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Determination of *d*-Methamphetamine in Urine After Administration of *d*- or *dl*-Methamphetamine to Rats by Radioimmunoassay Using Optically Sensitive Antiserum

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ABSTRACT: A radioimmunoassay was developed for the determination of *d*-methamphetamine in urine. Antiserum to *d*-methamphetamine was prepared in rabbits by immunization with *d*-*N*-4-aminobutylmethamphetamine conjugated with bovine serum albumin. *d*-1-[^{3}H]-Methamphetamine was used as a labeled compound for radioimmunoassay. The specificity of the antibody against *d*-methamphetamine was determined by cross-reaction studies with optical isomers of methamphetamine and its analogs. The antibody was specific for *d*-methamphetamine and exhibited no significant cross-reaction with the *l*-isomers. This stereoselective assay was applied to determination of *d*-methamphetamine excreted in urine after oral administration of *d*- or *dl*-methamphetamine to rats.

KEYWORDS: toxicology, *d*-methamphetamine, radioimmunoassay

Cheng et al [*1*] reported that rabbits immunized with *N*-4-aminobutylmethamphetamine coupled to bovine serum albumin (BSA) produced an antibody to methamphetamine, and Faraj et al [*2*] proved the specificity of the antibody by competitive binding assays with metabolites, homologs, and analogs of amphetamine. Recently, specific antibodies to *dl*-methamphetamine was prepared by immunization of rabbits with conjugates of *dl*-*N*-carboxymethylmethamphetamine or *dl*-*N*-carboxypropylmethamphetamine with BSA, and these antibodies were used for the detection and determination of methamphetamine in urine by the hemagglutination-inhibition test and radioimmunoassay [*3,4*]. Niwaguchi et al [*5*] showed that an antibody to *d*- or *l*-methamphetamine was able to discriminate between *d*- and *l*-methamphetamine.

The optical specificity of an antibody to *d*-methamphetamine is discussed. In addition, it is shown that *d*-methamphetamine excreted in the urine of rats after oral administration of *d*- or *dl*-methamphetamine can be detected by radioimmunoassay using *d*-l-[^{3}H]-meth-

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amphetamine as a labeled compound. It is well known that *d*- and *dl*-methamphetamine are widely abused owing to the potent stimulant effects of *d*-methamphetamine.

Materials and Methods

Reagents

d-Methamphetamine hydrochloride, *l*-ephedrine hydrochloride, and *l*-methylephedrine hydrochloride were purchased from Dainippon Pharmaceutical Co. Ltd., Osaka, Japan. *dl*-Amphetamine sulfate, *dl*-ephedrine hydrochloride, and *dl*-methylephedrine hydrochloride were obtained from Takeda Chemical Industries, Ltd., Osaka, Japan, Fuji Chemical Industries, Ltd., Toyama, Japan, and Hoei Pharmaceutical Co. Ltd., Osaka, Japan, respectively. *dl*-Methamphetamine hydrochloride was prepared from *dl*-ephedrine by the method of Emde [*6*], and the purity of the product was checked by thin-layer chromatography (TLC) and chemical ionization mass spectrometry (CIMS). The results of these analyses, as well as the melting point and specific optical rotation of the product, are shown in Table 1. *l*-Methamphetamine hydrochloride was obtained by optical resolution of *dl*-methamphetamine using L-(+)-tartaric acid, and analytical data for the product are also given in Table 1. *d*-(*S*)-ψ-1-Phenyl-1-chloro-2-methylaminopropane [*d*-(*S*)-ψ-chloroephedrine] hydrochloride and its racemate, and *d*-(*s*)-ψ-1-phenyl-1-chloro-2-dimethylaminopropane [*d*-(*S*)-ψ-chloromethylephedrine] hydrochloride and its racemate were prepared from *l*-(*S*)-ephedrine, *dl*-ephedrine, *l*-(*S*)-methylephedrine, and *dl*-methylephedrine hydrochloride, respectively, by the method of Emde [*7*]. The analytical data are listed in Table 1.

d-l-[^{3}H]-Methamphetamine was prepared from *d*-(*S*)-chloroephedrine by catalytic reduction with $^3H_2$ and Pd·$BaSO_4$ [*6*]. Chromatographic analysis of the product, using an Aloka JTC-203 radio thin-layer chromatoscanner, yielded a single peak. The developing solvents are shown in Table 1. The specific radioactivity of the product was 170 mCi/mmol.

TABLE 1—*Analytical data for materials.*

Compound	Melting Point, °C	$[\alpha]_D^{20}$, °[a]	R_f Value on Thin-Layer Chromatograms[b]		Chemical Ionization Mass Spectrometry[c]
			Solvent A	Solvent B	QM^+, *m/e*
dl-Methamphetamine	132	0	0.70	0.58	150
l-(*R*)-Methamphetamine	172	−17	0.70	0.58	150
d-(*S*)-ψ-1-Phenyl-1-chloro-2-methylaminopropane	201	+115	0.87	0.85	184, 186
dl-ψ-1-Phenyl-1-chloro-2-methylaminopropane	186	0	0.87	0.85	184, 186
d-(*S*)-ψ-1-Phenyl-1-chloro-2-dimethylaminopropane	210–212	+104	0.72	0.65	198, 200
dl-ψ-1-Phenyl-1-chloro-2-dimethylaminopropane	157	0	0.72	0.65	198, 200
d-(*S*)-*N*-4-Aminobutyl-methamphetamine	...	...	0.06	0.16	221

[a]Specific optical rotation at 20°C for D (sodium) line.

[b]Thin-layer chromatography was performed on 250 μm of silica gel GF_{254} (E. Merck). The solvent systems used for development were (A) isopropanol-28% aqueous ammonia (95:5) and (B) chloroform-methanol (95:5) saturated with 28% aqueous ammonia.

[c]Chemical ionization mass spectrometry was performed using isobutane as a reactant gas; QM^+ = quasi-molecular ion.

Preparation of Antigen

d-N-4-Aminobutylmethamphetamine was prepared by the method of Cheng et al [*1*,*5*]. The purity of the product was checked by TLC and CIMS, as shown in Table 1. The *d-N*-4-aminobutylmethamphetamine-BSA conjugate was prepared by a carbodiimide method [*1*,*5*].

Preparation of Antiserum

The antigen (2 mg) was dissolved in 1 mL of physiological saline solution and emulsified with an equal volume of Freund's complete adjuvant. This emulsion was injected subcutaneously at multiple sites into the backs of male albino rabbits. A booster injection was given every two weeks twice and then every four weeks three times. The animals were bled two weeks after the last injection.

Radioimmunoassay Procedure

For dilution of antiserum and normal rabbit serum, 0.02*M* tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer (pH 7.2) was used. The reaction mixture, which contained 100 μL of antiserum diluted 80-fold, normal rabbit serum diluted fivefold, 0.02*M* Tris-HCl buffer, *d*-1-[^{3}H]-methamphetamine solution (approximately 0.01 μCi), and an unknown or standard solution, was incubated at 25°C for 1 h. Then 500 μL of saturated ammonium sulfate solution was added, and the mixture was centrifuged. The pellet was dissolved in 100 μL of buffer, 100 μL of saturated ammonium sulfate solution was added, and the mixture was recentrifuged. The pellet was dissolved in 300 μL of Soluene 350 (Packard Instrument Co., Inc.), and the bound radioactivity was counted in a liquid scintillation counter (Beckman LS-9000).

The percent cross-reaction of antibody was determined according to the method of Abraham [*8*].

Animal Experiment

Male Wistar rats weighing 100 to 150 g were used. Either *d*- or *dl*-methamphetamine hydrochloride in aqueous solution was administered orally at a dose of 10 or 20 mg/kg body weight. Rat urine was collected every 24 h after administration of the drug. The amount of *d*-methamphetamine was determined after dilution of the urine with water.

Results and Discussion

The standard curve for *d*-methamphetamine is shown in Fig. 1. The curve was linear up to 200 ng/mL of *d*-methamphetamine.

The specificity of the antibody against *d*-methamphetamine was given by cross-reaction studies with optical isomers of methamphetamine and its analogs. It is obvious from the data shown in Table 2 that the antibody in this system was more specific for *d*-methamphetamine than that prepared by Faraj et al [*2*]. No significant cross-reactions with *l*-methamphetamine and *dl*-amphetamine were observed.

Since urine is liable to cause nonspecific inhibition of binding, urine obtained from untreated rats was diluted twofold to 100-fold with water, then *d*-methamphetamine hydrochloride was added to each diluted urine to bring the concentration to 70 ng/mL, and the amounts of *d*-methamphetamine were determined by radioimmunoassay. Table 3 shows that rat urine must be diluted more than 20-fold with water to prevent nonspecific inhibition in the assay.

To investigate the effects of *l*-methamphetamine on the accuracy of the radioim-

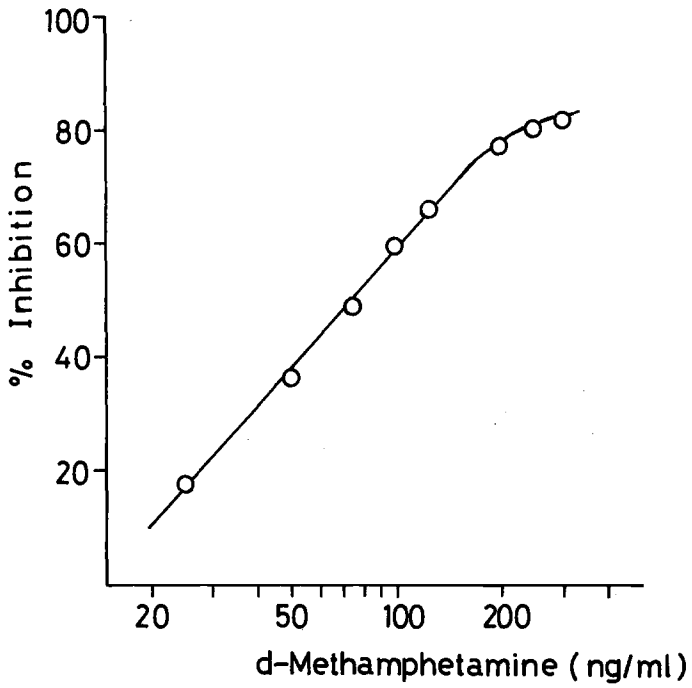

FIG. 1—*Inhibition of binding of* d-*1-[^{3}H]-methamphetamine to antibody against* d-*methamphetamine by nonradioactive* d-*methamphetamine.*

TABLE 2—*Percent cross-reaction of antibody to* d-*methamphetamine with the optical isomers and analogs.*

Compound	Percent Cross-Reaction
d-(*S*)-Methamphetamine	100
dl-Methamphetamine	52
l-(*R*)-Methamphetamine	3.9
dl-Amphetamine	0.9
l-(*S*)-Methylephedrine	44
dl-Methylephedrine	26
l-(*S*)-Ephedrine	0.4
dl-Ephedrine	0.8
d-(*S*)-ψ-1-Phenyl-1-chloro-2-dimethylaminopropane	1.1
dl-ψ-1-Phenyl-1-chloro-2-dimethylaminopropane	1.2
d-(*S*)-ψ-1-Phenyl-1-chloro-2-methylaminpropane	0.04
dl-ψ-1-Phenyl-1-chloro-2-methylaminopropane	0.2

munoassay for *d*-methamphetamine, 70 ng/mL of *d*-methamphetamine hydrochloride and varying concentrations of *l*-methamphetamine hydrochloride were added to the 50-fold diluted urine obtained from untreated rats. As shown in Table 4, the presence of more than 100 ng/mL of *l*-methamphetamine hydrochloride in the solution containing 70 ng/mL of *d*-methamphetamine hydrochloride interfered with the results of the assay for

TABLE 3—*Effect of dilution of rat urine.*

Dilution of Urine with Water, Times by Volume	*d*-Methamphetamine Hydrochloride Found,[a] ng/mL
2	124 ± 2
5	90 ± 2
10	76 ± 4
20	70 ± 3
40	74 ± 3
60	73 ± 2
100	70 ± 3

[a]Sufficient *d*-methamphetamine hydrochloride was added to each dilution of urine obtained from untreated rats to bring the concentration to 70 ng/mL.

TABLE 4—*Effect of* l-*methamphetamine on radioimmunoassay for* d-*methamphetamine.*

l-Methamphetamine Hydrochloride Added,[a] ng/mL	*d*-Methamphetamine Hydrochloride Found, ng/mL
0	70 ± 3
20	68 ± 5
50	72 ± 4
100	74 ± 3
200	96 ± 3
500	130 ± 5

[a]*l*-Methamphetamine hydrochloride and 70 ng/mL of *d*-methamphetamine hydrochloride were added to 50-fold diluted urine obtained from untreated rats.

d-methamphetamine. The recovery of *d*-methamphetamine, which was added to 50-fold diluted rat urine at concentrations of 33.3, 66.7, and 100.0 ng/mL as hydrochloride, ranged from 93 to 100% for nine determinations.

The amounts of *d*-methamphetamine excreted in urine after oral administration of *d*- or *dl*-methamphetamine are shown in Table 5. The urine had a pH of 8.5 to 9.0. The amount of *d*-methamphetamine excreted in urine after oral administration of *d*-methamphetamine was higher than that excreted after oral administration of *dl*-methamphetamine.

For resolution and determination of optical isomers of amphetamine, gas chromatographic techniques using *N*-trifluoroacetyl-*l*-prolyl chloride or *N*-pentafluoropropionyl-*l*-propyl chloride as the resolving agent have already been developed [*9–11*], and the methods have been applied to the determination of optical isomers of amphetamine and methamphetamine in biological materials [*12,13*]. The radioimmunoassay established here for the determination of *d*-methamphetamine in urine after administration of *d*- or *dl*-methamphetamine makes special pretreatment of the specimens unnecessary.

TABLE 5—*Urinary excretion of* d-*methamphetamine after oral administration of* d- *or* dl-*methamphetamine to rat.*

Day after Administration	Percent Dose		
	d-Methamphetamine		*dl*-Methamphetamine,[a]
	10 mg/kg	20 mg/kg	20 mg/kg
1	8.5 ± 0.6	10.9 ± 1.9	4.7 ± 0.2
2	0.9 ± 0.2	0.6 ± 0.3	0.6 ± 0.2
3	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1

[a]Percentage of *d*-methamphetamine in the urine to the *d*-isomer in the racemate administered.

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