH MDMA (5 mg/kg, *i.p.*, rat 1).

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

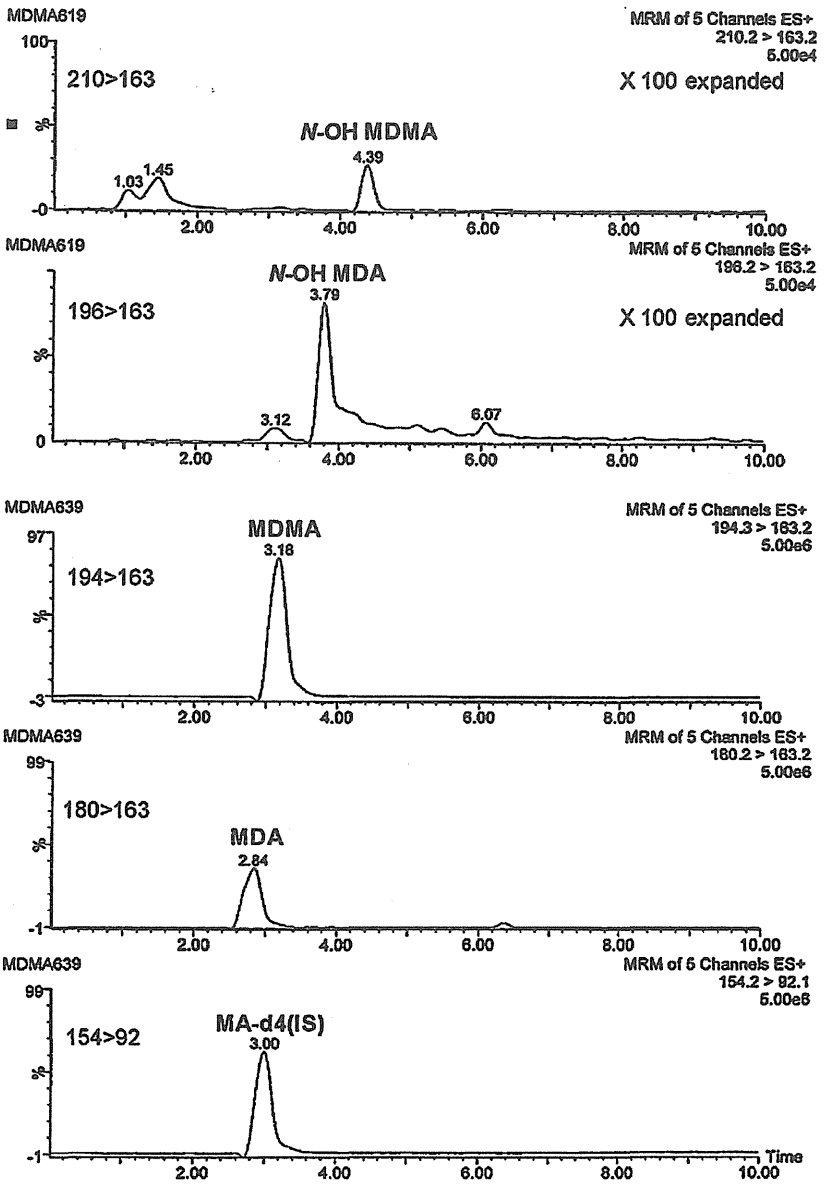

Fig. 7. UPLC–MS/MS MRM chromatograms of the extract from the rat hair collected 4 weeks after the first administration of *N*-OH MDMA (5 mg/kg × 10 days, *i.p.*, rat 1).

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

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

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

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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

* Corresponding author. Tel.: +81 3 3700 8764; fax: +81 3 3707 6950.
E-mail address: kikura@nihs.go.jp (R. Kikura-Hanajiri).

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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.

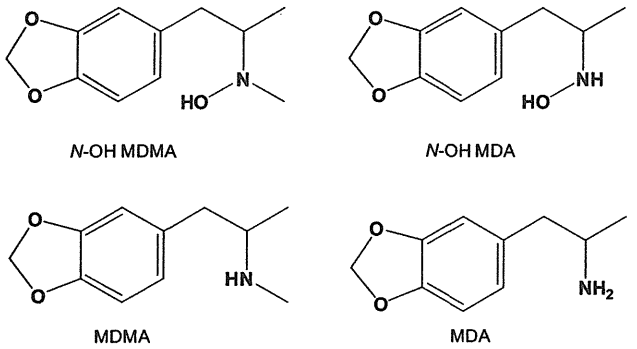
2. Materials and methods

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).



N-OH MDMA *N*-OH MDA MDMA MDA

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.

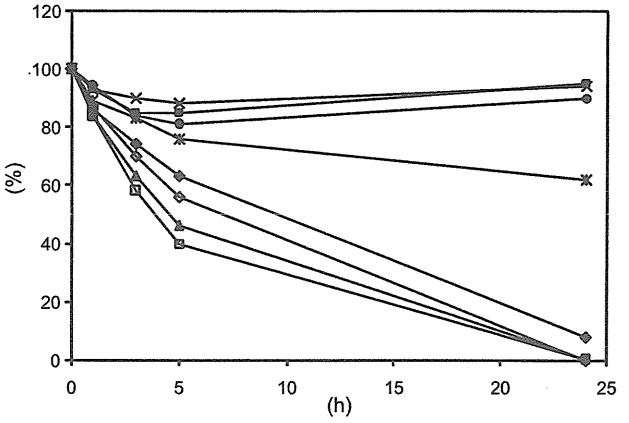


Fig. 2. Stabilities of *N*-OH MDMA oxalate in phosphate buffers at different pHs. ■, pH 3.0; ×, pH 4.0; ●, pH 5.0; *, pH 6.0; ◆, pH 7.0; ▲, pH 8.0; □, pH 9.0; ◇, pH 10.0.

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

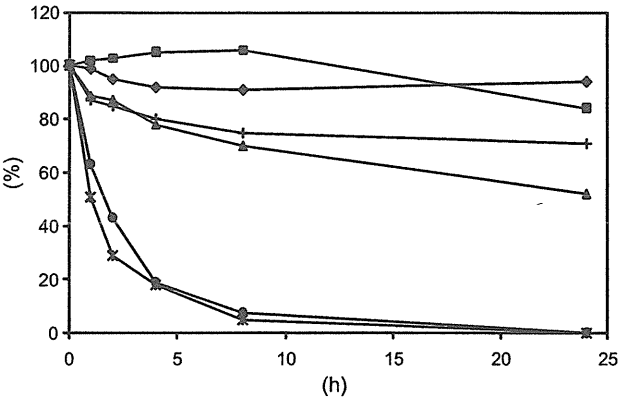


Fig. 3. Stabilities of *N*-OH MDMA oxalate in urine samples kept under different conditions. ●, kept at room temperature; ■, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept at room temperature; ▲, kept on ice; ◆, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept on ice; *, the control urine was 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.

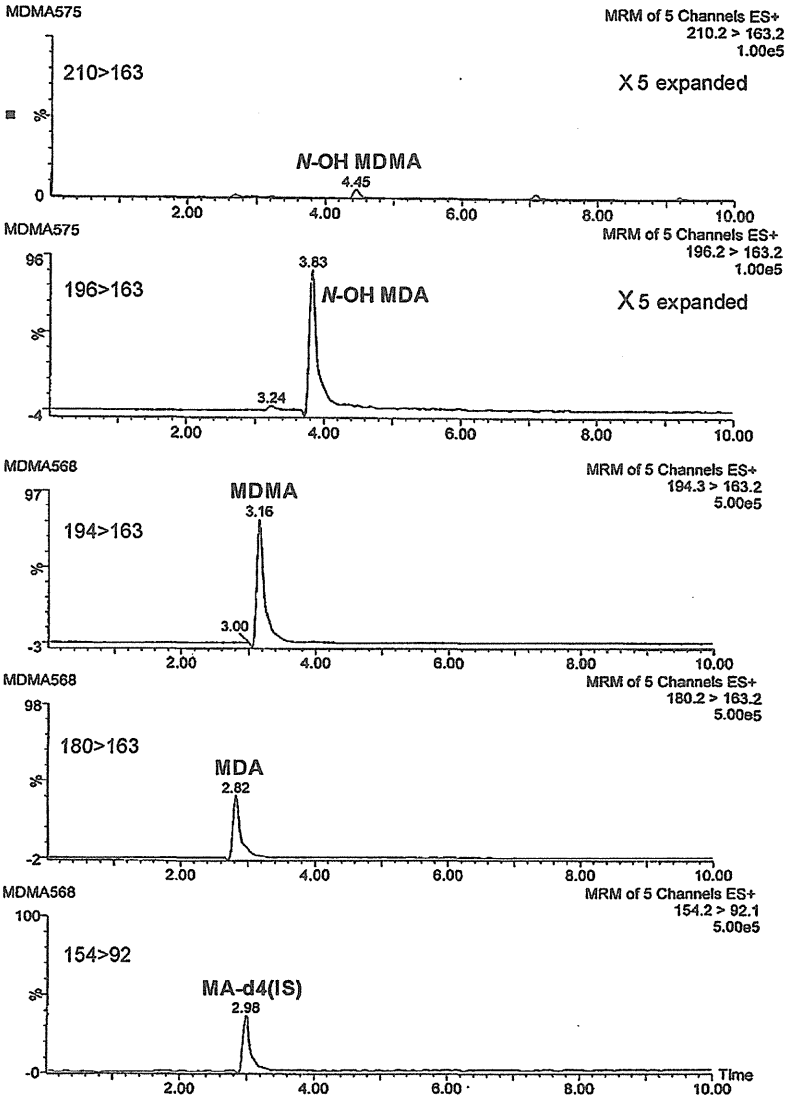


Fig. 4. UPLC–MS/MS MRM chromatograms of the extract from the rat urine 0–10 h after the last administration of *N*-OH MDMA (5 mg/kg, *i.p.*, rat 1).

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^{TM}) 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.

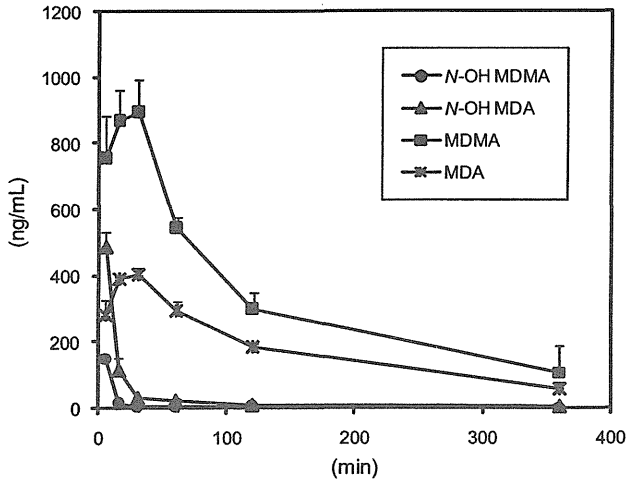


Fig. 5. Time courses of rat plasma drug concentrations after the first administration of *N*-OH MDMA (5 mg/kg, *i.p.*, $n = 3$). The error bar indicates each standard deviation.

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.

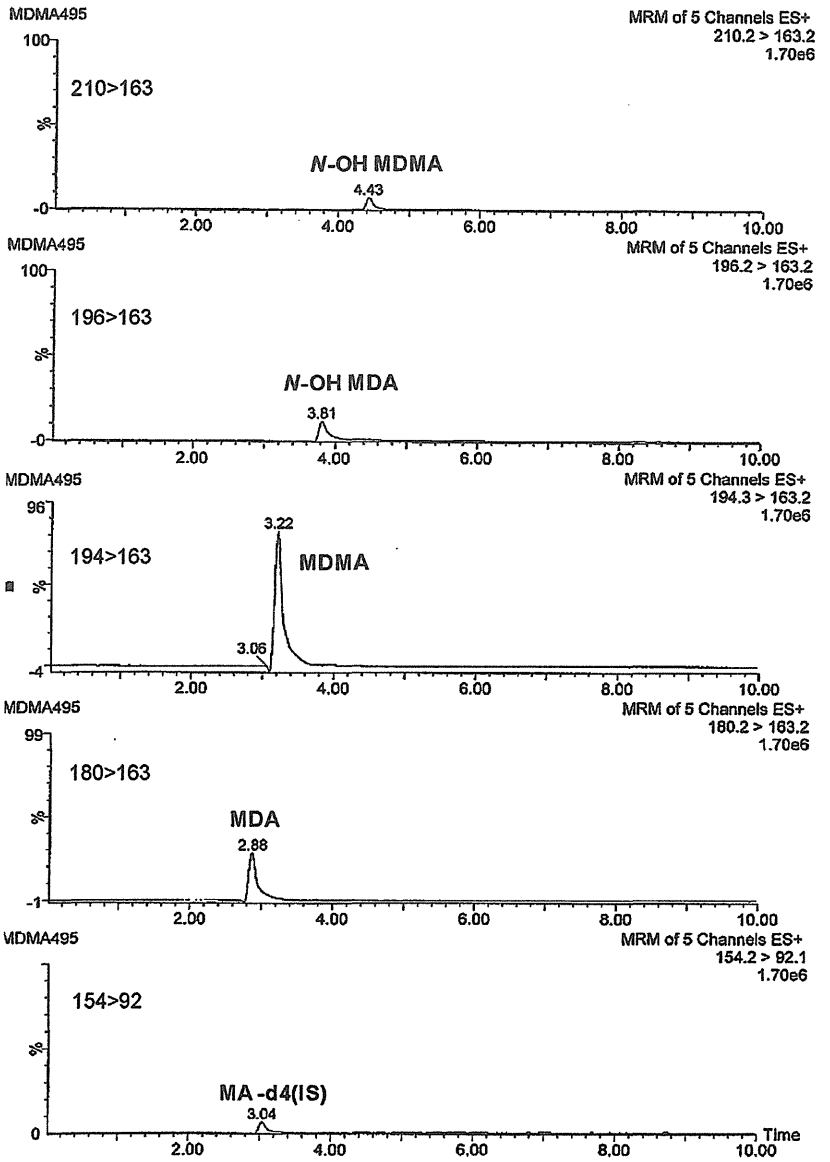


Fig. 6. UPLC–MS/MS MRM chromatograms of the extract from the rat plasma 5 min after the first administration of *N*-OH MDMA (5 mg/kg, *i.p.*, rat 1).

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 ± 17.3
	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

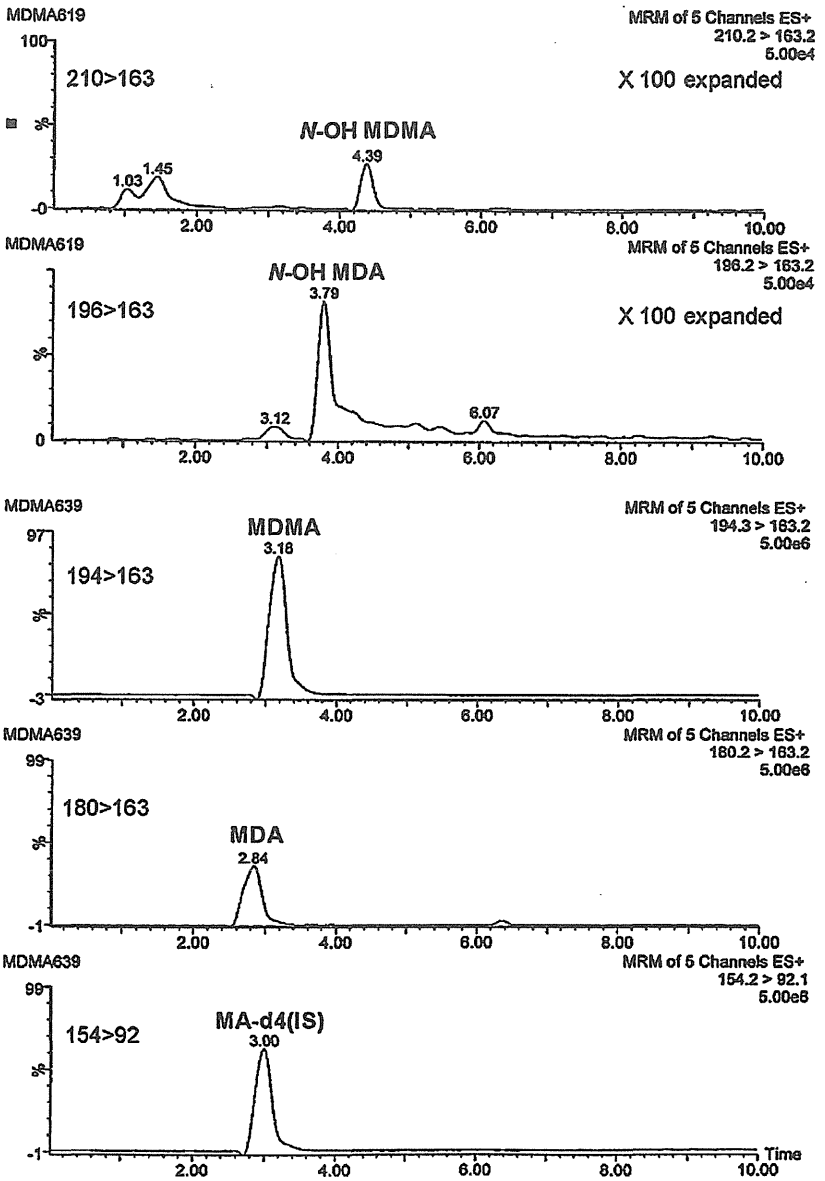


Fig. 7. UPLC–MS/MS MRM chromatograms of the extract from the rat hair collected 4 weeks after the first administration of *N*-OH MDMA (5 mg/kg × 10 days, *i.p.*, rat 1).