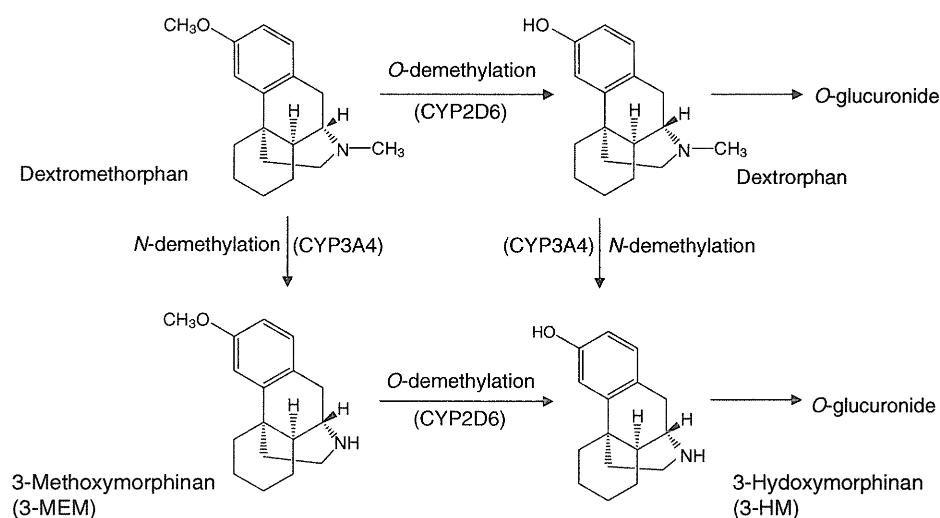

Fig. 2 Main metabolic pathway of dextromethorphan in humans

spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface. Quantification was performed using multiple reaction monitoring of the transitions of precursor ions to product ions with each cone voltage and collision energy as shown in Table 1. The optimal MS parameters obtained were as follows: capillary 3.0 kV, source temperature 120 °C, and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas, with a flow rate of 800 and 50 L/h, respectively. Argon was used as the collision gas, with a flow rate of 0.25 mL/min. All data collected in the centroid mode were processed using MassLynx™ NT4.1 software with a QuanLynx™ program (Waters, Milford, MA, USA).

Since the standard compounds of (−)-3-MEM and (−)-3-HM were not available, these peaks were confirmed by comparison of their retention times and mass fragmentations with those of the standard compounds of the dextro forms ((+)-3-MEM and (+)-3-HM) using an ODS column. The analyses were performed using an Acquity HSS T3 column (100×2.1 mm i.d., 1.8 μm) from Waters (Milford, MA, USA). The column temperature was maintained at 40 °C, and the following gradient system was used with a mobile phase A (1% formic acid) and mobile phase B (1% formic acid/acetonitrile) delivered at 0.3 mL/min: 90% A/10% B (0 min)–70% A/30% B (8 min). The MS parameters were the same as for the analyses using the chiral column described above.

Animal experiments

The animal experimental model was designed as shown in our previous reports [33, 34]. All experiments were carried out with the approval of the Committee for Animal Care and Use of the National Institute of Health Sciences, Japan. Dextromethorphan hydrobromide (dissolved in an isotonic sodium chloride solution, 2.5 mg/mL, rat 1–3) or levomethorphan (dissolved in a mixed solution of 5% Emulphor™ EL-620/5% ethanol/90% isotonic sodium chloride solution, 2.5 mg/mL, rat 4–6) was administered to male DA pigmented rats, which were 5 weeks old and around 90 g mean weight (Japan SLC, Shizuoka, Japan). The drugs were given once daily at 5 mg/kg by intraperitoneal injection for ten successive days. Blood samples were collected 5, 15, 30, 60, 120, and 360 min after the first administration from the orbital vein plexus. Plasma samples were prepared by centrifugation at 10,000×*g* for 3 min and stored at −20 °C until analysis. The area under the plasma concentration time curve (AUC) was calculated by the conventional method. Urine samples were collected 0–24, 24–48, and 48–72 h after the last administration and stored at −20 °C. Each animal had been shaved on the back just before the first drug administration. The new growing hair samples were collected 28 days after the first administration.

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

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

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

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

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

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

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

Extraction of parent compounds and their metabolites from rat hair samples

Hair samples were washed three times with 0.1% sodium dodecyl sulfate under ultrasonication, followed by washing three times with water under the same condition. After the sample was dried under a nitrogen stream at room temperature, approximately 10 mg of finely cut hair was precisely weighed and extracted with 1 mL of methanol/ 5 M hydrochloric acid mixed solution (20:1) containing 50 μL of the IS methanol solution for 1 h under ultrasonication. Following overnight storage at room temperature, the hair was filtered off, the filtrate was evaporated with a nitrogen stream, and the residue was dissolved in 1 mL of distilled water. The solution was treated with an OASIS HLB column and analyzed as described above.

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

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

The drug concentrations in the samples were calculated using the peak–area ratios of the ions monitored for the target compounds versus IS. The calibration curves for the determination were constructed by analyzing extracted drug-free control samples spiked with the standard solution, as described above. The calibration samples containing 0, 1, 2, 4, 20, 40, 200, and 400 ng/mL of the target drugs for the rat plasma, 0, 5, 10, 50, 100, 500, 1,000 2,500, 5,000, and 10,000 ng/mL for the urine samples and 0, 0.1, 0.5, 1.0, 5.0, 10, 25, and 50 ng/mg for the hair samples were prepared just before analysis. The limit of quantitation (LOQ) of each drug was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance, while the limit of detection (LOD) was defined as concentrations in a sample matrix resulting in peak areas with signal-to-noise ratios (*S/N*) of 3.

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

Demethylation of dextromethorphan/levomethorphan in rat and human liver microsomes

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

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

Results

Chiral separation of dextromethorphan/levomethorphan and their metabolites

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

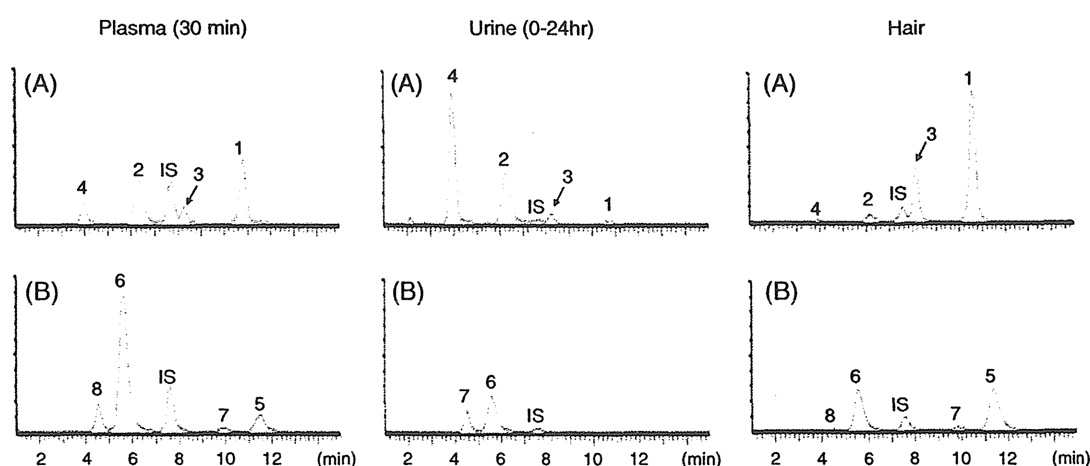

Fig. 3 LC-MS/MS total ion current (TIC) chromatograms (MRM mode) of the extracts from plasma, urine, and hair of rats administered with (**A**) dextromethorphan and (**B**) levomethorphan using a chiral column. *1* Dextromethorphan, *2* Dextrorphan, *3* (+)-3-MEM, *4* (+)-3-HM, *5* Levomethorphan, *6*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

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

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

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

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

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

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

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

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

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

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

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

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

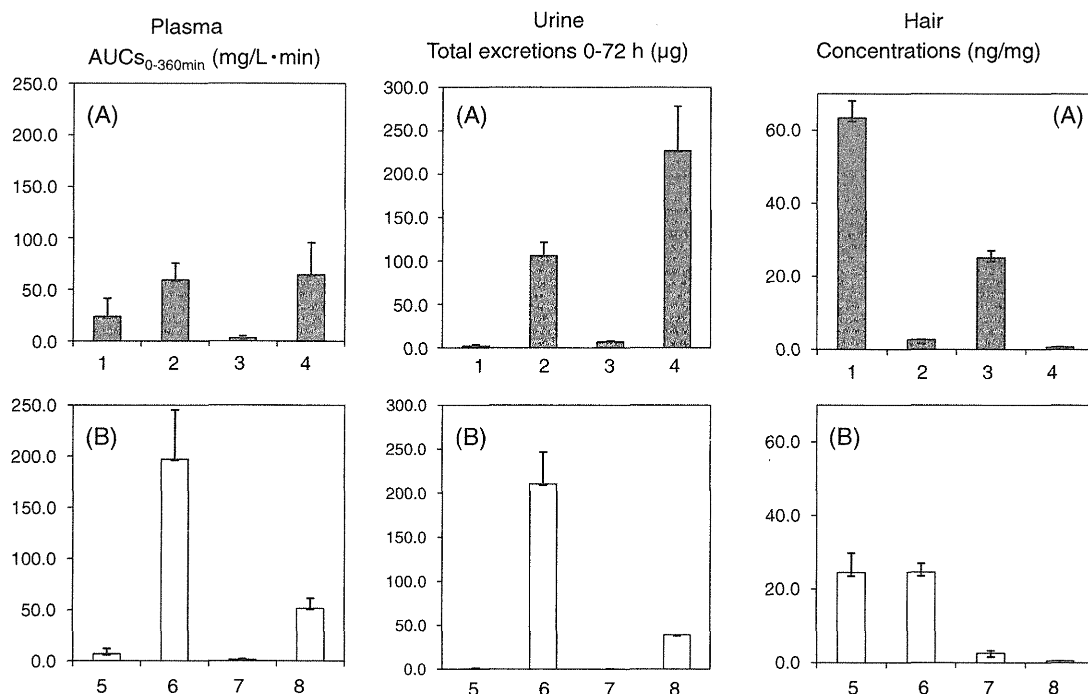

Fig. 4 Rat plasma $AUCs_{0-360min}$, total excretions into rat urine, and concentrations in rat hair of parent compounds and their metabolites after administration of (**A**) dextromethorphan and (**B**) levomethorphan. *1* Dextromethorphan, *2* Dextrorphan, *3* (+)-3-MEM, *4* (+)-3-HM, *5* Levomethorphan,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

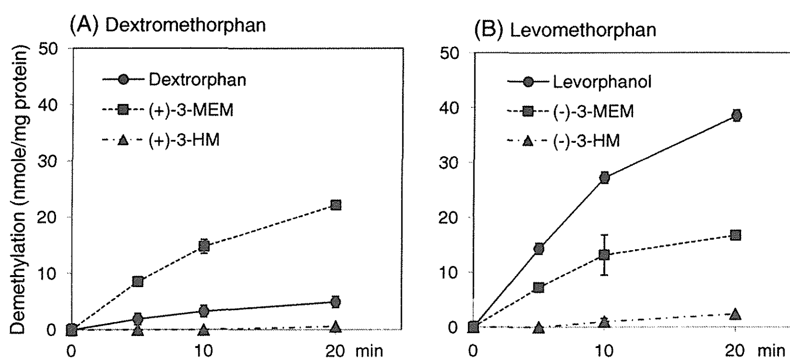

Fig. 5 Demethylation of (**A**) dextromethorphan and (**B**) levomethorphan in DA rat liver microsomes

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

Demethylation of dextromethorphan/levomethorphan in DA rat liver microsomes

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

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

Demethylation of dextromethorphan/levomethorphan in pooled human liver microsomes

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

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

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

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

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

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

Discussion

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

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

In the DA rat samples, obvious differences in the ratios of the metabolites were found between the dextro and levo forms. These differences were most clearly detected in the hair samples. The concentrations of the parent compounds, their *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites were 63.4, 2.7, 25.1, and 0.7 ng/mg for the dextro forms and 24.5, 24.6, 2.6, and 0.5 ng/mg for the levo forms, respectively. In order to investigate the differences of their metabolic properties between dextromethorphan and levomethorphan, DA rat and human liver microsomes were studied. As a result, we have shown the enantioselective metabolism of levomethorphan, not only in DA rats but also in human liver microsomes, especially with regards to the *O*-demethylation. Because it is well-known that CYP2D6 (mainly related to *O*-demethylation of dextromethorphan) is polymorphically expressed in humans, it may be difficult to discuss the enantioselective metabolism in humans who can be classified as poor, intermediate and extensive metabolizers of dextromethorphan. In future studies, the metabolic properties of these drugs using CYP2D6 enzymes (having a variety of phenotypes) should be examined to clarify the effects of their genotypes on the enantioselective *O*-demethylation of levomethorphan observed in this study.

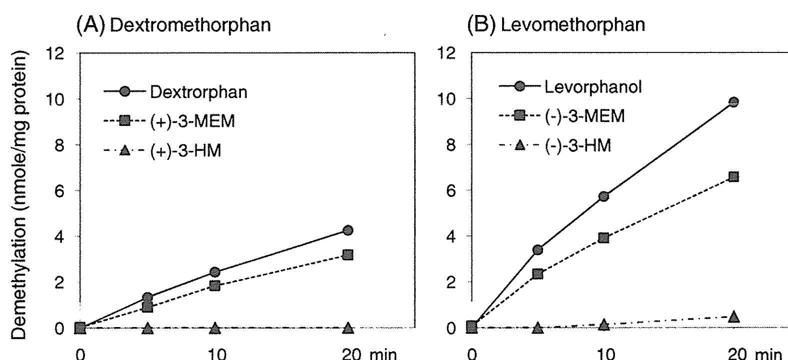

Fig. 6 Demethylation of (**A**) dextromethorphan and (**B**) levomethorphan in human liver microsomes

Conclusions

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

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—Note—

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

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

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

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

緒　　言

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

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

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

実 験 方 法

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

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

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

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

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

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

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

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

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

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

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

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

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

結果及び考察

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

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

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

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

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

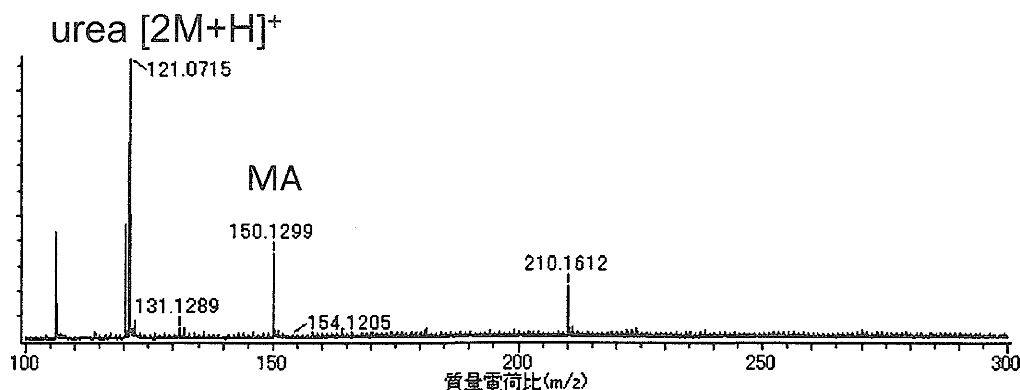

Fig. 1. DART-TOFMS Mass Spectrum of the Urine Sample Spiked with MA (20 μg/ml) without Any Pretreatments

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

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

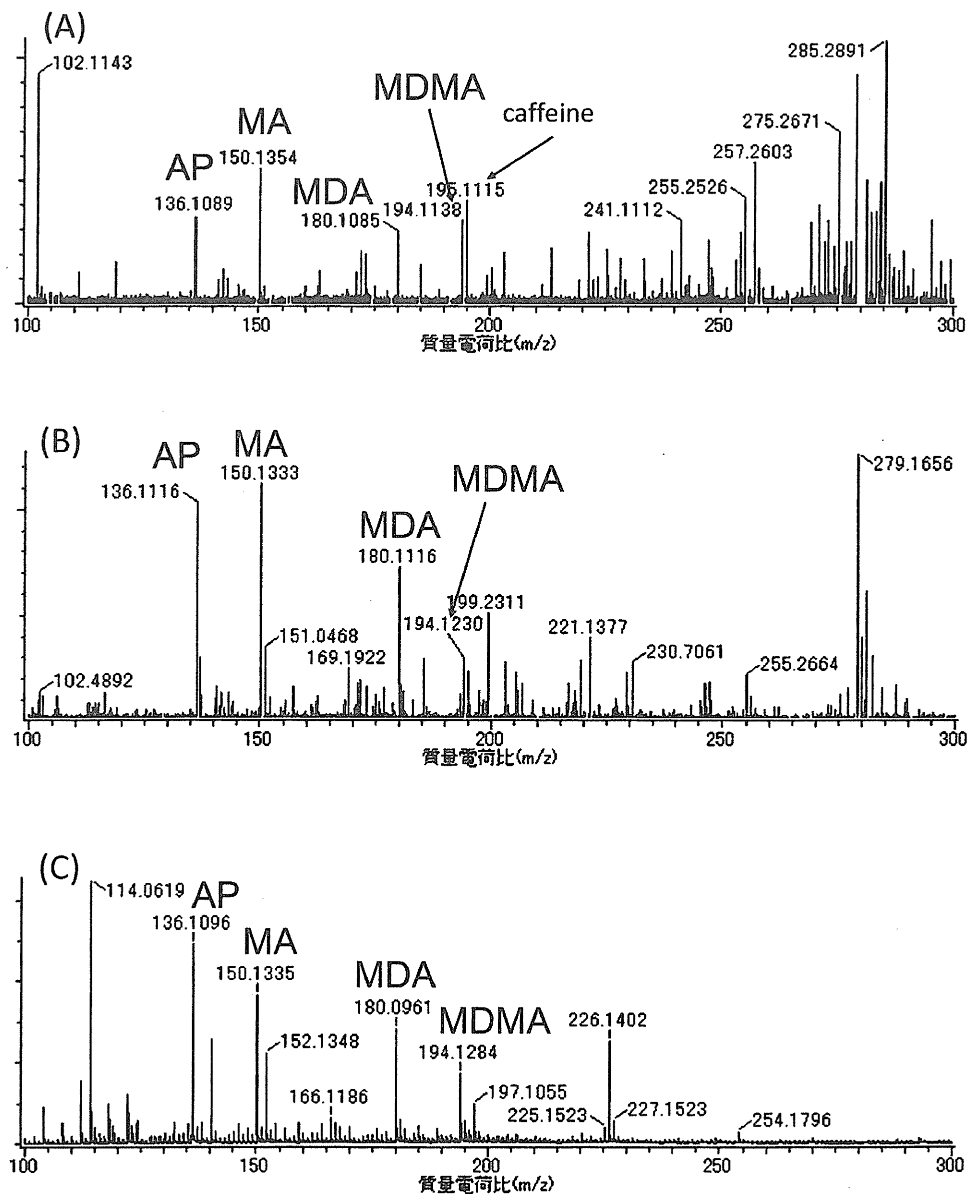

Fig. 2. DART-TOFMS Mass Spectra of the Extracts from the Urine

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

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

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

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

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

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

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

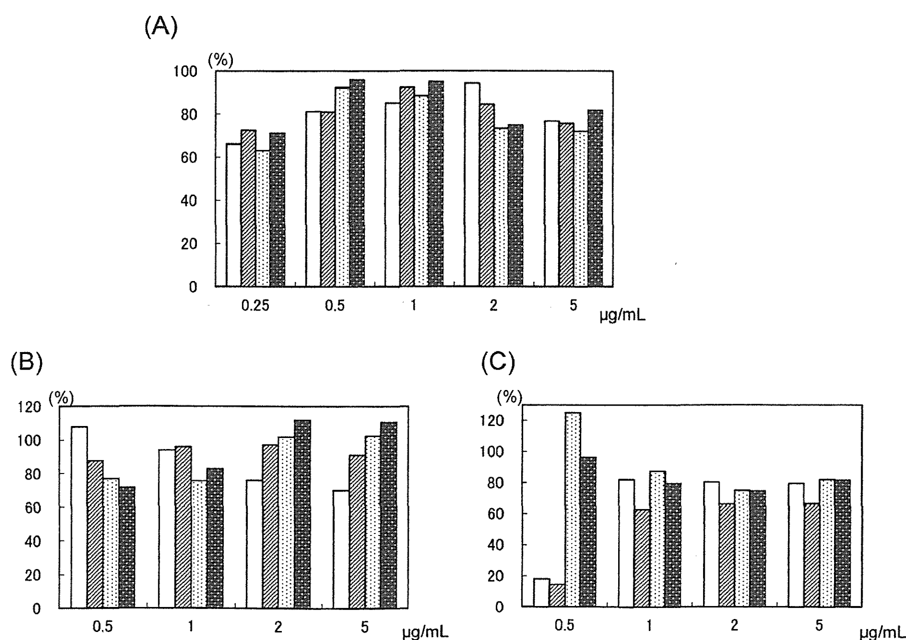

Fig. 3. Recoveries of Targeted Compounds
AP (□), MA (▨), MDA (⬚) and MDMA (▩). (A) from urine samples extracted by C18 pipette tip-type solid-phase extraction, (B) a liquid-liquid extraction with a mixed solution of dichloromethane and hexane and (C) treated with urease.

各化合物の回収率が大きくばらついた [Fig. 3(C)].

結　　論

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

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

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A survey of the potency of Japanese illicit cannabis in fiscal year 2010

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ABSTRACT

In recent years, increased 'cannabis potency', or Δ^9-tetrahydrocannabinol (THC) content in cannabis products, has been reported in many countries. A survey of Japanese illicit cannabis was conducted from April 2010 to March 2011. In Japan, all cannabis evidence is transferred to the Minister of Health, Labour and Welfare after criminal trials. The evidence was observed at Narcotics Control Department offices in major 11 cities. The total number of cannabis samples observed was 9072, of which 6376 were marijuana. The marijuana seizures were further classified, and it was found that in terms of the number of samples, 65.2% of them were seedless buds, and by weight 73.0% of them were seedless buds. Seedless buds were supposed to be 'sinsemilla', a potent class of marijuana. THC, cannabinol (CBN) and cannabidiol (CBD) in marijuana seizures exceeding 1 g were quantified. The number of samples analyzed was 1115. Many of them were shown to contain CBN, an oxidative product from THC. This was a sign of long-term storage of the cannabis and of the degradation of THC. Relatively fresh cannabis, defined by a CBN/THC ratio of less than or equal to 0.1, was chosen for analysis. Fresh seedless buds (335 samples) contained an average of 11.2% and a maximum of 22.6% THC. These values are comparable to those of 'high potency cannabis' as defined in previous studies. Thus, this study shows that highly potent cannabis products are distributed in Japan as in other countries.

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1. Introduction

In recent years, increased 'cannabis potency', or Δ^9-tetrahydrocannabinol (THC) content in cannabis products, has been reported in many countries such as the USA [1], UK [2,3], Netherlands [4], Germany [5], Italy [6], and New Zealand [7]. The World Drug Report 2011 by United Nations Office on Drugs and Crime (UNODC) noted that the average concentration of THC is presently at higher levels than 10–15 years ago; however, data for the past five years show a stable trend in some countries, although the pattern is not consistent for all products and all countries [5]. Two reviews on cannabis potency mention that the data on this issue are still not adequate and further research is required [8,9].

The higher potency of cannabis is attributed to genetic factors (selected seed varieties and cultivation of female plants), environmental factors (cultivation techniques), and freshness (production sites are close to the consumer and storage degradation of THC is avoided) [10]. Several papers report an increased ratio of non-fermented flowers, called 'sinsemilla', in the street market [1,11,12]. The World Drug Report 2006 noted that 'sinsemilla is distinct enough in appearance and potency to be considered a separate drug' [12]. Some reports mention the possibility of increases in mental disorders or emergency calls due to increased cannabis potency [5,11–13]. Driving under the

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influence of drugs (DUID) is also a significant issue of public concern that relates to cannabis use [12].

In Japan, cannabis is the second-most-often abused psychoactive drug next to methamphetamine, and its abuse is rapidly increasing. The number of arrested individuals in 2010 was 2367 [14], double that in 2000, 1224 [15]. Furthermore, several large-scale indoor cultivations of sinsemilla have been found in recent years.

The THC contents of Japanese cannabis were previously surveyed by the Ministry of Health and Welfare in 1970 [16]. However, this survey was of hemp used for fiber production, or wild cannabis. There are no sufficient data on the THC levels of abused cannabis in Japan. The aim of this study is to survey cannabis potency in Japan and compare it with the results of previous studies.

In Japan, all cannabis evidence is transferred after criminal trials from regional prosecutors' offices to the Minister of Health, Labour and Welfare via regional Narcotics Control Department (NCD). The survey of these seizures was conducted in NCD offices located in 11 major cities all over Japan.

UNODC has pointed out several problems with the comparison of THC level data from different countries or periods, such as differences in the classification of cannabis and in the analytical method, or a lack of randomness in sampling [12]. So the authors used UNODC's recommended method [17] for quantification and set clear criteria for sampling or classifying marijuana seizures to avoid possible bias or ambiguity.

Users of psychoactive drugs determine the dose of the drug by reference to its potency. So high potency cannabis would not necessarily be harmful, but a 'change' or 'difference' in the potency can cause unexpected overdoses and health problems [11]. So we compared our results with the THC levels, which were regarded to be higher than the previous ones. And we also determined the pattern of THC contents in marijuana samples. The present paper is the first report for cannabis potency in Asian area, covering almost whole seizures in one nation, using standardized methods for quantification.

2. Material and methods

2.1. Cannabis surveyed

Seized cannabis plants or products were surveyed after criminal trials. They were transferred to the Minister of Health, Labour and Welfare via regional NCD offices within a few months or years after seizure. Eleven major prosecutors' offices were targeted. The survey period was from April 2010 to March 2011. Seizures from the Sendai Regional Prosecutor's Office were not completely surveyed. They were examined only when exceeding 1 g in weight, and the survey of them started in October 2010.

2.2. Cannabinoids reference materials, reagents and devices

Cannabinoids reference materials: methanol solutions (1 mg/mL) of THC, cannabinol (CBN), and cannabidiol (CBD) were purchased from Cerilliant Co, Round Rock, TX, USA.

Internal standard solution (ISTD): tribenzylamine (TBA) in ethanol (0.5 mg/mL).
Ethanol: 99.5%, analytical grade
Mortar and pestle: made of agate or porcelain.
Finger musher: Assist AM. 79340.
Stainless steel sieve: 1 mm mesh, 75 mm diameter.
Membrane filter: 0.45 μm pore size.
Dry bath: heating ability of up to 150 °C.

2.3. Analytical procedures

2.3.1. Sample preparation

Quantification of cannabinoids was accomplished according to the UNODC's 'Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products' [17]. This method aims the quantification of total THC (THC + THC acid), so it has a heating process to convert THCA into THC. Stems, twigs, or seeds were removed from marijuana samples. The remaining buds and leaves were crushed by a finger masher or a mortar and pestle. The crushed marijuana was filtered through a sieve. A 200-mg quantity of sieved sample powder was weighed and 20 mL of ethanol was added. It was subjected to ultrasonic extraction for 15 min. The solution was filtered through a membrane filter, and 500 μL of filtrate was placed in a 2 mL vial and heated at 150 °C for 12 min. To the residue, 1.5 mL of ethanol was added and stirred. The calibration solutions were CBN solutions with ISTD. Their concentrations were 7 steps between 3.33 and 533.3 μg/mL, corresponding to 0.1–16% in the marijuana sample.

2.3.2. Gas chromatography (GC) conditions for cannabinoids

Instrument: Agilent 7890 or Shimadzu GC2010
Column: HP-5 or HP-5MS (30 m × 0.25 mm, 0.25 μm)
Carrier gas: He, 1.1 mL/min (constant flow)
Inlet temperature: 250 °C (original temp. of 280 °C was changed by technical requirements of our laboratories)
Oven temperature: 2 min at 200 °C, 10 °C/min 200–240 °C, 15 min at 240 °C
Split ratio: 20:1
Detector: flame ionization detector, 300 °C
Detector gas: H_2 35 mL/min, Air 350 mL/min
Injected volume: 1 μL
CBN was used as the reference material for the quantification of THC and CBD.
The correlation factor was 1. The cutoff level was 0.1% for each cannabinoid.

2.3.3. Gas chromatography–mass spectrometry (GC–MS) conditions for discrimination of CBD and CBC

Instrument: GCMS-QP2010 Plus
Column: HP-5MS (30 m × 0.25 mm, 0.25 μm)
Scan range: m/z 40–400
Ionization conditions: electron ionization, 70 eV, 200 °C
The other conditions were same as those for GC.

2.4. Checking reference materials and GC response factor

The exact content level of each reference solution was tested at every purchase of a new lot. Test solutions were prepared by mixing 150 μL methanol solution of THC, CBN or CBD (1 mg/mL), 500 μL ISTD solution and 850 μL ethanol. They were injected onto GC, and the chromatographic peak area of each compound was compared to that of ISTD.

2.5. Method validation

2.5.1. Recovery tests

The accuracy of the quantification method for cannabinoids in marijuana was tested at three laboratories. Marijuana of low THC or CBN content was chosen and extracted with ethanol. A 500-μL volume of extract was taken and fortified with cannabinoids followed by heating and quantification using GC. Test solutions were prepared separately for each cannabinoid, and the fortification level was equal to 1% in marijuana.

2.5.2. Interlaboratory testing

The analytical method was tested at eight laboratories. Three marijuana seizures of adequate amounts were chosen. They were cut into 0.5–1-cm pieces and mixed well. These samples were packed in plastic bags in ca 1 g portions. They were transferred to the laboratories and analyzed in triplicate. The average, standard deviation and z-score of the cannabinoid levels detected in each laboratory were calculated.

2.6. Observation and classification of cannabis

Cannabis seizures were classified into 5 groups: marijuana, whole plant, hashish, hash oil and others (mixture with tobacco or herbs, burned residue, etc.). Marijuana samples were further classified into 4 groups: seeded buds, seedless buds, leaves and others (stems or twigs). A document describing the amount of cannabis was attached to every set of seizures. The amount of marijuana, hashish or hash oil was expressed in terms of weight. On the other hand, the amount of the whole plant was expressed in either weight or number for analytical or handling reasons. The number or weight of each seizure was recorded.

2.7. Selection of quantification samples

Marijuana seizures exceeding 1 g were subjected to quantifications of THC, CBN, and CBD in eight laboratories. Packages of marijuana with the same appearance and identical kinds of bags were assumed to have originated from identical lots, and one of the packages was picked up for analysis. If each package of the same origin contained less than 1 g but their sum total exceeded 1 g, two or more packages were combined to exceed 1 g. If one package contained much more than 1 g, 1 g was taken. When a large package contained a mixture of buds and leaves, the buds were taken.

Table 1
Comparison of experimental and theoretical GC responses of cannabinoid reference solutions.

ID	Compound	Lot No.	Expiration date	Description in certification		Experimental GC response		Theoretical peak area [18] (CBN = 1)
				Prepared concentration (mg/mL)	Analyzed concentration (mg/mL)	Peak area (D = 1)	Peak area normalized by 'analyzed concentration' (D = 1)	
A[a]	THC	FC080603-01F	July 2010	1.000	1.004	0.993	0.980	0.982–1.00
B	THC	FE021710-01	February 2015	1.000	1.018	1.028	1.000	
C	CBN	FE061208-01	June 2012	1.000	0.990	0.998	0.999	1
D	CBN	FE111210-01	November 2014	1.000	0.991	1	1	
E	CBD	FE100108-01	December 2011	1.000	1.005	1.013	0.999	0.97–0.990
F	CBD	FE111510-02	June 2015	1.000	1.002	1.006	0.995	

[a] This solution was quantified in August 2010, a month later than the expiration date.

3. Results and discussion

3.1. Checking of reference materials and GC response factor

The GC responses of the lots of cannabinoid reference solution were examined and compared with each other to confirm the concentration and response factors. Table 1 shows the features of each reference solution and the experimental results. The provider of the reference solutions describes 'prepared concentration' and 'analyzed concentration' in the certification documents. 'Prepared concentration' was 1.000 mg/mL for all lots and the supplier recommended use of this value for the measurement of the concentration.

As THC is an unstable compound, the UNODC analytical manual recommends the use of CBN as a reference material for THC [17]. Poortman-van der Meer et al. mentioned that theoretical THC/CBN and CBD/CBN were 0.982–1.00 and 0.97–0.990, respectively, depending on the theoretical model adopted [18].

Among the six lots, THC solution A was tested after the expiration date, and the GC response was significantly lower than the theoretical one, so it was excluded from consideration. The other lots, B, E, and F, all gave GC responses exceeding the theoretical values. On the other hand, when the GC responses were normalized by the 'analyzed concentration', they were in better agreement with the theoretical values. The factors for THC/CBN and CBD/CBN were all in the range of 0.995–1.000. Thus, a factor of 1 and a concentration of 1.000 mg/mL were used to calculate THC and CBD concentrations based on CBN calibration curves in the following experiments and survey.

Quantification using CBN as a reference material can be somewhat inaccurate, but this method has many merits because THC is strictly controlled in Japan and it is very difficult to obtain, distribute and store. Furthermore, cannabinoid reference solutions are expensive.

3.2. Method validation

3.2.1. Discrimination of CBD and CBC

It is known that CBD has a retention time very close to that of cannabichromene (CBC) in GC [19]. Though the mass spectra of CBD and CBC resemble each other, they are distinguishable by the existence of the m/z 246 peak (CBD) or the absence of it (CBC) as shown in many commercial databases. Some of the samples in the present research were tested using gas chromatography–mass spectrometry and a chromatographic peak considered to be CBC was obtained, though it was not confirmed because of a lack of reference material.

One of these marijuana extracts was mixed with CBD reference solution to make equal concentrations of CBD and supposed CBC. The mixture was analyzed, and it was shown that the peaks of CBD and supposed CBC did not separate, even with a column of 30 m in length instead of the 15 m column recommended by UNODC [17]. However, CBD showed a retention time 0.04 min shorter than supposed CBC, so they were distinguishable on chromatogram. All samples in the survey were split into 2 groups by their retention time for CBD or CBC and none of them gave ambiguous peak.

3.2.2. Recovery test

Table 2 shows the recoveries of cannabinoids added to marijuana extracts at three laboratories. Some values exceeded 120% in spite of usage of the internal standard. This finding is considered to be due to an enhanced response by matrices that originated from marijuana. On the other hand, one recovery value for THC was 90.8%, suggesting that loss occurred during the analytical process, possibly in the heating step. This experiment showed both the larger and smaller results for THC as two possibilities. No further experiment could be done because of a shortage of cannabinoids solution.

3.2.3. Interlaboratory test

Table 3 shows the results of the quantification of cannabinoids in divided marijuana samples at eight laboratories. All three samples contained no CBD, but supposed CBC was detected in them. The THC and CBN detected were all below 1%, and their standard deviations were equal to or below 0.2% except for THC in Sample 3. The THC level in Sample 3 was 6.2% on average, and the relative standard deviation (RSD) between laboratories was 9.2%, better than the reported RSD of 29% in similar interlaboratory practice in Europe [18]. Fig. 1 shows the z-scores of the THC values detected in Sample 3 at eight laboratories. The z values of all laboratories were below 2.

As the interlaboratory test was conducted without any pre-quantification of THC to keep blindness, the THC levels found in 2 of the 3 samples included in the test happened to be rather low. They were not ideal samples to be representative for surveyed marijuana. However, the test was not repeated because test sample distribution needed so complicated legal procedures that the opportunity was limited to be only once.

Table 2
Recovery (%) of cannabinoids.

	Lab X	Lab Y	Lab Z
THC	104.2 (1.0)	90.8 (1.1)	126.1 (3.4)
CBN	122.3 (5.3)	104.8 (2.4)	127.4 (3.6)
CBD	123.1 (6.0)	108.8 (2.7)	104.4 (3.2)

One % of cannabinoid was added to extract of marijuana sample.
Standard deviation is shown in parentheses.
n = 3.

Table 3
Cannabinoids quantification results in 8 laboratories.

	THC (%)			CBN (%)		
	Average	Range	SD	Average	Range	SD
Sample 1	0.3	0.2–0.4	0.1	0.2	0.1–0.3	0.1
Sample 2	0.6	0.4–0.8	0.1	0.8	0.5–1.1	0.2
Sample 3	6.2	5.3–7.1	0.6	0.4	0.3–0.6	0.1

3.3. Collection and classification of cannabis

The total number of post-trial cannabis seizures collected was 9072. Fig. 2 shows the sample numbers from each area. The number from Tokyo was the largest, 1800, and the second largest was from Yokohama, 1687. Cannabis was incompletely collected in the Sendai area, so the number for Sendai does not reflect the true number of seizures in this area.

Table 4 shows the number and weight of each cannabis class: 1257 were whole plants, 6376 marijuana, and 886 hashish. The total weight was 286.6 kg for marijuana and 36.0 kg for hashish. The whole amounts of marijuana and hashish seizures made from 2008 to 2010 have been reported to be 181.7–382.3 kg and 13.9–33.4 kg per year, respectively [14]. Therefore, the present research covered the majority of Japanese cannabis seizures.

Marijuana seizures were further classified, and the results are shown in Figs. 3 and 4. Fig. 3 is expressed in terms of the number of seizures, and Fig. 4 in weight. Seedless buds were dominant among the classes, consisting 65.2% of the total in number and 73.0% in weight.

A survey in the USA in 2008 showed that 46.8% of seized marijuana was 'sinsemilla' [1], and another survey in the UK in 2008 showed this number to be more than 97% [3]. The present results show that seedless buds, 'sinsemilla', represent the majority of marijuana seizures in Japan as in these other countries.

Fig. 5 shows the ratio of the cannabis classes in each area. The ratio of leaves or others (stem, twig, etc.) was higher in Sapporo, Hiroshima and Naha. Police sometimes seize waste of cannabis cultivation, so it is difficult to distinguish whether the leaves were intended to be consumed or thrown away.

The ratio of seeded buds to seedless buds was higher in Sapporo and Fukuoka. In Hokkaido Prefecture, where Sapporo City is located, huge numbers of wild cannabis are eradicated every year. About 920,000 plants were eradicated in 2010 all over Japan, of which 810,000 were in Hokkaido Prefecture [14]. Thus, some extent of seeded buds from Sapporo was supposed to be wild cannabis taken for consumption. On the other hand, in Kyushu area, where Fukuoka City is located, no wild cannabis has been eradicated in recent years. The reason for the frequent encounter with seeded buds in this area is not specified.

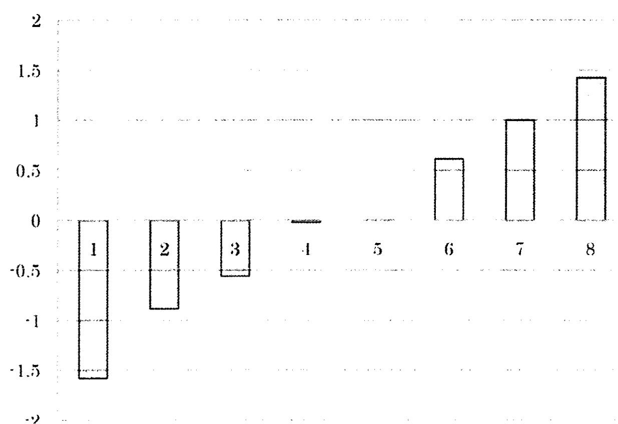

Fig. 1. z-Score of THC level in Sample 3 reported by 8 laboratories.

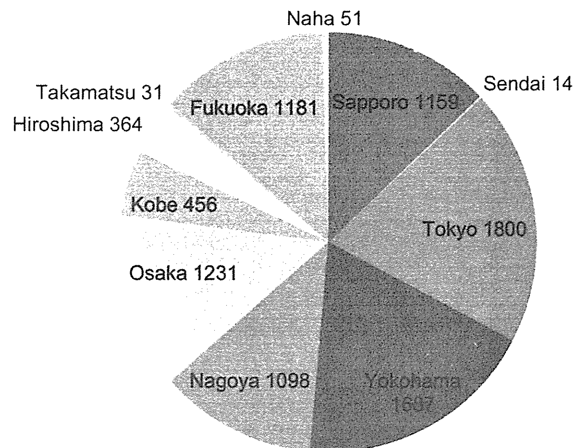

Fig. 2. Number of cannabis seizures in each area (total 9072).

Table 4
Cannabis seizures observed after criminal trials.

	Number of items	Weight (kg)		Number of plants
Whole plant	1257	8.300	+	2337
Marijuana	6376	286.559	+	5
Hashish	886	35.983		
Oil	5	0.026		
Others	548			
Total	9072	330.869		2342

Size of each item was expressed as weight or number of plants.
'Others' includes mixture with tobacco, burned residue, etc.

3.4. Cannabinoids levels in seized marijuana

3.4.1. THC and CBN

Marijuana seizures were selected for the quantification of cannabinoids. The total number quantified was 1115. The results for each class are shown in Table 5. The highest THC level was found in seedless buds, 8.3% average. The CBN level in these buds was 1.3% therefore the sum of THC and CBN was 9.6%.

Seized cannabis had been stored for long periods during criminal trials. It is known that CBN is not found in fresh cannabis. It is gradually produced by conversion from THC during storage time. The sum of THC and CBN levels is not equal to the initial THC level in marijuana, but is rather less than the initial level [20]. Thus, the sum is considered to be the lower limit of the initial THC level, but is not an exact value.

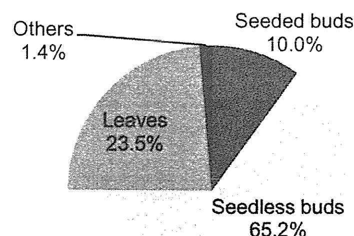

Fig. 3. Number of marijuana in each class (total 6376).