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Forensic Toxicologic Analysis of Methamphetamine and Amphetamine in Body Materials by Gas Chromatography/Mass Spectrometry*

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Summary. Qualitative and quantitative analysis of methamphetamine and amphetamine in biologic materials was carried out by gas chromatography/mass spectrometry. A deuterium-labeled methamphetamine was employed as an internal standard with a detection limit of 50 pg and absolute stability and reproducibility. Blood was found to be the best material for estimation of the toxicity of the stimulant drug. It can be replaced by muscle which contains methamphetamine concentrations close to those of blood. The authors' classification of the toxic blood levels of methamphetamine from therapeutic to fatal doses was confirmed by additional data obtained from new case studies.

Key words: Methamphetamine, toxicological analysis in biological material – Amphetamine, in body material – Toxicology, Methamphetamine, amphetamine

Zusammenfassung. Es wurden qualitative und quantitative Analysen von Methamphetamin und Amphetamin aus biologischem Material mittels Gaschromatographie/Massenspektrometrie durchgeführt. Als interner Standart wurde Deuterium-markiertes Methamphetamin mit einer Nachweisgrenze von 50 pg bei hoher Stabilität und Reproduzierbarkeit verwendet. Blut erwies sich als das beste Ausgangsmaterial zur toxikologischen Untersuchung dieses stimulierenden Arzneimittels. Es kann jedoch auch durch Muskulatur ersetzt werden, in der Methamphetamin-Konzentrationen ähnlich denen im Blut nachweisbar sind. Die vom Autor vorgenommene Klassifizierung von

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toxischen Methamphetamin-Blutkonzentrationen nach therapeutischen und tödlichen Dosen wurde durch weitere Daten aus neuen Fällen bestätigt.

Schlüsselwörter: Methamphetamin, Nachweis in biologischem Material – Amphetamin, Nachweis in biologischem Material – Toxikologie, Amphetamin und Methamphetamin

Introduction

Since abuse of stimulant drugs, primarily methamphetamine (MA), is one of the most serious social problems in Japan, the accurate and sensitive detection of the drugs in body materials is required to estimate the medico-legal aspects of their toxic effects before death. For the analysis of cadaver materials, special care must be taken to prevent possible interference from contamination or alteration by products of putrefaction. The authors have already reported a gas chromatography/mass spectrometry (GC/MS) method using 3-phenyl-1-propylamine (3-PPA) as an internal standard (IS) [1] and applied the technique to case studies with satisfactory reliablity. In the present paper, an improvement of the method was attempted by introducing deuterium-labeled methamphetamine (DMA) as the theoretically desirable IS for GC/MS. The technique using DMA, the distribution of MA in the body, and the choice of suitable samples in cases of unfavourable conditions, such as putrefaction and contamination, were investigated. The results of the new MA determinations were evaluated.

Materials and Methods

Agents

Amphetamine (A; 1-phenyl-2-aminopropane) was synthesized according to the method of Lindeke and Cho [2] and used as the sulfate. Methamphetamine (MA; 1-phenyl-2-methylaminopropane) was purchased as the hydrochloride salt from Dainippon Seiyaku Co. Ltd. Trifluoroacetic anhydride (TFAA) was purchased from Nakarai Chemicals, Ltd. 3-Phenyl-1-propylamine (3-PPA), employed as a regular IS, was obtained from Aldrich Chemical Co. Inc. in the form of the free base. Pentadeuterated methamphetamine (PDM; 1-phenyl-2-[methyl-d_3-amino]-propane-1,2-d_2, see Fig. 1), employed as an IS for GC/MS in this study, was synthesized according to the procedure described below. Standard solutions of these amines were dissolved in 1N-HCl and stored at −20°C in a refrigerator. They were stable (semi-permanently) for at least 2 years.

Preparation of PDM

Synthesis Procedure. Dideuterated amphetamine (DDA; 1-phenyl-2-aminopropane-1,2-d_2) synthesized according to the procedure of Lindeke and Cho [2] is preserved in form of cry-

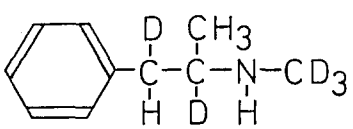


Fig. 1. Structural formula of 1-phenyl-2-[methyl-d_3-amino]-propane-1,2-d_2

stallized sulfate (DDA sulfate). DDA sulfate is extracted into diethylether from alkalized aqueous solution. The ether phase is washed with a small volume of water. The organic solvent is removed under a stream of nitrogen. To the cooled DDA solution are added excess volume of 10N aqueous NaOH and an equivalent amount of dimethyl-d_6 sulfate for methylation. The products including PDM are extracted with diethylether, then finally dried with potassium carbonate.

Purification. Active alumina, aluminium oxide (90 active neutral made by Merck), is placed in the dried ether solution to adsorb the amines, such as PDM, dimethylamphetamine-d_8 (DMA-d8; 1-phenyl-2-[dimethyl-d_6-amino]propane-1-2-d_2) and unreacted DDA. After filtration, the alumina phase is washed with 3 times volume of diethylether, twice volume of 1% ethanol-diethylether, and twice volume of diethylether successively so that DMA-d8 with low polarity is removed first. Then, PDM is eluted from the alumina phase by washing with 6 times of 20% ethanol-diethylether, leaving the other highly polar amines adsorbed. The elutant is acidified with a small amount of concentrated hydrochloric acid, evaporated in vacuum, dissolved in water, and alkalized, then extracted into diethylether. The organic solvent layer is dried with potassium carbonate. The above procedure is repeated twice.

PDM-hydrochloride is crystallized by adding HCl-diethylether to the dried ether solution of PDM in a cold bath. The PDM-HCl crystals are collected, washed with a small volume of diethylether, and dried.

Extraction

Samples of blood (<2 g) and other related materials (<1 g) are homogenized in 5 ml 0.01 N HCl by a Polytron homogenizer. Shortly before the end of homogenization, 5 µl 3-PPA (1.0 µg/µl) is added. When PDM is used as IS, 5 µl (0.20 µg/µl) is added to the sample before the homogenization. The homogenates are alkalized with 0.5 ml 2 N NaOH and extracted with 12 ml diethylether. If an emulsion forms, the extraction container is cooled at a temperature below −5°C for 30 min and centrifuged. The organic phase is transferred into another tube and back-extracted with 2 ml of 1 N HCl. The aqueous phase is placed into another glass tube and re-extracted with 1.2 ml of dichloromethane after alkalization with 0.4 ml of 10 N NaOH. To the organic phase in a 2 ml glass-container are added 0.1 ml of ethyl acetate and 0.05 ml of TFAA. Ethyl acetate facilitates trifluoroacetylation by increasing the solubility of TFAA. The mixture is shaken, stored overnight, and the solvent is carefully evaporated to ca. 0.05 ml under a gentle stream of nitrogen at room temperature. One microlitre of the concentrated solution is injected into a GC/MS apparatus for quantitative determination. The sample prepared by this procedure is semi-permanently stable.

Conditions of GC/MS

The EI and CI systems were applied by using two different instruments:

EI System. Shimadzu LKB 9000 Gas Chromatograph/Mass Spectrometer [electron impact (EI) mode] controlled through the data system GCMSPAC 500D. Ionization energy: 20 eV. Accelerating voltage: 3,500 V. Ion source temperature: 270°C. Monitoring ions: m/z 140 for A, m/z 154 for MA, m/z 158 for PDM, and m/z 162 for 3-PPA.

CI System. Shimadzu LKB 9000A Gas Chromatograph/Mass Spectrometer [chemical ionization (CI) mode] equipped with High Speed MIDPM 9060S. Ionization energy: 150 eV. Box current: 500 µÅ. Accelerating voltage: 3,500 V. Reagent gas: iso-butane, 1 torr. Ion source temperature: 270°C. Monitoring ions: m/z 232 for A, m/z 246 for MA, and m/z 251 for PDM.

The same GC conditions were employed with both systems: Column: 2 m × 3 mm i.d. glass tube packed with 5% OV-17 on chromosorb W, HP (80/100 mesh). Temperatures: column oven 180°C, injection block 220°C, separator 240°C. Carrier gas: helium at a flow rate of 30 ml/min.

Standard Curves

Blood samples containing MA and A at the concentrations of 0.05, 0.10, 0.50, and 1.0 µg per 2 g were prepared and extracted by the above procedure. These samples were trifluoroacetylated. The

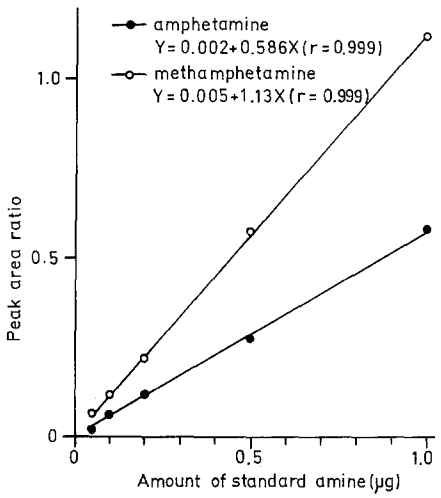


Fig. 2. Standard curves for determining methamphetamine and amphetamine in biologic materials in the EI mode

standard curves were obtained by plotting the peak area or height ratio between each amine and IS. The straight lines were found in the concentration range from 0.05 to 1.0 μg amine/2 g blood. Figure 2 shows the curves in the EI mode using PDM as IS.

Results

Mass Spectra and Gas Chromatograms with SIM

The EI and CI mass spectra of PDM-base as well as PDM-TFA are compared with the spectra of MAs in Fig. 3A, B. The quasi molecular ion of PDM-base was observed at m/z 155 and that of PDM-TFA at m/z 251 on the CI mass spectra. The mass spectra as well as SIM gas chromatograms of PDM showed cleaner patterns than those of 3-PPA, which often has background interference. The troubles were observed particularly when a large amount of nicotine was involved. An unidentified peak often causes interference in the CI patterns of 3-PPA.

The ions m/z 158, 120, 119 and 113 on the EI spectrum correspond to m/z 154, 118, and 110 of unlabeled MA-TFA whose fragmentation was described by Kamei [3]. The spectra of A- and 3-PPA-TFA have been reported previously [1, 3–6]. Figure 4 shows the chromatograms of EI-GC/SIM of MA and A extracted from biologic sample with PDM as IS, and Fig. 5 that of CI-GC/SIM. No background peak appeared in connection with body materials.

Purity of Prepared PDM

The melting point of PDM-hydrochloride was 134.5–138.0°C. The purity of PDM calculated by GC/SIM was above 98%, as compared with purchased MA. The yield of PDM from DDA was about 5%. It contained less than 0.5% as an impurity. No interfering peaks were observed on a GC/SIM chromatogram for amine analysis. The stability of deuterium on PDM remained above 99% in 1 N-HCl solution over 2 years, as checked by MS.

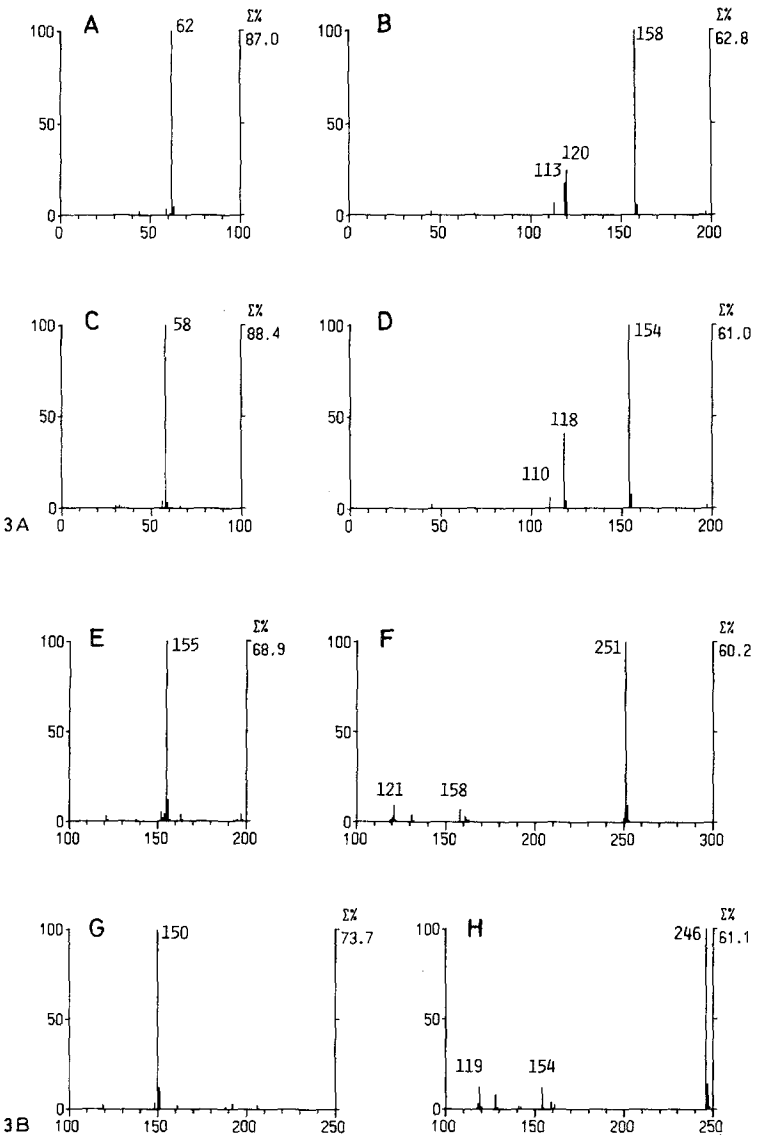
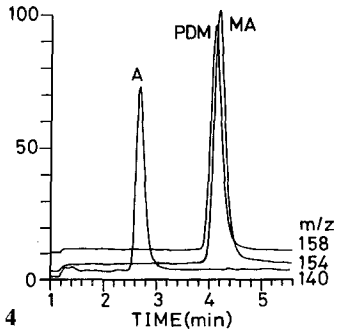


Fig. 3 A. Mass spectra of methamphetamine *(MA)* and pentadeuterated and methamphetamine *(PDM)* in the EI mode; **A:** PDM-base, **B:** PDM-TFA, **C:** MA-base, and **D:** MA-TFA

Fig. 3 B. Mass spectra of methamphetamine *(MA)* and pentadeuterated methamphetamine *(PDM)* in the CI mode; **E:** PDM-base, **F:** PDM-TFA, **G:** MA-base, and **H:** MA-TFA

Reproducibility of the Methods

Since both EI and CI methods are considered to have about equal sensitivity for quantitative determination of amines, the reproducibility was measured only on the EI system. Samples for five series were prepared from 2 ml blood, 1 g liver, and 1 g muscle, each of which was spiked with 0.5 µg A and MA. The results



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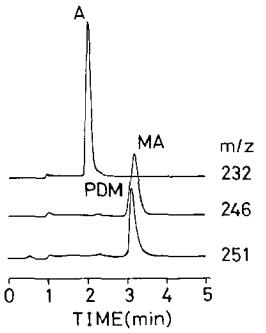

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Fig. 4. Gas chromatogram with selected ion monitoring in the EI mode from blood sample spiked with 1 μg of each of three amines: *A* amphetamine, *MA* methamphetamine, *PDM* pentadeuterated methamphetamine

Fig. 5. Gas chromatogram with selected ion monitoring in the CI mode from blood sample spiked with 1 μg of each of three amines; *A* amphetamine, *MA* methamphetamine, *PDM* pentadeuterated methamphetamine

Table 1. Reproducibility of the method using pentadeuterated methamphetamine as internal standard in the EI mode

Material	M ± SD (cv) unit: μg	
	Amphetamine	Methamphetamine
Blood ($n = 5$)	0.510 ± 0.012 (2.4%)	0.505 ± 0.007 (1.4%)
Liver ($n = 5$)	0.525 ± 0.006 (1.1%)	0.512 ± 0.008 (1.6%)
Muscle ($n = 5$)	0.527 ± 0.015 (2.8%)	0.524 ± 0.007 (1.3%)

M Mean value; *SD* standard deviation; *cv* coefficient of variation

Table 2. Reproducibility of the method using 3-phenyl-1-propylamine as internal standard in the EI mode

Material	M ± SD (cv) unit: μg	
	Amphetamine	Methamphetamine
Blood ($n = 5$)	0.494 ± 0.016 (3.2%)	0.519 ± 0.031 (6.0%)
Liver ($n = 5$)	0.479 ± 0.025 (5.2%)	0.484 ± 0.022 (4.5%)
Muscle ($n = 5$)	0.484 ± 0.021 (4.3%)	0.502 ± 0.031 (6.2%)

M Mean value; *SD* standard deviation; *cv* coefficient of variation

Table 3. Relationship between blood level and toxicity of methamphetamine in humans

Grade of toxicity	Range of blood level μmol/100 g
Fatal	Above 3
Serious	Above 2
Intermediate	Above 0.3–0.4
Mild	Below 0.2

determined on the standard curves are shown in Table 1 with standard deviations (SD). Apparent recovery was over 100%.

The same blood sample was tested for 16 days to determine its daily variation. The reproducibilities amounted to 2.2% for A and 2.7% for MA in terms of coefficients of variations. These results indicate that the technique is reliable enough to be used in practice. The reproducibility of the technique when 3-PPA was used as IS was also compared. The results given in Table 2 indicate that 3-PPA possesses almost the equal qualities as PDM when used as IS for case work.

The lower limit of detection was about 50 pg in both EI- and CI-GC/SIM. The CI method was found to be slightly more sensitive than the EI on account of the clearer base line on chromatograms with lower background.

Analysis of Practical Samples

Ten autopsy cases were examined to check the validity of the toxic level standards set up by one of the authors [7] in Table 3. Detected A amounting to about 10% of MA was taken as a metabolite of MA and included in the total MA amount expressed as mole units. The mole unit concept was chosen to express the toxicity as joint effects of A and MA. The expression of "number" of concerned toxic substances can be understood easier than units of "weight" of mixtures with different molecular weight.

The results of case studies with body fluids and tissues are illustrated in Table 4. The data present values agree with the authors' former criteria on toxicity levels. The distribution of MA in the body was almost the same as reported previously [7]. The MA concentration in the body tissues was about 2–3 times that of blood and muscle. Urine showed the highest levels and widest variations in the individual cases.

Distribution of MA in the Body and Selection of a Sample for Practical Analysis

The MA distribution ratio between each body tissue and blood, obtained from data of autopsy cases, was checked by animal experiments. Normal male Wistar rats, weighing 250–300 g, were given 5 mg MA/kg b.wt. by i.v. injection and oral administration, and killed by pithing after 1/6, 1/3, 1/2, 1, 2, 3, 4, and 5 h. Figure 6 shows the blood levels of MA plotted against time on oral and i.v. administration. For comparison, the changes in the level of human blood [8] were plotted

Table 4. Methamphetamine concentration in body fluids and tissues of autopsy cases

No.	Age	Sex	Concentration (μmol/100 g or ml)								Toxicity	Remarks
			Bl	Ms	Lv	Br	Sp	Kd	Lg	Ft	Ur	
1	25	M	14.1	9.7	69.7	13.9	22.7	14.3	18.1	0.86	90	F Excitement; per os.
2	29	M	8.8	6.5	13.3	14.0	39.4	10.8	–	1.0	–	F Unexpected death; per os.
3	45	F	3.1	4.6	9.6	8.2	6.5	9.9	5.4	0.62	30	F-S Excitement; i.v. inj.; Basedow's disease
4	22	F	2.2	2.4	7.6	5.8	5.7	5.0	13.8	0.24	117	S Intensive confusion; i.v.inj.; Liver cirrhosis
5	33	M	1.5	–	–	–	–	–	–	–	17	I Accident due to reckless driving
6	68	M	1.4	–	–	–	–	–	–	–	–	I Hit by a car; ignoring traffic rules
7	51	M	1.1	0.73	3.3	2.5	2.3	0.83	2.3	0.26	6.1	I Found dead in a bath, naked on the lower part of body
8	24	F	–	0.36	0.90	–	0.93	0.80	1.12	0.03	–	I Decayed body; murdered; reportedly abuser
9	33	M	0.013	–	0.0032	0.016	0.018	0.025	0.095	–	0.17	M Gangster murdered
10	45	M	–	–	–	–	–	–	–	–	0.01	M Shot by police while violent act; abuser

Bl blood; *Ms* muscle; *Lv* liver; *Br* brain; *Sp* spleen; *Kd* kidney; *Lg lung; Ft* fat; *Ur* urine; Sex: *F* female; *M* male; Toxicity: *F* fatal; *S* serious; *I* intermediate; *M* mild; Remarks: *i.v. inj.* intravenous injection

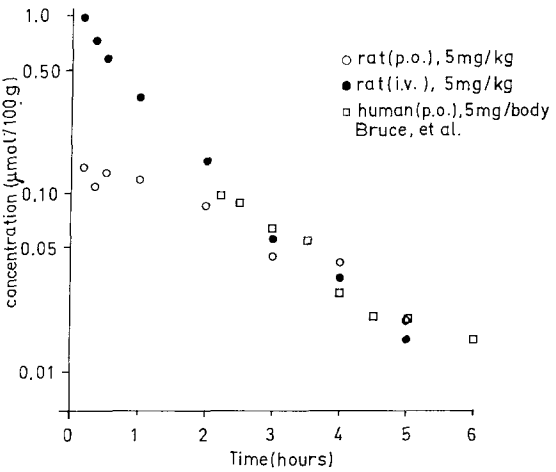

Fig. 6. Time course of blood levels after administration of methamphetamine to rats, additionally plotting the change of human blood levels taken from the paper by Bruce and Maynard [8]

on the same graph. These data were considered to be related to our data in the distribution volume, dose per initial blood level. The blood level decreased rapidly with a half-life time of 0.88 h from the time of intravenous injection and 1.6 h when given orally. The ratios of MA concentration between body materials and blood are given in Table 5. Table 6 shows the apparent distribution volumes and half-life times of the blood levels from data in Fig. 6.

As to MA distribution in the body, liver and kidney showed slightly larger variations than other samples. The liver level rapidly reached equilibrium in case of i.v. administration, whereas it markedly varied on oral dosage. The kidney usually gave the highest level of other organ tissues; however, this was not true for human kidney. Other tissues showed similar levels. Muscle agree closest with blood. The results support the opinion one of the authors [7] that muscle could replace blood when the latter cannot be used due to putrefaction.

Discussion

The advantage of gas chromatography with selected ion monitoring, mass fragmentography, for trace quantitative analysis of chemical substances stems from its high sensitivity and selectivity. The choice of IS often plays an important role in obtaining an accurate measurement by this technique. The use of stable-isotopically labeled IS has given good results [9–11]. Such stable isotopes, however, are not readily available due to their high price and complicated syntheses. For these reasons, the authors have been using, in their prior work, 3-PPA as the IS for routine determination of amphetamines. However, since 3-PPA can supposedly be bound to protein, errors are possible. These can be prevented by replacing 3-PPA by an isotope as IS, which is ideal for GC/MS analysis. The PDM (see formula in Fig. 1) prepared in this study was shown to be a desirable IS on account of its distinct separation from unlabeled MA and since it permits the use of EI as well as CI mass spectrometry. PDM can be synthesized at a lower expense and by a simpler procedure than by introducing deuterium

Table 5. Proportion of tissue level to blood level in human and rat samples

Species	Tissue level/blood level [M ± SD, *n*, cv %]						
	Muscle	Liver	Kidney	Brain	Spleen	Lung	Fat
Human	0.82 ± 0.29	3.16 ± 1.63	2.73 ± 1.61	2.23 ± 1.20	2.36 ± 2.22	3.76 ± 2.22	0.20 ± 0.13
	18	28	26	22	21	12	20
	35.8	51.7	59.0	54.1	56.9	58.9	63.8
Rat (i.v.)	2.89 ± 0.78	6.92 ± 4.20	27.4 ± 17.1	6.64 ± 1.29	8.68 ± 2.30	11.6 ± 5.17	0.41 ± 0.17
	22	22	22	22	22	22	20
	26.9	60.7	62.3	19.5	26.4	44.6	41.2
Rat (p.o.)	2.11 ± 0.76	19.9 ± 11.5	36.4 ± 13.4	6.15 ± 1.55	8.44 ± 2.16	11.2 ± 4.46	0.37 ± 0.12
	24	24	24	24	24	24	24
	36.2	57.8	36.8	25.1	25.6	39.9	31.3

M Mean value; *SD* standard deviation; *n* number of subjects; *cv* coefficient of variation

Table 6. The value of half-life and apparent distribution volume of methamphetamine in rats as compared with humans in the other papers

Species	Observed period	Dose μmol/kg	Half life h	Distribution vol. l/kg
Rat (i.v.)	1 –5 h	33.6	0.88	4.62
Rat (p.o.)	1 –5 h	33.6	1.6	17.9
Human (Bruce (and Maynard [8])	2.2–6 h	0.56	1.3	0.18
Human (Une [18])	Few days	Various	Av. 18.2 (9.8–33)	Av. 9.43 (2–26)

In humans cases, the values are calculated after converting the weights into 60 kg

into other positions of the amine, such as the 3-carbon of propyl group and the phenyl ring [2, 11]. The cost was approximately one third of that of previous methods. The yield, 5% of PDM from DDA, appears low; however, this is insignificant: 10 mg PDM is sufficient to prepare 10,000 test samples.

The method using PDM as IS might be called "direct identification" based on the reliable information power with excellent separation by GC and presentation of the molecular construction by both EI and CI methods. Other methods, such as radioimmunoassay [12–15], should be called "indirect identifications" on account of their lower information capability, although they may be sensitive and handy.

Losses of volatile amines during extraction [16] are avoided by addition of ethyl acetate before trifluoroacetylation. The presence of ethyl acetate until the final stage of evaporation suppresses the volatility of the amine derivatives that are formed. The detection power of the CI mode is superior to that of the EI mode due to a higher specificity of the ionizing mechanism as described before [5, 6]. Attention should be paid to column contamination which often occurs artificially. In practice, the control samples without any amines show high peaks of A or MA as contaminants, especially after the examination of large amounts of amines. The contaminated amines are usually corresponded to below 0.003 μmol/100 g, or 0.002 μg/sample. Although this is far lower than the toxic level, this source of error should be taken into consideration in cases of abuse. It is desirable, therefore, to apply both the EI and CI modes in parallel using PDM for measuring very small amounts of the stimulant amines.

As to the MA distribution in the body, the larger variations for liver are supposedly caused by metabolic influence. Mizoi [17] also found the highest levels in kidney in animal experiments but could not detect differences in human cases.

In animals experiments, reasonable values, as compared with human tests [8], were obtained during a half-life period of 5 h after administration. The MA concentration in the blood was too low to be detected after more than 5 h. The data observed in abusers by Une [18] are worthwhile to be taken into consideration.

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