研究成果の刊行に関する一覧表

原著論文

発表者氏名	論文タイトル名	発表誌名	巻、号	ページ	出版年
Kikura-Hanajiri, R. 他	The disposition into hair of a new designer drug; methylone, MBDB, methcathinone	Journal of Chromatography B	855	121-126	2007
Matsumoto, T. 他	Evaluation of characteristic deuterium distributions of ephedrines and methamphetamines by NMR spectroscopy for drug profiling	Analytical Chemistry	80 (4)	1176-1181	2008
Ogata J. 他	Detection method for the ability of hemp (Cannabis sativa L.) seed germination by the use of 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC)	Yakugaku Zasshi	128 (11)	1707-1711	2008
Shoda, T. 他	4-Hydroxy-3-methoxymethamphetamine glucuronide as a Phase II metabolite of MDMA: Enzyme-assisted synthesis and involvement of human hepatic UGT2B15 in the glucuronidation	Chem Pharm. Bull.	57 (5)	472-475	2009
Kawamura, M. 他	Simple and rapid screening for psychotropic natural products using Direct Analysis in Real Time (DART)-TOFMS	Yakugaku Zasshi	129 (6)	719-725	2009
Matsushima, Y. 他	Effects of Psilocybe argentipes on marble-burying behavior in mice	Biosci. Biotech. Biochem.	73 (8)	1866-1868	2009
Kikura-Hanajiri, R. 他	Determination of a new designer drug, N-hydroxy-3,4-methylenedioxymethamphetamine and its metabolites in rats using ultra-performance liquid chromatography–tandem mass spectrometry	Forensic Science International	on–line available		2010

総説

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花尻瑠理	毛髪を中心とした代替生体試料中薬物分析	ぶんせき	(2)	76-81	2009
Kikura-Hanajiri, R.	Simple and rapid screening method using Direct Analysis in Real Time (DART)-MS	FFI Journal	215 (2)	137-143	2010
Shirota, O.	Charged Aerosol Detection (CAD): A new universal approach for HPLC	FFI Journal	215 (2)	144-153	2010

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The disposition into hair of new designer drugs; methylone, MBDB and methcathinone☆

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Abstract

The disposition into hair of methylone and other new designer drugs, methcathinone and MBDB, was studied with the animal model. Moreover, the incorporation rates of these drugs were compared with those of their related eight compounds previously studied in order to evaluate their incorporation tendency into hair and the usefulness of hair specimens for the retrospective confirmation of the use of these drugs. When the ratio of hair concentration to AUC in plasma ([Hair]/AUC) was represented as an index of the incorporation rate of drugs into hair, the [Hair]/AUC of methylone was 14 times higher than that of methcathinone. It might support earlier findings that the methylenedioxy group on the benzene ring leads to considerably higher incorporation rates. However, [Hair]/AUC of methylone was five-sevenths times lower in comparison with that of MDMA. This suggested that the beta-carbonyl group leads to lower incorporation rates. Although methylone has both groups in its structure, the positive effect of the methylenedioxy group may be stronger than the negative effect of the beta-carbonyl group. On the other hand, the [Hair]/AUC of MBDB, which has methylenedioxyphenyl-2-butanamine structure, was higher than that of MDMA while that of methcathinone, having beta-ketone in its structure, was extremely low. In conclusion, as with MA and MDMA, the incorporation tendency of methylone and MBDB (except for methcathinone) into hair is relatively high, and a hair sample would be a good specimen for the confirmation of retrospective use of these drugs.

Keywords: Methylone; Hair analysis; Drug disposition into hair; GC–MS

1. Introduction

It is difficult to evaluate the drug incorporation tendency into hair only by hair concentrations because they vary according to the total doses administered and the bioavailability of the drugs. Even if a defined dose is used in the experimental design, it may not be enough because there are large individual differences in both metabolism and bioavailability. One reasonable method for controlling these problems is to consider the total amount of drugs in whole blood or plasma. We have proposed that the drug incorporation rates into hair could be measured as the comparison between the total amount of drugs in the blood and those in the hair [1,2]. The total amount of drugs in the blood can be represented by area under the plasma concentration curve (AUC). On the other hand, in practice, it is difficult to estimate the total amount of drugs in the hair. Therefore, this measure may theoretically be replaced by drug concentrations in the hair. These concentrations would be determined by comparisons with equal lengths of hair that have grown for a definite duration. Based on the above reason, we have used the ratio of drug concentration in hair to plasma AUC ([Hair]/AUC) as one of the indexes of the incorporation rate of a drug from blood into hair in the animal experimental model [1,2].

In a previous report [2], we have studied the structural factors of drugs on the incorporation rate into hair from blood using 32 amphetamine analogs. As a result, [Hair]/AUC of 3,4-methylenedioxyamphetamine (MDA) which has a 3,4-methylenedioxy group to the benzene ring of amphetamine (AP) was 5.5 times higher than that of AP; 3,4-methylenedioxymethamphetamine (MDMA) corresponding to

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methamphetamine (MA) analog was 5.9 times higher than that of MA, and 3,4-methylenedioxyethylamphetamine (MDEA) corresponding to *N*-ethylamphetamine (EAP) analog was 6.1 times higher than that of EAP. Moreover, the [Hair]/AUC of 3-methoxy-4,5-methylenedioxyamphetamine (MMDA) was 2.3 times higher than that of MDA [3]. These results suggested that a methylenedioxy or a methoxy group on the benzene ring leads to considerably higher incorporation rates. On the other hand, the result of the [Hair]/AUC measurement of cathinone containing a carbonyl group at the benzyl position of AP gave unusually low values compared with that of AP. It is clear that the beta-ketoamphetamines (e.g., cathinone) would show very low incorporation rates.

Methylone (2-amino-1-(3,4- methylenedioxyphenyl)propan-1-one) is a new ecstasy-type designer drug that recently appeared in the Japanese drug market as well as in Europe (Fig. 1). Although only a little is known about the harmfulness of this drug, risks common to MDMA cannot be excluded because of the similarities between these drugs [4–6]. Methylone has the methylenedioxy and the beta-carbonyl groups in its structure. As mentioned above, these two functional groups would have contrary effects on the incorporation rate into hair of the drug. Therefore, it may be difficult to guess the incorporation tendency compared to its corresponding compound, MA.

In this study, the dispositions into hair of methylone and other new designer drugs, methcathinone and *N*-methyl-1-(3,4-methylenedioxyphenyl)butan-2-amine (MBDB), were studied with the animal model. Moreover, the incorporation rates of these drugs were compared with those of the related compounds previously studied, cathinone, AP, MA, MDA, MDMA, MDEA and MMDA, in order to evaluate their incorporation tendency into hair and the usefulness of hair specimens for the confirmation of the retrospective use of these drugs. Its chemical structure and those of related drugs are shown in Fig. 1.

2. Experimental

2.1. Chemicals and reagents

MBDB hydrochloride and MBDB-d5 (1,2-dideutero-*N*-trideuteromethyl-1-(3,4-methylenedioxyphenyl)-2-butanamine) were obtained from Cerilliant (Round Rock, TX, USA). MDMA-d3 hydrochloride [3] and methamphetamine-d4 hydrochloride [7] used as internal standards were prepared as previously reported. Methcathinone hydrochloride was prepared by the oxidation of pseudoephedrine [8]. Methylone hydrochloride was synthesized according to the procedure of Kamata et al. [9]. Its structure and purity were confirmed by a melting point (degradation, 225 °C), TLC, GC–MS [9] and ^{1}H nuclear magnetic resonance. Solid-phase extraction column (Bond Elut Certify, 10 mL/300 mg) was obtained from Varian (Harbor City, CA, USA). Pentafluoropropionic anhydride was purchased from Aldrich (Steinheim, Germany). All other chemicals and solvents were of analytical reagent grade or HPLC grade (Wako Chemicals, Osaka, Japan).

2.2. Instrumentation

For the quantitative analysis of the drugs in plasma and hair samples, GC–MS in the electron impact (EI) mode at 70 eV of electron energy was used. The instrument consisted of a Hewlett-Packard 5890 series II plus GC with a 5972 mass selective detector. Helium was used as the carrier gas through the fused silica capillary column (DB35-MS capillary, 30 m × 0.25 mm i.d., 0.25 μm film thickness) at 1 mL/min. The injector temperature was 200 °C, and splitless injection was employed with a split valve for 1.0 min. The oven temperature was started at 100 °C (held for 1.0 min), followed by a 10 °C/min ramp to 280 °C (held for 5 min) for the analyses of methylone and methcathinone. It

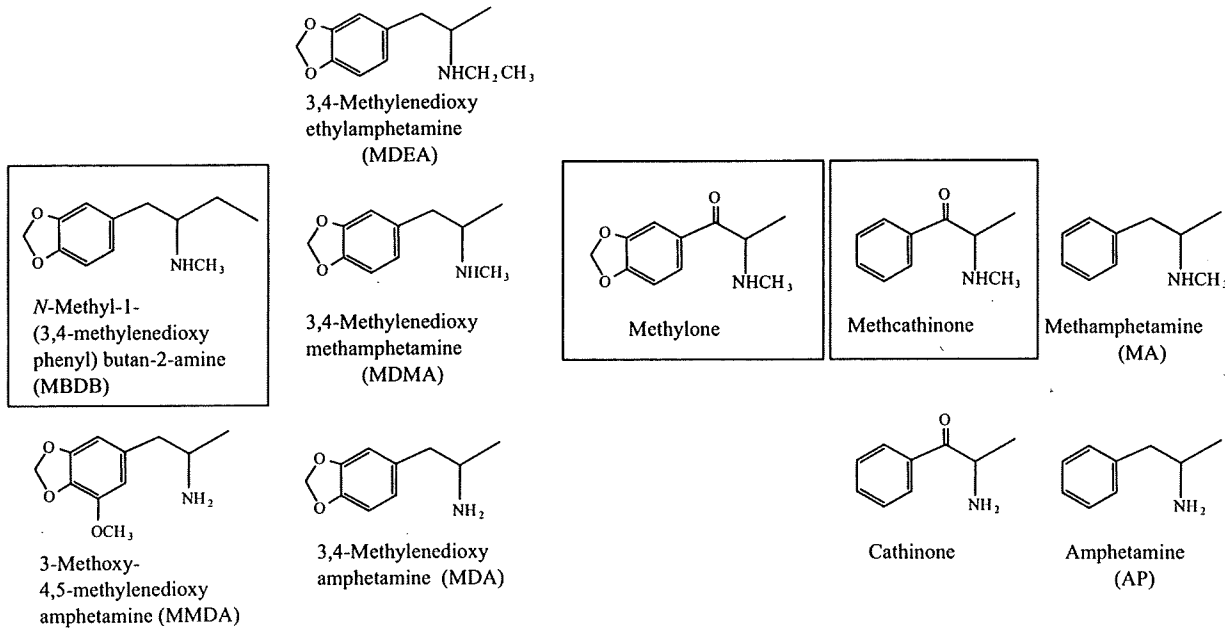

Fig. 1. Chemical structures of methylone, MBDB, methocathinone and its related compounds.

was programmed from 100 °C (3 min hold) to 280 °C (5 min) at a rate of 20 °C/min for the analysis of MBDB. The mass selective detector was kept at 280 °C. Drugs in biological specimens were investigated by monitoring the selected ions as follows: *m/z* 149 (a base peak ion), 204, 121, 160, 353 (M^+) for pentafluoropropionyl (PFP)–methylone, *m/z* 218 (a base peak ion), 176, 135, 353(M^+) for PFP–MBDB, *m/z* 204 (a base peak ion), 105, 160, 77, 119 for PFP–methcathinone, *m/z* 207 for PFP–MDMA-d3 (as an internal standard for the analysis of methylone), *m/z* 222 for PFP–MBDB-d5 (for the analysis of MBDB) and *m/z* 208 for MA-d4 (for the analysis of methcathinone).

2.3. Animal experiments

The animal experimental model was designed to compare the ratio of hair to AUC in many kinds of drugs as shown in our previous report [1,2]. The experimental drugs were administered to male dark agouti (DA) pigmented rats ($n = 3$), which were 5 weeks old and around 100 g mean weight (Japan SLC, Shizuoka, Japan). The drugs were given once a day at 5 mg/kg by intraperitoneal injection for 10 successive days. Two hundreds μL of Blood samples were collected 5, 15, 30, 60 120 and 360 min after the drug administration and plasma samples were prepared. The AUC was calculated by the conventional method [1]. Each animal had been shaved on the back just before the first drug administration. The newly growing hair samples were collected 28 days after the first administration. The ratio of the drug concentration in the hair to the AUC of the plasma was calculated as the index of the incorporation rate into hair.

2.4. Analytical methods

2.4.1. Stock solution

An individual standard solution of 1.0 mg/mL of each drug, methylone, methathinone and MBDB was prepared in methanol and distilled water, and stored at 4 °C. The IS solutions of 10 μg/mL of MDMA-d3, 1 μg/mL of MA-d4 and 5 μg/mL of MBDB-d5 in methanol for the analysis of hair samples and those of 1 μg/mL of MDMA-d3, 2 μg/mL of MA-d4 and 1 μg/mL of MBDB-d5 in distilled water for plasma samples were also prepared.

2.4.2. Plasma

To 100 μL of plasma sample was added 50 μL of the IS aqueous solution and 3 mL of 0.1 M potassium hydrogen phosphate buffer (pH 6.0). After a Bond Elut Certify was pre-activated with methanol and the buffer, the sample solution was applied to the Bond Elut Certify and the column was washed with 1 mL of distilled water and 1 M acetic acid, successively. The column was dried under vacuum for 5 min. After the column was rinsed with 1 mL of methanol and dried under vacuum for 2 min, 3 mL of mixed solution of dichloromethane, methanol and hydrochloric acid (60:40:1) was passed through the column to elute the target drugs. Following evaporation of the solvent under a nitrogen stream, the residue was dissolved in 200 μL of PFPA/ethylacetate (1:1) and heated at 60 °C for 20 min. The reaction solution was evaporated with a nitrogen stream, and the residue was re-dissolved in 50 μL of ethylacetate. One μL of the solution was automatically injected into the GC–MS.

2.4.3. Hair

Hair samples were washed three times with 0.1% sodium dodecyl sulfate (SDS) under ultrasonication, followed by washing three times with water under the same condition. After the sample was dried under a nitrogen stream at room temperature, approximately 15 mg of finely cut hair was precisely weighed and extracted with 3 mL of methanol/5 M hydrochloric acid mixed solution (20:1) containing 100 μL of each IS methanol solution for 1 h under ultrasonication. Following storage at room temperature overnight, the hair was filtered off, and the filtrate was evaporated with a nitrogen stream and the residue was dissolved in 3 mL of 0.1 M potassium hydrogen phosphate buffer (pH 6.0). The solution was treated with Bond Elut Certify, derivatized and analyzed as above.

2.5. Calibration curves

The drug concentrations in the samples were calculated using the peak-area ratios of the base peak ions of 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, 10, 50, 100, 500, 1000, 2000 ng/mL (methylone or methcathinone) and 0, 50, 100, 250, 500, 1000, 2000 ng/mL (MBDB) for the rat plasma samples were prepared just before analysis. The samples containing 0, 5,10, 25, 50, 100, 200 ng/mg (methylone or MBDB) and 0, 1.0, 2.5, 5.0, 10, 25, 50 ng/mg (methcathinone) for the hair samples were also prepared. The limit of quantitation of each drug was chosen to be the concentration of the lowest calibration standard with an acceptable limit of 20% for both precision and accuracy.

2.6. Precision and accuracy of the method

The precision and accuracy of the method were evaluated by analyzing triplicates of the plasma samples which were spiked with the standard solutions containing 10, 50, 2000 ng/mL (methylone or methcathinone) and 50, 500, 2000 ng/mL (MBDB), respectively. For the analyses of hair, the control samples, spiked with the standard solutions each containing 5, 50, 200 ng/mg (methylone or MBDB) and 1, 10, 50 ng/mg (methcathinone), were evaluated. Accuracy, expressed as bias, was calculated as the difference between the amount of each drug added and recovered.

3. Results and discussion

3.1. Accuracy and precision of the methods

Under the chromatographic conditions used, there was no interference with all drugs or the deuterated internal standards

Table 1
Validation of results of the GC–MS analysis of methylone, MBDB and methcathinone for rat plasma and hair samples

	Compounds	Linear range	Linearity	Precision (%)		Accuracy (%)	
				Concentration	%	Concentration	%
Plasma (ng/mL)	Methylone	10–2000	$y=0.0031x+0.0815$, $R^2=0.9966$	10	11.6	10	7.3
				500	11.4	500	−1.0
				2000	17.3	2000	−2.7
	MBDB	50–2000	$y=0.0019x+0.0051$, $R^2=0.9999$	50	2.6	50	13.2
				500	0.2	500	−1.3
				2000	2.8	2000	0.9
	Methcathinone	10–2000	$y=0.0011x+0.0141$, $R^2=0.9992$	10	10.6	10	4.3
				500	2.4	500	−0.9
				2000	4.4	2000	6.5
Hair (ng/mg)	Methylone	5.0–200.0	$y=0.0193x+0.0358$, $R^2=0.9966$	5.0	1.8	5.0	14.0
				50.0	9.6	50.0	−1.5
				200.0	2.3	200.0	−1.6
	MBDB	5.0–200.0	$y=0.0267x+0.0649$, $R^2=0.9987$	5.0	13.9	5.0	4.2
				50.0	0.9	50.0	−1.4
				200.0	1.8	200.0	−4.4
	Methcathinone	1.0–50.0	$y=0.0906x+0.0059$, $R^2=0.9999$	1.0	5.1	1.0	13.3
				10.0	4.4	10.0	4.8
				50.0	4.7	50.0	−2.1

by any extractable endogenous materials in the control rat plasma and control rat hair. The calibration curves were linear over the concentration range 10–2000 ng/mL (except for MBDB; 50–2000 ng/mL) for rat plasma and 5.0–200.0 ng/mg (except for methcathinone; 1.0–50.0 ng/mg) for rat hair with good coefficients of determination of $r^2 \geq 0.997$. The precision and accuracy data of the analytical procedure for rat plasma and hair samples, spiked with standard solution of methylone, MBDB and methcathinone, are presented in Table 1. The precision of these drugs ranged from 0.2 to 17.3% and the accuracy ranged from −4.4 to 14.0%. These values are almost below 15% although the precision data for methylone in the plasma sample at 2000 ng/mL are a little higher.

3.2. Drug concentrations in rat plasma

After intraperitoneal administration of methylone hydrocloride, MBDB hydrocloride and methcathinone hydrocloride to DA rats at 5 mg/kg, the concentrations of each drug in rat plasma over 360 min were monitored using GC–MS-SIM. Time courses of the rat plasma concentrations of these drugs are shown in Fig. 2. Half-lives of methylone, MBDB and methcathinone in the plasma were 120, 170 and 220 min, respectively. Average peak plasma concentrations of these drugs were approximately 700–1500 ng/mL 15 or 30 min after the administration. The plasma AUCs of methylone, MBDB and methcathinone were 147 ± 12, 151 ± 17 and 191 ± 59 μg min/mL, respectively, and

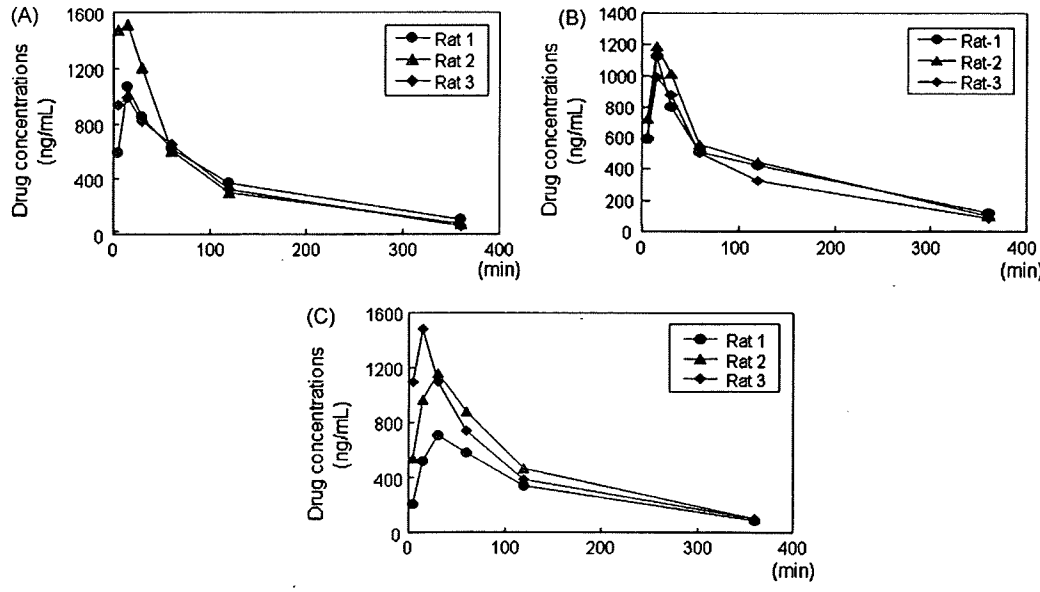

Fig. 2. The time courses of the rat plasma concentrations after administration of (A) methylone HCl, (B) MBDB HCl and (C) methcathinone HCl (i.p., 5 mg/kg, $n=3$).

Table 2
Rat plasma AUCs, drug concentrations in rat hair and incorporation rates ([Hair]/AUC)

Compounds	Plasma AUC (μg min/mL)	Hair (ng/mg)	[Hair]/AUC
Cathinone*	291 ± 20	4.2 ± 0.9	0.01 ± 0.00
Methcathinone	191 ± 59	6.6 ± 1.9	0.04 ± 0.01
AP*	182 ± 7	18.1 ± 0.1	0.10 ± 0.02
MA*	125 ± 21	16.3 ± 2.3	0.13 ± 0.02
MDA*	411 ± 59	121.9 ± 27.5	0.30 ± 0.05
Methylone	147 ± 12	79.8 ± 22.3	0.55 ± 0.19
MDMA*	121 ± 16	93.4 ± 10.9	0.77 ± 0.05
MDEA*	165 ± 10	138.4 ± 4.4	0.85 ± 0.03
MMDA*	183 ± 50	215.0 ± 20.5	1.24 ± 0.06
MBDB	151 ± 17	164.5 ± 25.0	1.10 ± 0.25

$n = 3$.
* The data is from [3].

these values were close to those of their related compounds (AP, MA, MDMA, MDEA and MMDA) whose data have been reported in our previous study, as shown in Table 2.

3.3. Drug concentrations in rat hair

The concentrations of each drug in the rat hair are shown in Table 2. The results of GC–MS analyses of PFP-derivatized extracts from the control rat hair and the hair of rats after intraperitoneal administration of methylone hydrochloride at 5 mg/kg for 10 successive days were shown in Fig. 3. The concentrations of methylone and MBDB in the hair were 79.8 ± 22.3 and 164.5 ± 25.0 ng/mg, respectively. The value of MBDB was the second highest of all drugs shown in Table 2. However, the concentration of methcathinone in the hair was extremely low (6.6 ± 1.9 ng/mg) in spite of its relatively high AUC.

3.4. Incorporation rates of drugs into rat hair from rat plasma

As mentioned, we propose that the ratio of drug concentration in hair to AUC in plasma ([Hair]/AUC) could be one of the indexes that indicate the incorporation rate of drug into hair from plasma [1,2]. According to our proposal, [Hair]/AUC of methylone, MBDB and methcathinone were 0.55, 1.10 and 0.04, respectively. When these values were compared with those of their related compounds that have been studied previously [2,3], the order of the [Hair]/AUC was cathinone < methcathinone < AP < MA < EAP < MDA < methylone < MDMA < MDEA < MBDB < MMDA (Fig. 4). The [Hair]/AUC of MDA, MDMA and methylone was 3–40 times higher than those of AP, MA and methcathinone, suggesting that the methylenedioxy group on the benzene ring leads to consid-

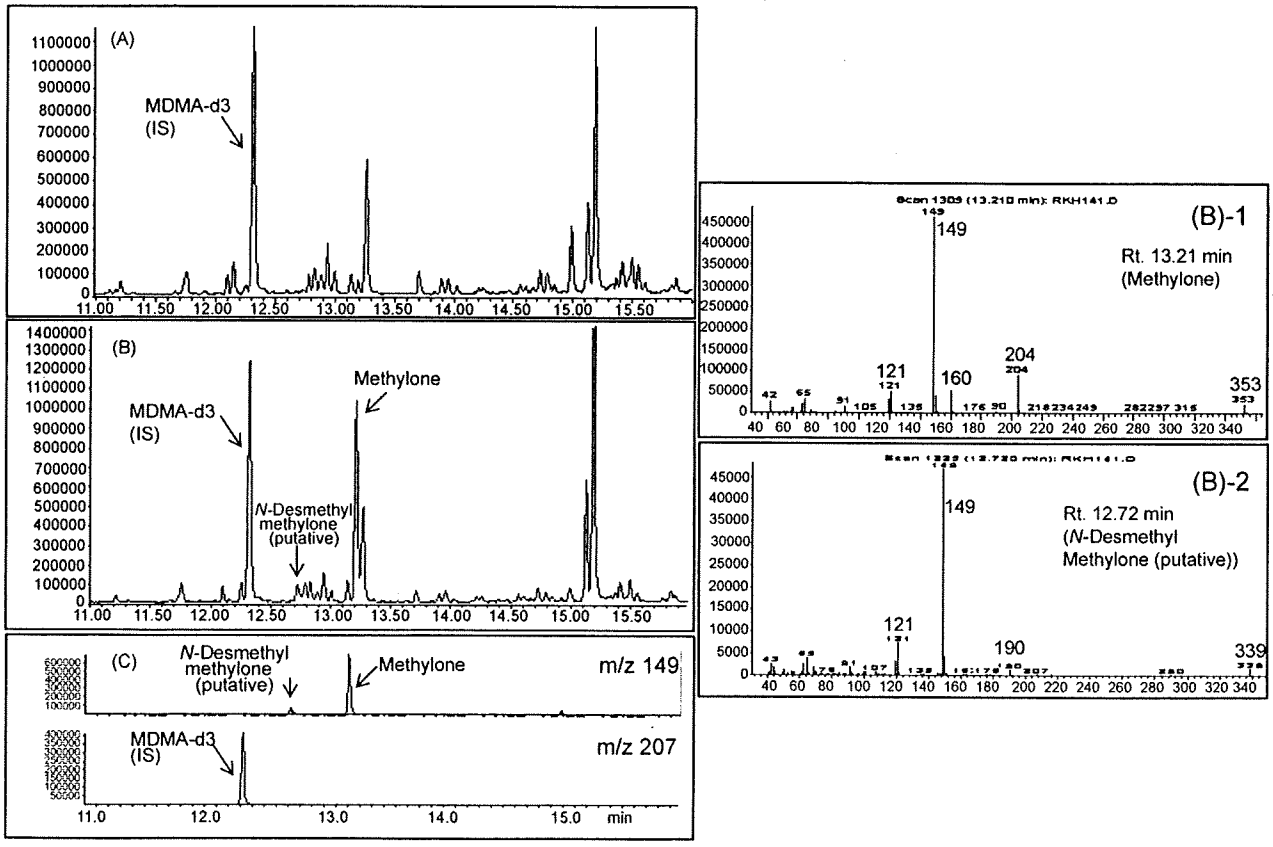

Fig. 3. GC–MS total ion chromatograms of PFP-derivatized extracts from (A) the control rat hair and (B) the hair of rat administered methylone HCl (i.p., 5 mg/kg × 10 days). Mass spectra of the peaks at 13.21 and 12.72 min of the chromatogram (B) are shown in (B)-1 (PFP-methylone) and (B)-2 (PFP-*N*-desmethylmethylone (putative)), respectively. The chromatograms of the selected monitoring ions of *m/z* 149 (the base peak ions of PFP-methylone and PFP-*N*-desmethylmethylone (putative)) and *m/z* 207 (the base peak ion of PFP-MDMA (IS)) are also shown in (C).

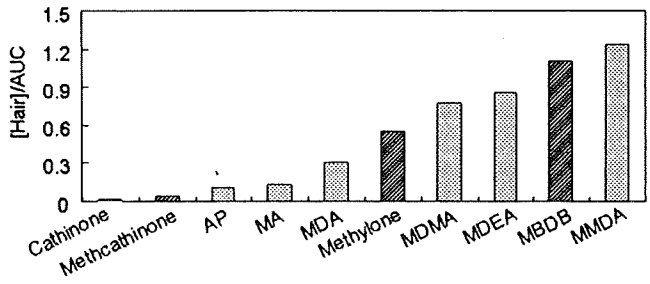

Fig. 4. Incorporation rates ([Hair]/AUC) of methylone, MBDB, methocathinone and their related compounds previously studied.

erably higher incorporation rates. However, the [Hair]/AUC of beta-ketoamphetamines, cathinone, methcathinone and methylone was 1/2–1/10 times lower in comparison with those of their corresponding amphetamines, AP, MA and MDMA, respectively. It is expected that the beta-carbonyl group might lead to lower incorporation rates. The [Hair]/AUC of methylone was lower than that of MDMA but higher than MA. Although methylone has both groups in its structure, the positive effect of the methylenedioxy group may be stronger than the negative effect of the beta-carbonyl group. On the other hand, the [Hair]/AUC of MBDB, which has methylenedioxyphenyl-2-butanamine structure, was higher than that of MDMA and the second highest to MMDA, although that of methcathinone, having beta-ketone in its structure, was extremely low.

4. Conclusions

As a result of an animal experiment, the incorporation rate ([Hair]/AUC) of MBDB was 1.4 times higher than that of MDMA, and a phenyl-2-butanamine structure may be more easily incorporated into hair than a phenyl-2-propamine. On the other hand, the value of methcathinone was unusually low, similar to cathinone. It is thought that these compounds, which have beta-carbonyl groups, may not be suitable for hair analysis because of their relatively low concentrations in the hair. The incorporation rate of methylone was 0.55. This value was four times larger than that of MA, but a little smaller than that of MDMA. It is suggested that the positive effect of the methylenedioxy group may be stronger than the negative effect of the beta-carbonyl group, in this case.

In our previous study [1], we determined melanin affinity and lipophilicity of 20 abused drugs, and these values were compared to their [Hair]/AUCs as an index of the incorporation tendency into hair. As a result, the combination of melanin affinity and lipophilicity showed a high correlation with the [Hair]/AUCs. In consideration of that result, those physico-chemical properties of methylone, methcathinone and MBDB could be significantly related to their [Hair]/AUCs.

In conclusion, as with MA and MDMA, the incorporation tendency of new designer drugs, methylone and MBDB (except for methcathinone) into hair is relatively high, and a hair sample would be a good specimen for the confirmation of the retrospective use of these drugs.

Acknowledgement

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Evaluation of Characteristic Deuterium Distributions of Ephedrines and Methamphetamines by NMR Spectroscopy for Drug Profiling

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We have established a method for quantitative analysis of the deuterium contents (D/H) at the phenyl, methine, benzyl, *N*-methyl and methyl groups of *l*-ephedrine/HCl, *d*-pseudoephedrine/HCl and methamphetamine/HCl by ^{2}H NMR spectroscopy. Comparison of the 5 position-specific D/H values of *l*-ephedrine/HCl and *d*-pseudoephedrine/HCl prepared by three methods (chemical synthesis, semichemical synthesis, and biosynthesis) showed that chemically synthesized ephedrines and semisynthetic ephedrines have highly specific distributions of deuterium at the methine position and at the benzyl position, compared with the other positions. The classification of several methamphetamine samples seized in Japan in terms of the D/H values at these two positions clearly showed that the methamphetamine samples had been synthesized from ephedrines extracted from *Ephedra* plants or semisynthetic ephedrines but not from synthetic ephedrine. This isotope ratio analysis method should be useful to trace the origins of seized methamphetamine in Southeast Asia.

Methamphetamine, a stimulant drug that activates adrenergic nerve in the brain, is controlled by the Stimulant Control Law in Japan. The precursor of methamphetamine is mainly *l*-ephedrine or *d*-pseudoephedrine from *Ephedra* plants, which are used as a therapeutic drug in Southeast Asia, including Japan.[1] There are two major synthetic approaches for the preparation of methamphetamine: one is synthesis from 1-phenyl-2-propanone (P-2-P), which is a precursor for the Leuckart method or reductive amination, and the other is synthesis from *l*-ephedrine or *d*-pseudoephedrine via Birch reduction, the Nagai method, or Emde method (Figure 1). To prevent the production of illicit methamphetamine, it is important to control, monitor, and evaluate the precursors of seized methamphetamine. Impurity analysis for drug profiling has been used to enable the identification of the synthetic route for methamphetamine manufacture from ephedrines.[2–5] For example, route-specific impurities can be detected by HPLC and GC/MS. Chiral analysis of seized methamphetamine may also be useful; for example, if methamphetamine is racemic, P-2-P may have been used as a precursor. By the analysis with HPLC and GC/MS, it is impossible to get the differences among ephedrines produced by different methods. Stable isotope ratio mass spectrometry (IR-MS) has recently attached much interest for drug profiling. Natural isotope analyses of MDMA,[6–11] heroin,[12–16]

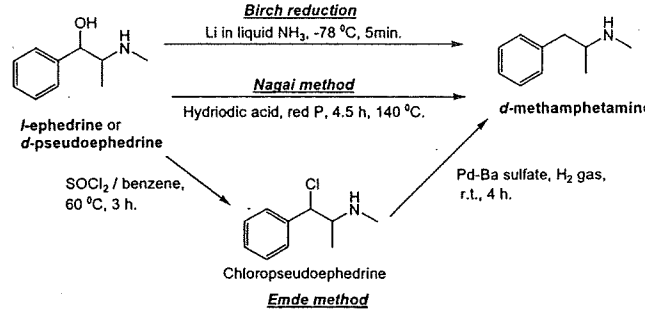

Figure 1. Three routes for methamphetamine manufacture from *l*-ephedrine or *d*-pseudoephedrine.

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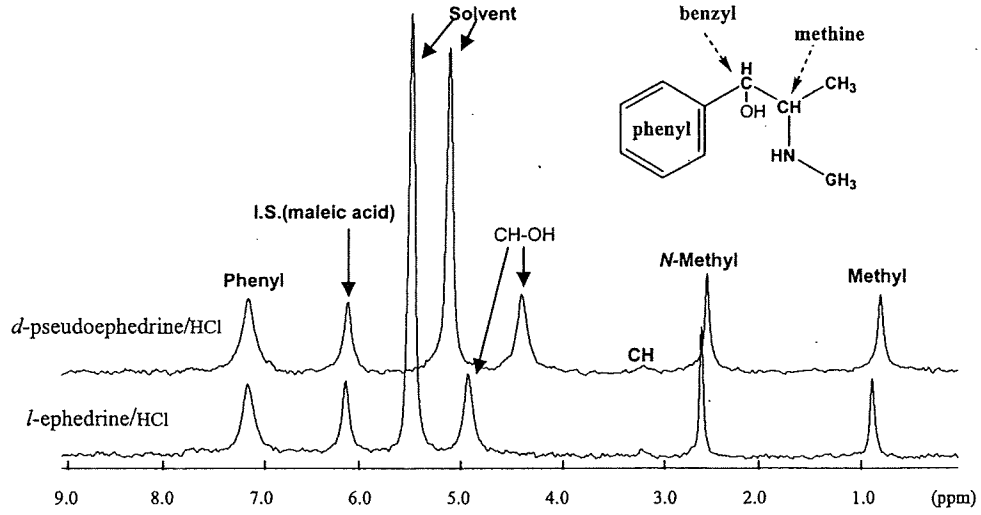

Figure 2. ^{2}H NMR spectra of *l*-ephedrine & *d*-pseudoephedrine using TFA as a shift reagent.

cocaine,[13,17] cannabis,[18,19] and methamphetamine[20] by IR-MS have been reported. The analysis of site-specific deuterium content by ^{2}H NMR is also potentially useful for drug profiling.[21,22] Armellin et al. have reported that the position-specific deuterium content of methamphetamine synthesized from P-2-P depends on the synthetic method.[23] However, the most popular methamphetamine synthetic routes employ *l*-ephedrine or *d*-pseudoephedrine as a precursor instead of P-2-P. Schmidt et al. employed ^{2}H NMR spectroscopy to discriminate the biosynthetic pathways of *l*-ephedrine.[24] However, the position-specific D/H values of *l*-ephedrines are likely to be a more powerful tool for discriminating the sources of ephedrines used as precursors for preparing illegal methamphetamine/HCl. In this paper, we focused on methamphetamine prepared from *l*-ephedrine and *d*-pseudoephedrine. We show that the methamphetamine precursor can be discriminated on the basis of the (D/H) values by using ^{2}H NMR, and we describe the relationship of the (D/H) values of the precursor ephedrines and the product methamphetamine.

EXPERIMENTAL SECTION

Materials and Chemicals. Six *l*-ephedrine/HCl and two *d*-pseudoephedrine/HCl samples were used. The origins of these chemicals were as follows: semichemical synthetic *d*-pseudoephedrine from Emmellen (India, sample a) and BASF (Germany, sample b); semichemical synthetic *l*-ephedrine from Emmellen (India, sample c) and BASF (Germany, sample d); synthetic *l*-ephedrine from Daiichi Fine Chemical Co. Ltd. (Japan, sample e); biosynthetic *l*-ephedrine from Dainippon Sumitomo Pharma Co. Ltd. (Japan, sample f), galenical market (People's Republic of China, sample g) and galenical market (Xinjian Uygur, sample h). *d*-Methamphetamine/HCl samples A–H were synthesized from *d*-pseudoephedrines and *l*-ephedrines by the Nagai method (Figure 1). Sample e was also used to examine the deuterium abundance in products obtained by the various synthetic methods, Nagai method (sample E), Emde method (sample I), and Birch reduction (sample J), as shown in Figure 1, in our laboratory. Twelve samples of *d*-methamphetamine crystals had been seized by law enforcement agencies in Japan, and sample P was *d*-methamphetamine/HCl from Dainippon Sumitomo Pharma Co. Ltd. All chemicals were reagent grade.

Synthesis. Samples A–H by Nagai method: A mixture of *l*-ephedrine/HCl or *d*-pseudoephedrine/HCl (10.0 g), aqueous hydroiodic acid (55~58 wt %, 20 mL), and red phosphorus (1.5 g) was heated at 140 °C for 4.5 h. After cooling, the mixture was basified (pH 12) with 2 N NaOH(aq) and *d*-methamphetamine was extracted with CH_2Cl_2. The organic phase was dried (Na_2SO_4) and evaporated under vacuum, and HCl-saturated Et_2O was added. After filtration, the crystals were dried under vacuum to give *d*-methamphetamine/HCl in 65% yield.

Sample I by Emde method: Step 1, to a solution of *l*-ephedrine/HCl (9.3 g) in benzene (50 mL) was added dropwise a solution of $SOCl_2$ (20 mL), and the mixture was kept at 60 °C for 3 h. After cooling, the precipitate was collected by filtration to afford chloropseudoephedrine/HCl in 94% yield. Step 2, to a solution of chloropseudoephedrine/HCl in pure water (20 mL) was added Pd–$BaSO_4$ (6 g). The mixture was stirred for 4 h at ambient temperature under H_2. After filtration, the solution was worked up as described under the Nagai method to give *d*-methamphetamine/HCl in 75% yield.

Sample J by Birch reduction: to liquid ammonia on a dry ice–acetone bath (−78 °C) was added dropwise an anhydrous THF solution of *l*-ephedrine free base (1.8 g), and the mixture was

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Table 1. D/H Values of the *d*-Pseudoephedrine and *l*-Ephedrine (ppm)[a]

sample	phenyl	methine	benzyl	*N*-methyl	methyl
a (semichemical synthesis)	158.4 ± 3.2	26.7 ± 5.4	641.1 ± 29.3	121.1 ± 2.4	118.6 ± 2.2
b (semichemical synthesis)	141.6 ± 1.6	33.3 ± 4.0	818.5 ± 10.7	140.7 ± 1.4	110.8 ± 0.9
c (semichemical synthesis)	150.5 ± 8.6	26.9 ± 10.0	665.0 ± 44.9	140.6 ± 2.4	109.3 ± 2.1
d (semichemical synthesis)	144.3 ± 7.0	13.7 ± 3.8	641.5 ± 23.3	132.5 ± 2.8	105.5 ± 1.9
e (chemical synthesis)	158.6 ± 2.8	192.1 ± 21.9	54.9 ± 27.2	139.0 ± 2.0	137.5 ± 1.2
f (biosynthesis)	138.0 ± 1.7	53.0 ± 8.4	118.2 ± 13.0	99.8 ± 1.5	129.5 ± 2.4
g (biosynthesis)	130.6 ± 2.9	52.9 ± 11.2	124.1 ± 6.9	99.1 ± 2.1	128.9 ± 1.7
h (biosynthesis)	128.7 ± 6.2	45.6 ± 6.6	120.0 ± 15.9	98.8 ± 1.4	125.9 ± 2.6

[a] $(D/H)_i$ ratios and standard deviations measured from eight spectra.

Table 2. D/H Values of Methamphetamines Synthesized by the Nagai Method (ppm)[a]

sample	phenyl	methine	benzyl	*N*-methyl	methyl
A	142.8 ± 2.3	44.7 ± 6.1	458.6 ± 4.4	137.2 ± 2.1	121.9 ± 0.9
B	142.3 ± 1.5	64.2 ± 3.9	617.8 ± 6.0	164.1 ± 1.5	107.3 ± 1.6
C	159.3 ± 3.9	27.1 ± 7.7	460.1 ± 13.2	151.3 ± 4.5	120.5 ± 1.7
D	141.1 ± 1.5	62.6 ± 0.6	492.5 ± 7.7	154.1 ± 2.7	107.2 ± 1.2
E	148.3 ± 1.4	250.7 ± 10.3	20.6 ± 4.5	106.6 ± 1.3	136.2 ± 1.2
F	136.8 ± 1.1	70.9 ± 2.4	72.3 ± 4.3	86.5 ± 0.5	134.9 ± 1.2
G	132.0 ± 1.7	61.2 ± 3.4	66.8 ± 3.6	79.9 ± 1.1	122.5 ± 0.8
H	130.8 ± 2.0	63.3 ± 3.0	62.4 ± 2.6	81.2 ± 0.6	126.2 ± 1.2

[a] The samples A–H were prepared from the ephedrines a–h, respectively, in Table 1. $(D/H)_i$ ratios and standard deviations measured from eight spectra.

Table 3. D/H Values of Methamphetamines Synthesized from Sample e by Various Methods (ppm)[a]

sample	phenyl	methine	benzyl	*N*-methyl	methyl
e	158.6 ± 2.8	192.1 ± 21.9	54.9 ± 27.2	139.0 ± 2.0	137.5 ± 1.2
E (Nagai method)	148.3 ± 1.4	250.7 ± 10.3	20.6 ± 4.5	106.6 ± 1.3	136.2 ± 1.2
I (Emde method)	144.4 ± 1.4	233.6 ± 3.0	72.6 ± 0.8	112.1 ± 0.8	136.3 ± 1.2
J (Birch reduction)	144.2 ± 2.4	199.5 ± 8.3	41.6 ± 5.7	105.8 ± 3.2	136.1 ± 2.9

[a] $(D/H)_i$ ratios and standard deviations measured from eight spectra.

stirred. Then, small pieces of lithium metal (300 mg) were added to the mixture during 5 min with stirring, retaining the blue color of the solution. To the reaction mixture was added ethanol (0.6 mL), followed by excess ammonium chloride as a quenching agent. The mixture was warmed to room temperature, ammonia was removed by evaporation, and the residue was dissolved in pure water (20 mL). *d*-Methamphetamine was extracted with CH_2Cl_2. The organic phase was worked up as described under the Nagai method to give *d*-methamphetamine/HCl in 50% yield.

Sample Preparation. An amount of 400 mg of *l*-ephedrine/HCl or 1 g of *d*-pseudoephedrine/HCl and 0.45 g of maleic acid (Wako Pure Chem. Ind. Ltd, Japan) as an internal standard were weighed into a 10 mm NMR sample tube (Shigemi Inc., Japan) and dissolved in 2.0 mL of deuterium-depleted water (Isotec Inc.). Then 1.0 or 0.4 mL of trifluoroacetic acid was added as a shift reagent. The signal of the benzyl position of *l*-ephedrine/HCl or *d*-pseudoephedrine/HCl was observed near that of H_2O by ^1H NMR. Though the ^2H NMR signal overlaps with the broad signal of DHO, it was possible to obtain quantitative values by using TFA as a shift reagent (Figure 2). In addition, 0.4–0.9 g of methamphetamine/HCl and 0.45 g maleic acid mixed in a 10 mm NMR tube were dissolved in 3.0 mL of deuterium-depleted water.

^2H NMR Spectroscopy. ^2H NMR spectra were recorded on an ECA–600 spectrometer (JEOL Ltd, Japan) at 92.13 MHz, using a 10 mm broad-band tunable probe, under CPD (WALTZ sequence) proton decoupling conditions. All samples were run unlocked at 298 K. At least eight spectra were run for each sample, collecting 7000 scans and using the following parameters: acquisition time of 5 times T_1 max, repetition time of 6 times T_1 max, 1843.11–2764.26 Hz spectral width, and 4K or 8K memory size, for adjustment of acquisition time and repetition time. Backward linear prediction was used to reconstruct the first few data points of the FID, replacing points which were corrupted by ringdown and Fourier-transformed with a line broadening of 2 Hz. The peak areas involving partially overlapped signals were determined through the deconvolution routine of the JEOL NMR software Delta using a Lorentzian line shape. The signals of *d*-pseudoephedrine/HCl and *l*-ephedrine/HCl, obtained from an analysis of the proton spectrum taken under the same conditions as the deuterium spectrum, were as follows: *d*-pseudoephedrine/HCl, 7.19–7.11 (5H, m, phenyl), 4.46 (1H, d, J = 9.7 Hz, benzyl), 3.23 (1H, dq, J = 6.9 Hz, 9.7 Hz, methine), 2.59 (3H, s, *N*-methyl), 0.84 (3H, d, J = 6.9 Hz, methyl); *l*-ephedrine/HCl, 7.20–7.10 (5H, m, phenyl), 5.00 (1H, d, J = 3.1 Hz, benzyl), 3.25 (1H, dq, J = 3.1 Hz, 6.9 Hz, methine), 2.63 (3H, s, *N*-methyl), 0.91 (3H, d, J = 6.9 Hz, methyl). The signal assignment of the spectrum of *d*-metham-

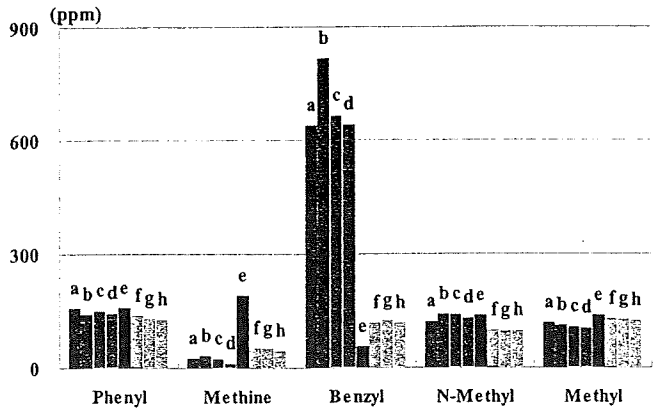

Figure 3. Distribution of D/H value of the *d*-pseudoephedrine and *l*-ephedrine.

phetamine/HCl was similarly assigned as follows (δ, ppm): 7.17–7.06 (5H, m, phenyl), 3.29 (1H, m, methine), 2.83 (1H, dd, $J = 6.2$ Hz, 13.7 Hz, benzyl), 2.64 (1H, dd, $J = 7.9$ Hz, 13.7 Hz, benzyl), 2.48 (3H, s, *N*-methyl), 1.03 (3H, d, $J = 6.6$ Hz, methyl). In the ^{2}H NMR experiment, the benzyl signal detected at 2.65 ppm by ^{1}H NMR in *d*-methamphetamine/HCl overlapped the signal based on the *N*-methyl group. Quantitative analysis of the spectra was done by curve fitting using the Lorentz function. The standard deviations of the quantitative values of phenyl, *N*-methyl, and methyl signals in the examined samples were less than 9 ppm {signal-to-noise ratio (S/N) > 30} as shown in Tables 1 –3. The standard deviations of the methine and benzyl signals of some samples were not so good due to the low deuterium abundance, but there was little quantitative overlap. The unlocked state during data acquisition in NMR experiments was found to have little effect on the quantitative analysis under the condition used.

Determination of the Site-Specific Isotope Ratio. The $(D/H)_i$ value for each spectrum was calculated from eq 1 using maleic acid as an internal reference. The isotopic ratio of maleic acid, having exchangeable protons within the molecule, $(D/H)_{MA}$, was precisely calibrated on the VSMOW scale (155.8 ppm) via the dimethylsulfoxide (146.3 ppm).

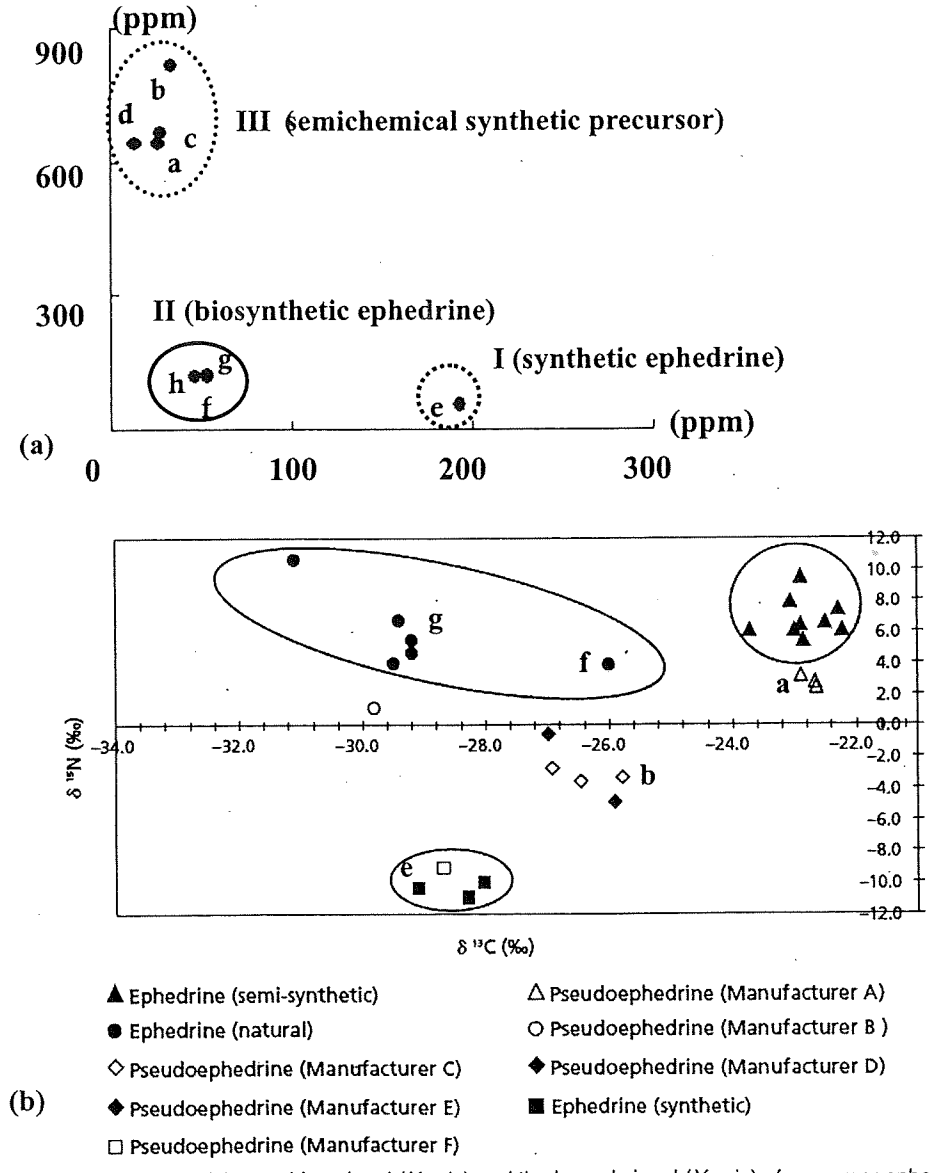

Figure 4. (a) Scatter plot of D/H values of the methine signal (*X* axis) and the benzyl signal (*Y* axis) of precursor ephedrines, (b) carbon and nitrogen isotope ratio of various ephedrines by IR-MS quoted from ref 26.

$$(D/H)_i = P_{ref} m_{ref} M_s S_i (D/H)_{ref} / P_i m_s M_{ref} S_{ref} \quad (1)$$

where P_i and P_{ref} are the stoichiometric numbers of hydrogens in position i and in the reference. S_i and S_{ref} are the surface areas of the signal. M_s, m_s, M_{ref} and m_{ref} are, respectively, the molecular weight and mass of the sample and the reference used.

RESULTS AND DISCUSSION

^{2}H Distribution of *d*-Pseudoephedrine/HCl and *l*-Ephedrine/HCl. The results of the quantitative analyses of *d*-pseudoephedrines/HCl and *l*-ephedrines/HCl by ^{2}H NMR spectroscopy are shown in Table 1 and Figure 3. All samples showed comparable D/H values at the phenyl, *N*-methyl, and methyl positions, while characteristic distributions of deuterium were observed at the methine and benzyl positions. Sample e showed the highest D/H value at the methine position and the smallest at the benzyl position, and samples a–d showed higher values at the benzyl position. Butzenlechner et al. reported that benzaldehyde from natural sources and benzaldehyde prepared by catalytic oxidation of toluene showed mean $\delta(^2H)$ values of -125 ± 14 ‰ and $+777 \pm 20$ ‰, respectively.[25] It is presumed that the higher values of samples a–d at the benzyl position may be due to synthesis via synthetic benzaldehyde from toluene as the precursor. Deuterium abundance of ephedrines differed greatly at the methine and benzyl positions from the other positions. The preparation method of ephedrines appears to affect the deuterium contents at both positions. In a scatter plot of absolute values (Figure 4a), three kinds of ephedrines could be distinctly separated into the individual groups. We previously reported that two groups of semichemical synthetic ephedrines could be discriminated on the basis of the values of $\delta^{15}N$ and $\delta^{13}C$ using IR-MS.[26] Five samples (a, b, e–g) in Figure 4a are the same as those in Figure 4b, which was reported in ref 26. In Figure 4b, it is not clear if semichemical synthetic pseudoephedrine (sample b) and biosynthetic ephedrine (sample f) belong to the same group or not. However, sample b (semichemical synthetic) and sample f (biosynthetic) were clearly discriminated by the D/H values at the benzyl and methine positions, as shown in Figure 4a. Thus, quantitative analysis by ^{2}H NMR appears to be more effective for discrimination of preparation methods of ephedrines.

^{2}H Distribution of *d*-Methamphetamine/HCl Prepared From *l*-Ephedrine/HCl or *d*-Pseudoephedrine/HCl. The results of the quantitative analyses of methamphetamines prepared from three kinds of ephedrine by ^{2}H NMR spectroscopy are reported in Table 2. All methamphetamines were synthesized by the Nagai method.

Figure 5a shows a scatter plot of deuterium abundance at the methine and benzyl positions in the synthesized methamphetamines. The distribution is clearly similar to that of the corresponding precursor shown in Figure 4a. Thus, the characteristics of deuterium abundance of the precursor were well-correlated with those of the product methamphetamine. It is therefore possible to obtain chemical information on the synthetic method of ephedrines used as a precursor, as we had expected.

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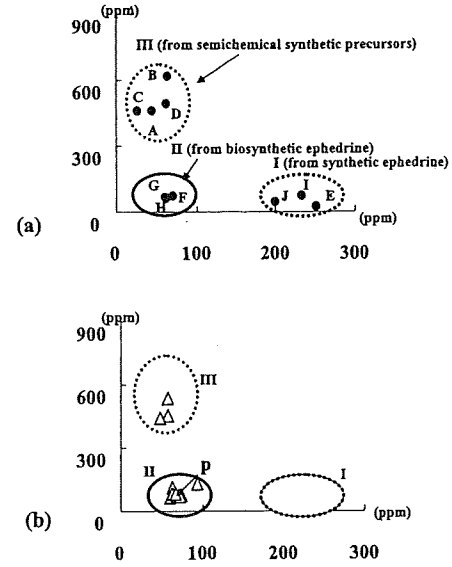

Figure 5. Scatter plot of D/H values of the methine signal (*X* axis) and the benzyl signal (*Y* axis) of (a) synthesized methamphetamines and (b) seized and commercial methamphetamines.

^{2}H Distribution of *d*-Methamphetamine Prepared from *l*-Ephedrine/HCl by Various Synthetic Methods. Table 3 shows the D/H values at each position of the precursor *l*-ephedrine e and the *d*-methamphetamines/HCl synthesized from *l*-ephedrine/HCl by three synthetic methods: Nagai method (E), Emde method (I), and Birch reduction (J). The cluster of sample E, I, and J in Figure 5a corresponds to cluster I in Figure 4a but not clusters II and III in Figure 4a. Thus, the variability of deuterium distribution by synthetic method of methamphetamine did not show large characteristic differences as compared with the variability of D/H values of precursors shown in Figure 4a.

^{2}H Distribution in Seized and Commercial Methamphetamines. Figure 5b shows a comprehensive scatter plot of deuterium abundance at the methine and benzyl positions for the 12 seized methamphetamine samples together with a commercial methamphetamine (P). From this plot, the studied methamphetamines could be grouped into two clusters. Cluster II includes methamphetamines synthesized from biosynthetic ephedrines. Cluster III includes methamphetamines which originated from precursors prepared by a semichemical synthetic process. The classification of several methamphetamine samples seized in Japan in terms of the D/H values at these two positions clearly showed that the methamphetamine samples had been synthesized from ephedrines extracted from *Ephedra* plants or semisynthetic ephedrines but not from synthetic ephedrine. Thus, our findings are expected to be helpful to monitor and identify the sources of ephedrines used for the manufacture of methamphetamine and thereby to assist enforcement authorities in the strategy of precursor control.

CONCLUSIONS

We have established a method for quantitative analysis of the position-specific deuterium contents (D/H) of ephedrines/HCl and methamphetamine/HCl by ^{2}H NMR spectroscopy. ^{2}H NMR spectroscopy is suitable for position-specific isotope detection, in contrast to IR-MS which gives only overall abundance. We compared five position-specific D/H values of *l*-ephedrine/HCl and

d-pseudoephedrine/HCl prepared by three methods (chemical synthesis, semichemical synthesis, and biosynthesis). Semichemical synthetic ephedrines characteristically showed a specific high distribution of deuterium at the benzyl position. As there is a large difference at the benzyl and methine positions compared with the other three positions, the D/H values at the benzyl and methine positions would be useful as indicators to classify the origin of ephedrines. The difference between biosynthetic and semichemical synthetic ephedrines was easier to discern from the combination of the D/H values at the benzyl and methine positions than from the combination of $\delta^{15}N$ and $\delta^{13}C$ obtained by IR-MS. The five position-specific D/H values of *l*-ephedrine used as a precursor were well-correlated with those for methamphetamine synthesized from it. The several seized and commercial methamphetamines could be classified into two groups by plotting the D/H values of methine versus benzyl position. The D/H values at these two positions of several methamphetamine samples seized in Japan suggested that the samples had been synthesized from ephedrines extracted from *Ephedra* plants or semisynthetic ephedrines but not from synthetic ephedrines. This isotope ratio analysis method should be useful to trace the origins of seized methamphetamine prepared from ephedrines.

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—Notes—

大麻種子の 2,3,5-Triphenyl-2*H*-tetrazolium Chloride (TTC) による発芽能力鑑定法

緒方 潤,[a] 花尻(木倉)瑠理,[a] 吉松嘉代,[b] 木内文之,[b] 合田幸広*,[a]

Detection Method for the Ability of Hemp (*Cannabis sativa* L.) Seed Germination by the Use of 2,3,5-Triphenyl-2*H*-tetrazolium Chloride (TTC)

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Cannabis plants show a high Δ^9-tetrahydrocannabinol content and are used as a psychoactive drug. Therefore the cultivation of hemp and its possession are prohibited by law in Japan. Meanwhile, *Cannabis* seeds have been used as a component of *shichimi-togarashi* (a Japanese spice), bird feed, or a crude drug (*mashinin*). To exclude the possibility of germination, it is officially noticed that hemp seeds must be killed. However, the number of violators has increased in recent years. To judge the ability of seed germination, a germination test is performed. However, the test requires several days and thus has not been used for on-site inspection. In this study, we developed a rapid detection method to determine the ability of *Cannabis* seeds to germinate using 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC). The principle of the assay is as follows. The endogenous respiratory enzymes in hemp seeds convert added colorless TTC into red 1,3,5-triphenylformazan. Consequently, a living embryo is stained red, while red does not appear in the dead seeds. The reaction was active over a pH range of 8.0–9.0, and the optimum activity was found from 40 to 50°C. Under the optimum conditions, we were able to determine the ability of seeds to germinate based on the presence of color within 20 min. Since this method is rapid and simple, it is applicable to on-site inspections. In addition, it could be used as an alternative technique to the germination test, because erroneous decisions is cannot occur under the assay principle.

Key words——*Cannabis sativa* L.; tetrazolium salt; germination test; hemp; 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC); marijuana

緒　　言

大麻 (cannabis, hemp, marijuana, marihuana) *Cannabis sativa* L. は中央アジア原産のアサ科の1年草で古くから世界各国で栽培され，繊維，食品，油，薬など様々な原料及び用途に利用されている.[1] 一方で，本植物には向精神作用があることが知られており，20世紀後半以降，幻覚剤としての濫用が日本を含め世界各国で大きな社会問題となっている.[2] 本植物は成分として麻酔性の強い Δ^9-テトラヒドロカンナビノール (酸) を含むことから，日本では大麻取締法により，その所持，栽培などが禁止されている.

しかし，本植物の種子 (果実：瘦果) は，江戸時代より「麻の実」として調味料 (七味唐辛子) に利用され，ペットショップなどでは「鳥のエサ」としても購入することができる．また，日本薬局方では，生薬「マシニン」(麻子仁：Hemp Fruit) が規定され，薬物として使用されている.[3] これらの点を踏まえ，大麻取締法では，大麻種子の所持や売買，流通に関しての規制を行ってはいないが，一般市場に流通している大麻種子 (果実) は加熱などによる発芽防止処理が施されており，水を与えても発芽することはない.

近年，大麻事犯は増加の一途を辿り，平成18年，大麻栽培による検挙者数は100人を超えている.[4] 一般に流通している大麻種子は上記の理由により発芽することはないが，発芽可能な種子が様々な方法で日本国内に持ち込まれていることも事実で

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ある．また，インターネット上などでは「麻の実標本」，「Cannabis Seeds」などとしてアサ（大麻）の種子が販売されており，これらの種子に高い発芽率が認められている．[5]

一般に種子の発芽力の有無は外観などからでは確認が困難であるため，発芽試験によって確認がなされている．当然ながら大麻種子の発芽能力を判定する方法としては，実際に発芽試験を行うことになるが，種を播種し，判定するにはある程度の日数が必要となる．したがって，大麻種子を所持した人間に対し，その場で，試験を実行することは不可能である．また，一度に多量の検体を処理するためには予試験法があることが望ましい．そこでこれらの諸事情を考慮し，一般市販試薬（テトラゾリウム塩類）を用いた，迅速かつ簡便な大麻種子の発芽能力（生死判別）を検定する方法（鑑別法）を確立したので報告する．

実験方法

1. 試料　発芽能力（発芽防止未処理）を有する大麻種子として，メキシコ産系統種子（以下，メキシコ種）(2007年収穫）及び栃木白の種子（以下，栃木白種）(2005年収穫）は㈱医薬基盤研究所薬用植物資源研究センター筑波研究部において栽培，収穫されたものを使用した．また，一般流通市場品試料（発芽防止処理済み）として都内ペットショップにて「鳥のエサ」（以下，飼料種子）及び食料品店にて「麻の実」（以下，食用種子）を購入し，実験に供試した．

2. 発芽試験　試料である各種子は流水にて十分に洗浄後，70%アルコールに1分間浸漬後，実験シャーレにMilliQ水を十分に含ませた脱脂綿をひいたものに播種し，常温にて放置した．培養時のカビの繁殖を防止するため極力，無菌的に操作した．

3. 呈色反応　2,3,5-Triphenyl-2*H*-tetrazolium chloride（TTC）は和光純薬工業より，1,3,5-triphenylformazan（TPF）は関東化学工業よりそれぞれ購入した．0.1%TTC水溶液を作成し，実験に使用する際の各種子は，胚を露出させるために粉砕し用いた．これらを直接，TTC水溶液500 μl中に入れ，60分間常温にて放置した．反応後は目視により発芽能力の有無を検討した．

4. 最適化条件の検討　反応温度，時間及びpHに関して詳細な検討を行った．反応温度に関する条件検討では反応時間を10分間とし，プレインキュベーション時間を2分間とした．反応時間の検討では，温度（4℃, 25℃及び45℃）についても検討に加えた．至適反応pHの測定には反応温度45℃，反応時間10分とし，酢酸–酢酸ナトリウム緩衝液（3–5），リン酸カリウム緩衝液（6, 7），トリス–塩酸緩衝液（8, 9），グリシン–水酸化ナトリウム緩衝液（10, 11）を用い，終濃度0.1Mとし測定を行った（カッコ内は使用pHを示す）．

これらすべての条件検討では，各種子1粒を1試験区とし，各種子を縦線に沿って2分割後，胚のみを全摘出して使用し，反応容量500 μlとして1.5 mlチューブ内で反応を行った．反応終了後，胚をMilliQ水にて3回洗浄し，胚に付着したTPFをメタノール：クロロホルム（2：1）混液1 mlを用いて溶出した．検出は島津UV-2550 UV-Visible Spectrophotometerを用い，480 nmの吸光度にて活性能力を検定した．

結果及び考察

1. 発芽試験　本研究で用いた各種大麻種子試料は，通常の発芽試験を行い，発芽能力の有無を確認した．その結果，発芽防止未処理の2種（メキシコ種及び栃木白種）は100%発芽が観察された（各20粒）．播種後10日間で発根及び根の伸長がすべての種子で確認された．特に収穫後の保存期間年数の短いメキシコ種は播種後の発芽速度は速く，5日程度で根の伸長までが確認された．また，一般流通市場品（発芽防止処理済み）としての飼料種子及び食用種子は播種後4週間を経過しても，発芽を確認することはできなかった（各200粒）．一般市場流通品の中には播種後，10%程度の確率で発根と思われる形態がみられたが，その後の根の伸長は観察されなかった．これは，播種，給水によって胚自体が膨潤し，種皮外に幼根が現れたものと考えられた．そこで，今回の発芽試験での能力検定は根の伸長までを確認して，その能力の有無を判断した．

2. 呈色反応　大麻種子試料4種（メキシコ種，栃木白種，飼料種子，食用種子）を用い，TTC[6–8]による呈色試験を行った．発芽能力を検討する方法としては，本実験のテトラゾリウム塩類［TTC，3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-

2*H*-tetrazolium bromide (MTT), 3,3′-[3,3′-dimetoxy-(1,1′-biphenyl)-4,4′-diyl]-bis[2-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride] (Nitro-TB) など] 以外に, iodine-potassium iodide (IKI)[8] 3-amino-7-dimethylamino-2-methyphenazine hydrochloride (Neutral Red),[9] Evans Blue[10] による呈色試験法があるが, 簡便性と正確性[8]においてTTCを含むテトラゾリウム塩類が優れていると考えられ, 今回はTTC法を用いることにした. 本法による鑑定は, 種子胚 (芽) 中の呼吸系酵素活性によってTTCが還元されTPF (赤色物質) に変換し (Fig. 1), 結果として胚が着色することを肉眼で判定するものである. その結果, メキシコ種 (54粒) 及び栃木白種 (180粒) において100%赤色の着色が観察された. これらの着色は明確に赤色と判定できるものであり, 「薄く」や「淡く」等の状態の着色はみられなかった (Fig. 2). また, 上記2種に着色の差はみられなかった. なお, 種子の成熟及び保存時に虫による食害にあったものや, 胚自体が腐敗や酸化によって褐色に変化したものについては除外した.

一般流通市場品の種子では100%着色がみられなかった (各100粒). また, オートクレーブ処理 (120℃, 10 min) したメキシコ種及び栃木白種でも同様に着色はみられなかった. これらの結果は胚の成分などが着色に影響を与えるものではないということを示している. 基質濃度0.1, 0.2, 0.5及び1.0%のTTC溶液を実験に用いたが, 陽性反応において違いはみられなかった. テトラゾリウム塩類は, 高濃度で染色が鋭敏になるものの, 陰性判定においても長時間で淡く着色がみられることから, 0.1% TTC溶液を標準液として以下の実験にも使用した (data not shown). 一連の実験でみられる着色は植物組織 (胚組織) のみで, 溶液の色が変化するものではない.

3. 最適化条件の検討 本法の大麻種子における最適条件を検討するために, 反応後の着色胚からTPFをメタノール：クロロホルム (2：1) で抽出し, 分光光度計 (480 nm) により定量する方法を採用した. 市販のTPFのスペクトル解析の結果においても480 nm付近に極大が存在した.

本反応は生きた細胞や組織が有する酵素による還元力を利用 (二次反応) したものである. そこで, この酵素反応の至適条件を検討するために, 反応温度, 時間及びpHについて調査した. 温度は20–70℃で10℃ずつ変化させ反応時間10分で測定した. 40–50℃の間で最大活性が得られることが分かった (Fig. 3).

反応時間は温度 (4℃, 25℃及び45℃) の条件も考慮して行った. 25℃及び45℃では時間の経過とともに活性 (着色) には直線的上昇がみられ, 25℃ (常温) よりも45℃の方が約3倍強い活性が得られている (Fig. 4). これらの結果を踏まえると鑑別法として加温することは早期判定にも重要であると考えられる. また, 胚全体をみると, 着色の部位的 (幼芽や幼根など) 差はみられないが, 胚の外側 (種皮と接している面) 部分に早期の着色がみられた. 4℃での反応では活性の時間的上昇がみられなかった. これは生細胞の酵素反応 (活動) が停止していることに起因するものと考えられる.

至適pHは各緩衝液を用いpH 3–11の間で検討した. pH 8–9付近で活性は最大となった (Fig. 5). これらの結果は一般的な植物由来酵素の性質と大きな違いはみられなかった. また, TTCが植物生体内酵素の直接的基質となることは考え難く, 本反応は細胞呼吸における電子伝達系酵素のプロトンの放出や流入などに関与しているとも考えられる.[11] な

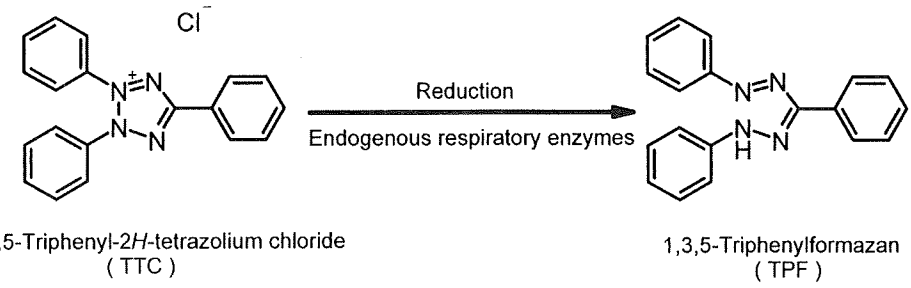

Fig. 1. Proposal Reaction Scheme of TTC to TPF
TTC is a colorless material and TPF is red. This reaction is caused by the endogenous respiration enzymes in embryo cells.

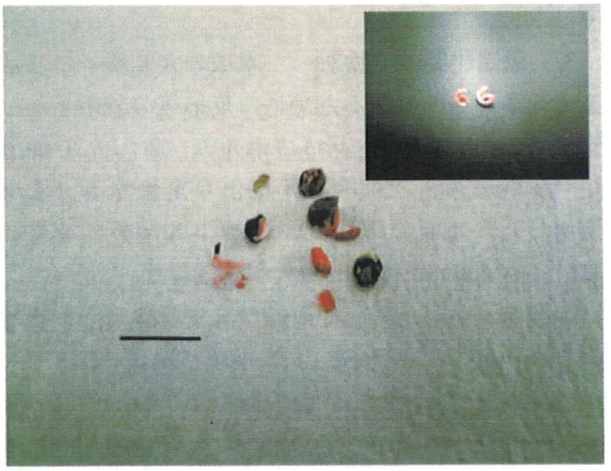

Fig. 2. Hemp Seed "Strain Mexico" by Use of TTC Method after 60 min at Room Temperature

The crushed seeds were directly entered into the TTC solution. The black bar shows a 1 cm scale. Right upper photo: an embryo sectioned from seed coat.

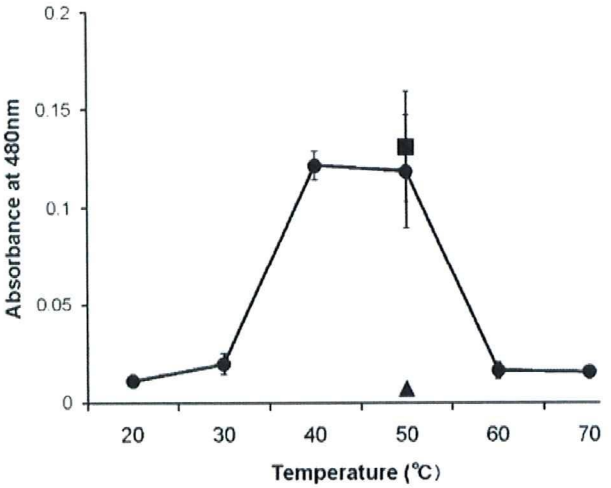

Fig. 3 Effect of Temperature on TTC Activity Using Hemp Seeds

The incubations were carried out under standard conditions. The data are the mean averages from six independent experiments ±S.D. Absorbance (480 nm) of 1 ml extract from an embryo (Ave. weight at 0.02 g). ●: strain Mexico, ■: cv. Tochigi-shiro, ▲: bird feed.

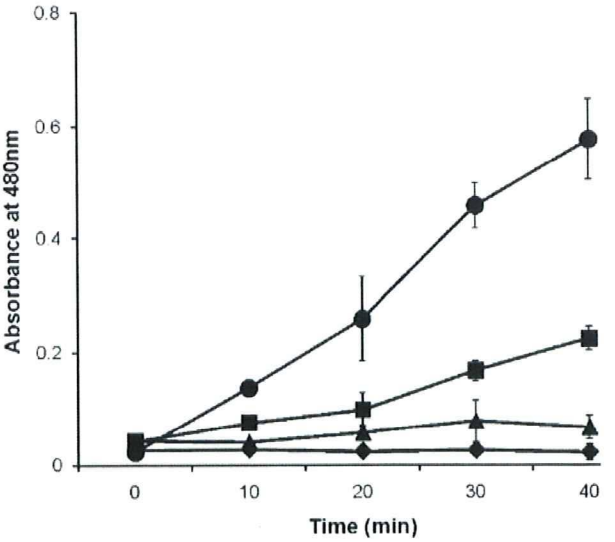

Fig. 4. Formation of TPF from TTC as a Function of Time

The incubations were carried out under standard conditions. The data are the mean averages from six independent experiments ±S.D. Absorbance (480 nm) of 1 ml extract from an embryo (Ave. weight at 0.035 g). ●: cv. Tochigi-shiro (45℃), ■: cv. Tochigi-shiro (25℃), ▲: cv. Tochigi-shiro (4℃), ◆: bird feed (45℃).

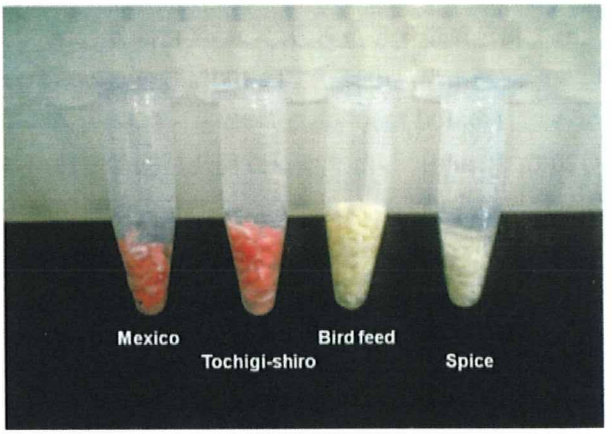

Fig. 6. The Visual Judgment of the Ability of Hemp Seeds Germination

The incubations were carried out below; 45℃, pH 9.0, 20 min.

お，強アルカリ条件下での保存では，溶液が淡い赤色に変化した．また，Evans Blue 試薬は細胞膜の選択透過性が失われると細胞内へ流入する染色剤であり，細胞の生死判別に用いられる試薬であるが，大麻種子での生死には明確な判定は下せなかった．現在までに TTC を含め，MTT や Nitro-TB など多くのテトラゾリウム塩類を用いた細胞生死判定指示薬が試薬メーカーから生産・販売されているが，試験研究機関以外の場所での，目視による迅速簡易鑑別という点では，反応指示液そのものが無色透明であること，その反応（判定）産物が水に不溶であること，陽性，陰性の差が色の違いではなく有無であること，誤判定を回避するために，反応産物の色が植物本来の組織の色と異なること等が挙げられる．これらの点を踏まえると，今回用いた TTC は大麻種子判定試験において，最も有効な試薬の 1 つであると考えられた．

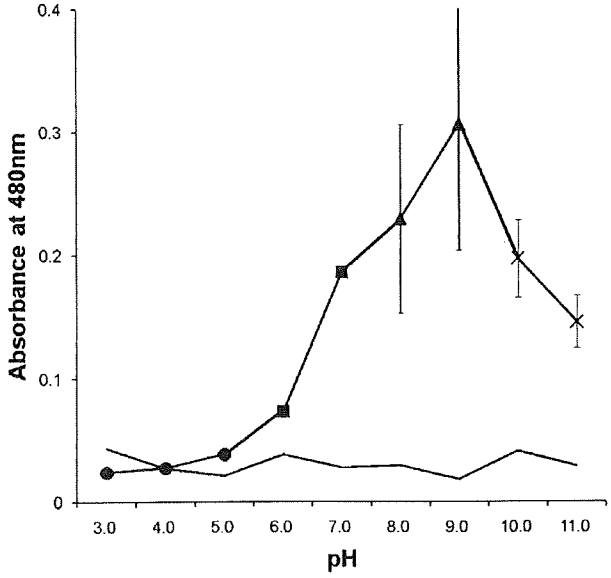

Fig. 5. Effect of pH on TPF Formation Using cv. Tochigi-shiro

The reaction mixture was incubated for 10 min and contained in a total volume 500 μl: An embryo was sectioned from a seed, 250 μl 0.2% TTC solution, 250 μl 0.2M each buffer. The data are the mean averages from six independent experiments ±S.D. Absorbance (480 nm) of 1 ml extract from an embryo (Ave. weight at 0.035 g). ●: Acetate buffer (3–5), ■: Phosphate buffer (6, 7), ▲: Tris-HCl buffer (8, 9), ×: Glycine-NaOH buffer (10, 11), —: Bird feed.

結　　論

TTC を用いた本法は，温度 45℃, pH 9.0, 20 分間の反応において迅速かつ明確に，その発芽能力を鑑定することが可能であると考えられた (Fig. 6). また，0.1%TTC 水溶液は暗所常温にて 2 ヵ月程度は使用に際して問題はなかった．種子を実際に発芽させる従来の試験では，発芽能力鑑定に数日以上かかる．一方，本法は，短時間で発芽能力を判定することができ，犯罪捜査の現場での試験に利用可能である．また，結果が非常に明確で，その原理を考えると誤判定の可能性がほとんどない．したがって，本法は，大麻種子の発芽能力鑑定法として，従来の発芽試験に変え得る可能性のある優れた手法であることが明らかとなった．

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4-Hydroxy-3-methoxymethamphetamine Glucuronide as a Phase II Metabolite of 3,4-Methylenedioxymethamphetamine: Enzyme-Assisted Synthesis and Involvement of Human Hepatic Uridine 5′-Diphosphate-Glucuronosyltransferase 2B15 in the Glucuronidation

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3,4-Methylenedioxymethamphetamine (MDMA), one of the most popular illicit recreational drugs, is metabolized primarily into 4-hydroxy-3-methoxymethamphetamine (HMMA) by drug-metabolizing enzymes. HMMA is further metabolized by phase II enzymes to give the glucuronide or sulfate which is excreted into urine. In the present study, enzyme kinetic studies with various microsomes showed that rat liver microsomes pretreated with Aroclor 1254 were most suitable for the enzyme-assisted synthesis of the glucuronide (HMMA-Gluc). This method selectively produced the β-anomer of HMMA-Gluc in a very high, isolated yield (71%), and with a purity that was sufficient for use in an analysis of MDMA intake and for enzyme kinetic studies. We also identified, by an LC-MS method, the human uridine 5′-diphosphate-glucuronosyltransferase (UGT) isoforms that catalyze the glucuronidation of HMMA. Among 12 isoforms of human recombinant UGT expressed in insect cells, UGT2B15 was the only isoform that showed adequate enzymatic activity in catalyzing HMMA glucuronidation with K_m and V_{max} values of 3.8 mM and 1.6 nmol/min/mg protein, respectively. The finding that UGT2B15 is capable of HMMA glucuronidation suggests this isoform may have an important *in vivo* role in human MDMA metabolism.

Key words 3,4-methylenedioxymethamphetamine; enzyme-assisted synthesis; glucuronide; uridine 5′-diphosphate-glucuronosyltransferase

The abuse of amphetamine-like designer drugs, which at present is a serious public health problem, is increasing among young adults. Although originally examined as an adjunct in psychotherapy, 3,4-methylenedioxymethamphetamine (MDMA), known in drug slang as "Ecstasy," has recently become the most popular recreational drug worldwide.[1—3]

The metabolic pathways of MDMA have been elucidated and over a dozen metabolites have been identified in animals and humans. The major MDMA metabolism pathway involves demethylation to 3,4-dihydroxymethamphetamine (DHMA) followed by *O*-methylation to 4-hydroxy-3-methoxymethamphetamine (HMMA) as shown in Chart 1. For unequivocal proof of MDMA use, detection of these metabolites along with MDMA from a urine sample analysis is required. HMMA exists as a major metabolite in urine,[4—8] whereas only a few studies concerning MDMA conjugates as phase II metabolites have been reported. The HMMA level in urine has been reported to increase after acid/enzymatic hydrolysis, suggesting that the majority of MDMA metabolites are excreted into urine as the glucuronide or sulfate of HMMA.[9] If these conjugates were more readily available, they could be used as analytical standards to obtain further unequivocal proof of MDMA use. The synthesis of HMMA glucuronide (HMMA-Gluc) was reported by Shima *et al.*,[10] but the yield by chemical synthesis was only 6%. For analysis of MDMA metabolites and for enzyme kinetic studies of the glucuronidation process, a more practical method to prepare HMMA-Gluc is needed. In this study, we describe the first enzyme-assisted synthesis of HMMA-Gluc using Aroclor 1254-induced rat liver microsomes. This method was easily performed and stereoselectively produced the β-anomer conjugate in very high yield. In addition, using the synthetic HMMA-Gluc, we investigated the glucuronidation activities of human uridine 5′-diphosphate-glucuronosyltransferase (UGT) isoforms toward HMMA. In a series of UGT1A and 2B isoforms, UGT2B15 was found to catalyze

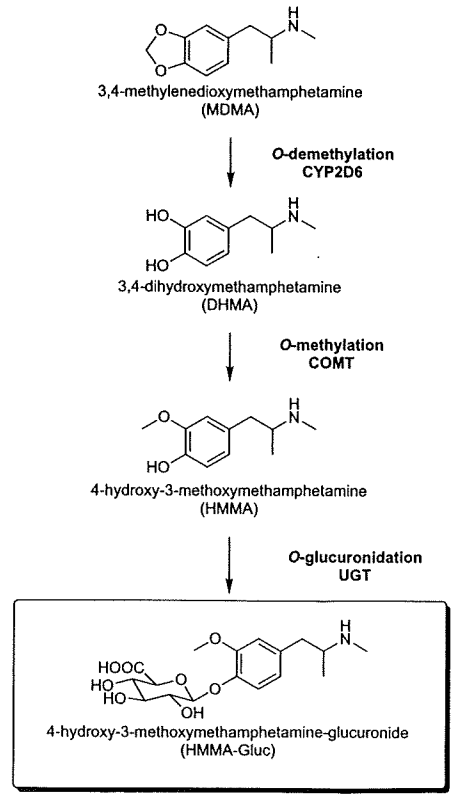

Chart 1. Principal Metabolic Pathway of MDMA in Humans

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the glucuronidation of HMMA.

Experimental

General Information Uridine 5′-diphosphoglucuronic acid (UDPGA) and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other reagents and solvents were purchased from Wako Pure Chemical (Osaka, Japan), Tokyo Kasei Kogyo (Tokyo, Japan), and Kanto Chemical (Tokyo, Japan), and were used without purification. Male Sprague-Dawley rat liver microsomes (Lot No. JJS) and Aroclor 1254-induced Male Sprague-Dawley rat liver microsomes (Lot No. ADM) were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.). Pooled human liver microsomes (Lot No. 70196) and microsomes from baculovirus-insect cells expressing UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17 were purchased from BD Gentest (Woburn, MA, U.S.A.). Control experiments were carried out using microsomes from insect cells infected with wild type baculovirus purchased from BD Gentest.

Analytical HPLC was performed using a CBM-20A system controller, LC-20A pump, SPD-M20A UV/Vis photodiode array detector, and CTO-10AC column oven (Shimadzu, Kyoto, Japan) equipped with a CAPCELL PAK C18 MGII 5 μm, 4.6×250 mm (Shiseido, Tokyo, Japan). The mobile phases were A: 0.1% trifluoroacetic acid (TFA)/H_2O and B: 0.1% TFA/CH_3CN. Preparative HPLC was performed using a SSC-6600 gradient controller, SSC-3465 pump, SSC-5410 UV/Vis detector and SSC-3465 column oven equipped with a SenshuPak PEGASIL ODS column, 5 μm 20×250 mm (Senshu Kagaku, Tokyo, Japan). The mobile phases were A: 0.1% TFA/H_2O and B: 0.1% TFA/CH_3CN. LC-MS was performed using a Dual λ Absorbance Detector 2487, micromass ZQ and an Alliance model 2695 (Waters, Milford, MA, U.S.A.) equipped with a CAPCELL PAK C18 MGII 5 μm, 4.6×250 mm (Shiseido). The mobile phases were composed of A: 0.1% HCOOH/H_2O and B: CH_3CN. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian AS 400 Mercury spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)-propionate-2,2,3,3,-d_4 (δ scale). High resolution mass spectra were obtained on a LTQ Orbitrap (Thermo Fischer Scientific, Waltham, MA, U.S.A.).

Synthesis of HMMA The synthesis of HMMA was carried out according to previous reports,[11,12] with slight modifications. A solution of 5.0 g of 4-hydroxy-3-methoxyphenylacetone in anhydrous methanol (50 ml) was added to a solution of 12 ml (117 mmol) of 40% methylamine in methanol solution, followed by the addition of 2.65 g (42 mmol) of $NaBH_3CN$ in several portion. The solution was adjusted to pH 6 with 2.0 M HCl and stirred at room temperature for 24 h under N_2. The mixture was then poured into 100 ml of water, adjusted to pH 2 with concentrated HCl, and stirred at room temperature for 1 h. After evaporation to remove methanol, the aqueous layer was washed with ether, basicified by the portionwise addition of NaOH until pH>12, and extracted twice with ether. The organic layer was dried over $MgSO_4$, concentrated *in vacuo*, and 2.0 M HCl in ether was added until efficient precipitation was observed. The precipitate was collected and recrystallized from ethanol to afford HMMA as a white solid (HCl salt, 3.44 g, yield 53%). mp 217—218 °C, ^{1}H-NMR (CD_3OD) δ: 1.01 (d, J=6.4 Hz, 3H), 2.34 (s, 3H), 2.46 (dd, J=7.0, 12.8 Hz, 1H), 2.66—2.77 (m, 2H), 3.81 (s, 3H), 6.60 (dd, J=2.0, 8.0 Hz, 1H), 6.72—6.74 (m, 2H), ^{13}C-NMR (CD_3OD) δ: 18.9, 33.5, 43.3, 56.3, 57.7, 113.8, 116.4, 122.7, 131.4, 146.4, 149.1, MS (ESI) 196 $[M+H]^+$, 165 $[M+H-NHCH_3]^+$, HR-MS (ESI) Found 196.1334, Calcd for $C_{11}H_{18}O_2N^+$ 196.1332.

Enzyme-Assisted Synthesis of HMMA-Gluc Ten milliliters of a buffer solution containing 50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 25 μg/ml alamethicin, 5 mM UDPGA, and 10 mM HMMA was stirred in a 37 °C water bath, and the reaction was started by the addition of 200 μl of Aroclor 1254-induced rat liver microsomes (24 mg protein/ml) and stirred continuously for 20 h. The reaction was stopped with 5 ml of 10% $HClO_4$. The precipitated proteins were removed by centrifugation (3000 rpm, 10 min, 4 °C), and the supernatant was filtered. The filtrate was purified by preparative HPLC, and after evaporation of the fraction containing the product, HMMG-Gluc was obtained as a white solid (13.2 mg, yield 71% from UDPGA). ^{1}H-NMR (D_2O) δ: 1.27 (d, J=6.4 Hz, 3H), 2.69 (s, 3H), 2.87 (dd, J=7.2, 14.0 Hz, 1H), 3.00 (dd, J=7.2, 14.0 Hz, 1H), 3.49—3.54 (m, 1H), 3.62—3.69 (m, 3H), 3.88 (s, 3H), 3.99 (d, J=9.2 Hz, 1H), 5.14 (d, J=7.2 Hz, 1H), 6.86 (dd, J=8.4, 2.0 Hz, 1H), 6.99 (d, J=2.0 Hz, 1H), 7.14 (d, J=8.4 Hz, 1H), ^{13}C-NMR (D_2O) δ: 15.0, 30.2, 38.5, 56.0, 56.5, 71.2, 72.6, 74.6, 75.2, 100.6, 114.0, 116.9, 122.3, 131.7, 144.5, 149.2, 172.0, MS, (ESI) 372 $[M+H]^+$, 196 $[M+H\text{-glucro}]^+$, 165 $[M+H\text{-glucro-}NHCH_3]^+$, 370 $[M-H]^-$, HR-MS (ESI) Found 372.1659, Calcd for $C_{17}H_{26}O_8N^+$ 372.1653.

Assay for HMMA Glucuronidation Using Microsomes Glucuronidation of HMMA by three kinds of microsomes was determined by HPLC. The incubation mixture (50 μl) contained 50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 25 μg/ml alamethicin, 20 mM UDPGA, microsomes and HMMA. Concentrations of microsomal proteins were 0.48 mg protein/ml for Aroclor 1254-induced rat liver microsomes, 0.48 mg protein/ml for non-induced rat liver microsomes, and 0.5 mg protein/ml for human liver microsomes. HMMA concentrations varied from 1.0 to 40 mM. Incubations were performed in a water bath at 37 °C, for 10 min with Aroclor 1254-induced and non-induced rat liver microsomes, and for 30 min with human liver microsomes. The enzyme assays were terminated by the addition of 50 μl of 10% $HClO_4$, briefly vortexed, and then centrifuged at 3000 rpm and 4 °C for 10 min. The supernatants were injected into the HPLC. The absorbance of HMMA-Gluc at 275 nm was detected, and the peak area was determined for kinetic analysis. The apparent K_m and V_{max} were estimated by analyzing Michaelis–Menten plots using KaleidaGraph ver. 4.0 software (Synergy Software, Reading, PA, U.S.A.).

UGT Assay for HMMA Glucuronidation Enzyme assay mixtures (50 μl final volume) contained 50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 25 μg/ml alamethicin, 5 mM UDPGA, 5 mM HMMA, and 1 mg protein/ml of microsomes expressing UGT isoforms or control microsomes. Incubation was performed for 120 min at 37 °C and was terminated by the addition of 50 μl of 10% $HClO_4$. The mixture was briefly vortexed, then centrifuged at 3000 rpm and 4 °C for 10 min. The supernatants were analyzed by LC-MS. The mass spectrometer was operated in the selected ion monitoring mode using the $[M+H]^+$ ion, m/z 372, for HMMA-Gluc.

The kinetic parameters for UGT2B15 processing of HMMA were estimated by analyzing Michaelis-Menten plots using KaleidaGraph ver. 4.0 software.

Results

Enzyme-Assisted Synthesis of HMMA-Gluc In the case of catechol derivatives, large scale (mg) syntheses of their glucuronides have been accomplished by an enzyme-associated method using rat liver microsomes pretreated with Aroclor 1254, a mixture of polychlorinated biphenyls, as biocatalyst.[13] We applied this method to the synthesis of HMMA-Gluc because of the structural similarities between HMMA and catechol. Concentrations of UDPGA and HMMA were 5 mM and 10 mM, respectively and alamethicin, a pore-forming peptide, was added to the reaction system to activate UGT in the liver microsomes without affecting CYP activity.[14]

A representative chromatogram of the reaction mixture is shown in Fig. 1. During the course of the reaction, peak C at 7.2 min was found to increase with a concomitant decrease in HMMA, peak D, at 9.2 min. The purification of peak C was therefore carried out by preparative HPLC after protein precipitation. After elimination of the solvent, a white solid was obtained. From analysis of ^{1}H-NMR, ^{13}C-NMR, and MS spectra, the structure of the product was determined to be HMMA-Gluc. The advantage of enzyme-assisted synthesis is that the formation solely of biologically relevant stereo- and

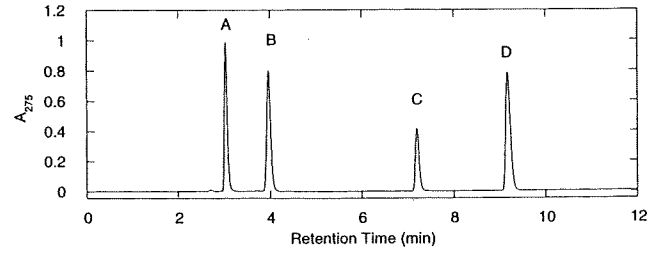

Fig. 1. HPLC Chromatogram after Enzymatic Synthesis

A and B, by-products derived from microsomes or UDPGA; C, HMMA-Gluc; D, HMMA.