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A Rapid and Simple Screening Method for Methamphetamine in Urine by Radioimmunoassay using a ^{125}I-Labeled Methamphetamine Derivative[1]

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N-Carboxymethylmethamphetamine, a derivative of methamphetamine, was prepared through a new synthetic pathway from ephedrine.

Specific antiserum was obtained by immunization of rabbits with the conjugate of N-carboxymethylmethamphetamine with bovine serum albumin. A radioimmunoassay procedure was established using this antibody (specific for methamphetamine) and a ^{125}I-methamphetamine derivative. A high degree of specificity of the antibody was confirmed by testing for cross-reaction with several methamphetamine analogs, and the sensitivity was found to be 1 ng/tube. The present micro method using radioimmunoassay is highly sensitive, rapid, simple and may be useful as a micro-scale primary screening test for methamphetamine excreted in human urine, for forensic and medical purposes.

Keywords——radioimmunoassay; methamphetamine; N-carboxymethylmethamphetamine; new synthetic method; antiserum; ^{125}I-labeled methamphetamine; specificity; sensitivity

dl-Methamphetamine(MA) is a well-known antihypnotic. Abuse of this drug is a serious social problem, and the preparation, storage and usage of this stimulant have always been strictly controlled in Japan. It is therefore desirable to have available a rapid and convenient method for the determination of micro amount of the compound in biological fluids of mammals, especially man.

MA in biological samples has been analyzed by gas and thin-layer chromatography, ultraviolet and fluorescence spectrometry and by an enzymatic method. [*cf.*3] However, these methods present many technical problems in the quantitative determination of this compound in human blood and urine. Recently, radioimmunoassay (RIA) has been developed as a highly specific and sensitive technique for the estimation of MA in biological samples using ^{3}H-labeled amphetamine.[3,4] We have applied the immunochemical assay technique using the more readily available ^{125}I-labeled MA derivative for the determination of MA in rat urine at the nanogram level. This RIA method appears to be suitable and convenient for rapid screening for foresic and clinical purposes.[1a,b] This report describes the method, including the preparation of specific antibodies to MA (I) with a haptenic derivative, N-car-

1) Preliminary communications have already appeared; *a*) S. Inayama, Y. Tokunaga, E. Hosoya, T. Nakadate, T. Niwaguchi, K. Aoki, and S. Saito, *Chem. Pharm. Bull.*, **25**, 838 (1977); *b*) S. Inayama, Y. Tokunaga, E. Hosoya, T. Nakadate, and T. Niwaguchi, *Chem. Pharm. Bull.*, **25**, 840 (1977).

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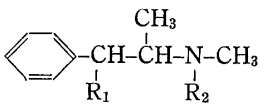
3) B.A. Faraj, Z.H. Israili, and N.E. Kight, *J. Med. Chem.*, **19**, 20 (1976).

4) L.T. Cheng, S.Y. Kim, A. Chang, and A. Castro, *FEBS Lett.*, **36**, 339 (1973).

boxymethylmethamphetamine (III), which is available through a new synthetic route from *dl*-ephedrine (IV).



I : $R_1=R_2=H$ (methamphetamine)
II : $R_1=H$, $R_2=CH_2COOCH_2CH_3$
III : $R_1=H$, $R_2=CH_2COOH$
IV : $R_1=OH$, $R_2=H$ (ephedrine)
V : $R_1=Cl$, $R_2=H$
VI : $R_1=SC_6H_5$, $R_2=H$
VII : $R_1=SC_6H_5$, $R_2=CH_2COOCH_2CH_3$
VIII : $R_1=H$, $R_2=CH_2CONH$-BSA[a]
IX : $R_1=H$, $R_2=(CH_2)_3NH_2$
X : $R_1=H$, $R_2=(CH_2)_3NHCOCH_2C_6H_4OCOCH_3(p)$
XI : $R_1=H$, $R_2=(CH_2)_3NHCOCH_2C_6H_4OH(p)$
a) BSA=bovine serum albumin

Chart 1. Structures of Methamphetamine Derivatives

Materials and Methods

Preparation of Antigen——All the new synthetic materials were well characterized by means of elemental analyses, as well as infrared (IR), proton nuclear magnetic resonance (PMR), and chemical ionization mass (CI-MS) spectroscopy.

Treatment of I with ethyl bromoacetate in benzene in the presence of anhydrous sodium carbonate (Na_2CO_3) at room temperature overnight afforded, in 71% yield, 1-phenyl-2-(N-ethoxycarbonylmethyl-N-methyl)aminopropane (II) as a colorless oil, bp 151—154° (5 Torr); $C_{14}H_{21}NO_2$. The subsequent hydrolysis of II with 5% potassium hydroxide (KOH) in methanol (MeOH) furnished, in 79% yield, III as colorless prisms, mp 142.5—143.5°; $C_{12}H_{17}NO_2$. *Anal.* Calcd C, 69.54; H, 8.27; N, 6.76. Found: C, 69.70; H, 8.23; N, 6.61.

A solution of pseudochloroephedrine (V), which was easily derived from IV and sodium thiophenoxide in tetrahydrofuran (THF) and ethanol (EtOH), was allowed to stand at room temperature for 10 hr. The usual work-up furnished 2-methylamino-1-phenyl-1-phenylthio-propane (VI) in 35—50% yield as an oil. Hydrochloride, mp 183—184°; $C_{16}H_{19}NS\cdot HCl$. *Anal.* Calcd C, 65.40; H, 6.86; N, 4.77. Found: C, 65.74; H, 6.95; N, 4.70. 2-(N-Ethoxycarbonylmethyl-N-methyl)amino-1-phenyl-1-phenylthio-propane (VII) was obtained as an oil in 86% yield when VI was treated with ethyl bromoacetate and anhydrous Na_2CO_3 at room temperature for 1 day. Hexachloroplatinate, mp 111—113°; $C_{20}H_{25}NO_2S\cdot 1/2H_2PtCl_6$. Desulfurization of VII in EtOH by means of Raney nickel was carried out in the usual fashion to give II in 68% yield; it was identical with an authentic sample of II prepared directly from I as mentioned above. Hydrolysis of II by the method described above furnished the corresponding acid, which was identical with an authentic sample of the above-mentioned III in every respect.

The conjugate of III with bovine serum albumin (III-BSA) (VIII) was prepared by conjugation of III to BSA using the mixed anhydride method[5,6] as follows. To 15 mg of III dissolved in 3 ml of dioxane, 17 μl of tri-*n*-butylamine and 9.1 μl of isobutyl chlorocarbonate were added. The mixture was stirred for 30 min in an ice bath. After adding 1 N sodium hydroxide solution dropwise to 70 mg of BSA in 50% dioxane-water, the mixture was adjusted to pH 8.0 and stirred for 30 min. The above two mixtures were combined, then 3 ml of water was added and the whole was stirred for 4 hr in an ice bath. The reaction mixture was concentrated by ultrafiltration using a Diaflo UM-2 membrane and purified by Sephadex G-25 gel chromatography. The conjugate fraction was lyophilized. Ten to twenty hapten molecules were found to combine with one BSA molecule in VIII by fluorometric determination in the presence of Marquis reagent[7] (Ex 400 nm; Em 465 nm).

Preparation of Specific Antisera——The antigen mentioned above (VIII) (2—5 mg) was dissolved in 1 ml of saline and emulsified with an equal volume of Freund's complete adjuvant. This emulsion was subcutaneously inoculated into male albino rabbits at multiple sites along the back. A booster injection was given every 2 weeks. The animals were bled 10—16 weeks after the initial injection. The sera thus obtained were tested for antibody production by means of the ring test or Ouchterlony analysis.

Synthesis of ^{125}I-MA Derivative——A solution of N-(3-aminopropyl)methamphetamine (IX) (412 mg) in THF (5 ml) was allowed to stand overnight at room temperature with *p*-acetoxyphenylacetylchloride

5) *a*) J.R. Vaughan, Jr., and R.L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952); *b*) B.F. Erlanger, F. Boreck, S.M. Beiser, and S. Lieberman, *J. Biol. Chem.*, **228**, 713 (1957).
6) B.H. Wainer, F.W. Fitch, R.M. Rothberg, and J. Fried, *Science*, **176**, 1143 (1972).
7) *a*) T. Ueda, A. Takada, and Y. Sakamoto, *Yakugaku Zasshi*, **93**, 535 (1973); *b*) H. Kozuka and S. Kawae, *Reports of Nat'l Res. Inst. of Police Science* (Tokyo), **20**, 252 (1967).

(530 mg) in the presence of anhydrous Na_2CO_3 (318 mg). The resulting solution was poured into dil HCl. The aqueous layer was washed with benzene, basified with saturated Na_2CO_3 and extracted with chloroform ($CHCl_3$). The organic layer was concentrated to give N-[3-(*p*-acetoxyphenylacetylamino)propyl]methamphetamine (X) (598 mg) as a pale yellow oil, $C_{23}H_{30}N_2O_3$. A solution of X (248 mg) in 5% KOH–MeOH was stirred for 24 hr at room temperature. After acidifying the reaction mixture with 2 N HCl and washing with benzene, the aqueous layer was basified with sodium bicarbonate and extracted with $CHCl_3$. The extract gave N-[3-(*p*-hydroxyphenylacetylamino)propyl]methamphetamine (XI) (187 mg) as a pale yellow oil, $C_{21}H_{28}N_2O_2$. *Anal.* Calcd C, 74.07; H, 8.29; N, 8.23. Found: C, 73.65; H, 8.63; N, 7.76.

^{125}I-labeled XI was prepared according to the method of Greenwood *et al.*[8] and purified by SP-Sephadex C-25 column chromatography. To a small test tube containing 0.25 μg of XI and 250 μCi of carrier-free $Na^{125}I$ (150 μl of 0.02 M Tris-HCl buffered saline solution, pH 7.4), 100 μg of chloramine T (in 25 μl of buffered solution) was added. Sodium metabisulfite (240 μg) solution was poured dropwise into the reaction mixture 2 min later. The reaction mixture was transferred to a small SP-Sephadex C-25 column. After washing the column with water, ^{125}I-labeled XI was eluted with 0.5 N ammonium hydroxide solution. An aliquot (5 μl) of the fraction was used for radioactivity measurement. The ^{125}I-XI fraction was stored and used for the RIA described below.

Radioimmunoassay Procedure——One hundred μl of the sample was incubated at 37° for 1 hr with 300 μl of the antiserum diluted 600 times with Tris-HCl buffered saline (0.5 M, pH 7.4) containing a fixed amount of ^{125}I-XI (*ca.* 10000 cpm). After the incubation, 400 μl of saturated ammonium sulfate solution was added to all the reaction mixtures. After centrifugation at 3000 rpm for 15 min at 5°, the radioactivity of the supernatant containing the unbound labeled compound was counted.

Urinary Excretion of MA in Rats——This radioimmunoassay method for the detection of MA in biological samples was used to examine the excretion of MA into urine in rats. Male Wister rats weighing 200—300 g were subcutaneously administered with MA hydrochloride at a dose of 5, 1, or 0.2 mg/kg. Urine of the rats (kept in metabolic cages) was collected at intervals, as shown in Fig. 2(a) and 2(b). The amount of MA in the urine was determined as described above after adequate dilution of the sample with water.

Results and Discussion

The radioimmunoassay of MA was carried out with the antibodies and the labeled compound, prepared by the methods described above. The standard curve for MA is shown in Fig. 1. The sensitivity of the present RIA was better (1 ng/tube) than that reported in our preliminary communication (8 ng/tube)[1b] due to improvement of the ^{125}I-iodination of XI, giving ^{125}I-MA derivative with higher specific activity (*ca.* 100 Ci/mmol). The specificity of the antibody directed against MA was examined by competitive binding assay with several

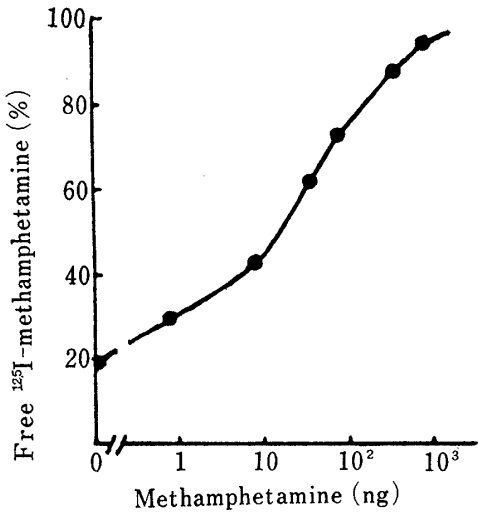


Fig. 1. Standard Curve for the Inhibition of Binding of a ^{125}I-Labeled Methamphetamine Derivative with the Antibody for Methamphetamine by Cold Methamphetamine

The abscissa and ordinate represent the amount of cold methamphetamine added and the percentage of unbound ^{125}I-labled methamphetamine derivative, respectively. Each point is the average of three determinations.

Table I. Specificity of the Antiserum for Methamphetamine

Compound	Cross reactivity[a] (%)
Methamphetamine (I)	100
Chloroephedrine (V)	8.9
Ephedrine (IV)	4.5
Amphetamine	4.2
Methoxyphenamine (*o*-Methoxymethamphetamine)	1.0
p-Hydroxymethamphetamine	0.8

a) cross reactivity: the amount of the substance which displaced ^{125}I-methamphetamine derivative equivalent to the displaced by 50 ng (half-maximal inhibition value) of unlabeled methamphetamine was taken as 100 using 1:600 dilution of antiserum.

8) F.C. Greenwood, W.M. Hunter, and J.S. Glover, *Biochem. J.*, **89**, 114 (1963).

analogs of MA. The results are summarized in Table I; the antiserum is clearly specific to MA. The inhibition data indicate that the specificity of the antibody for MA is directed towards the aromatic ring more than to the side chain region, because analogs substituted on the aromatic ring, such as *p*-hydroxymethamphetamine and methoxyphenamine, showed markedly reduced binding affinity. This finding supports the view that the specificity of the antibody for the hapten molecule is largely related to the region furthest away from the conjugating site of the hapten with the carrier protein. Endogenous substances such as catecholamine, phenylalanine and tyrosine exhibited no affinity for this antibody.

As an example of application of this RIA, the excretion of MA into urine in rats was analyzed, as shown in Fig. 2.

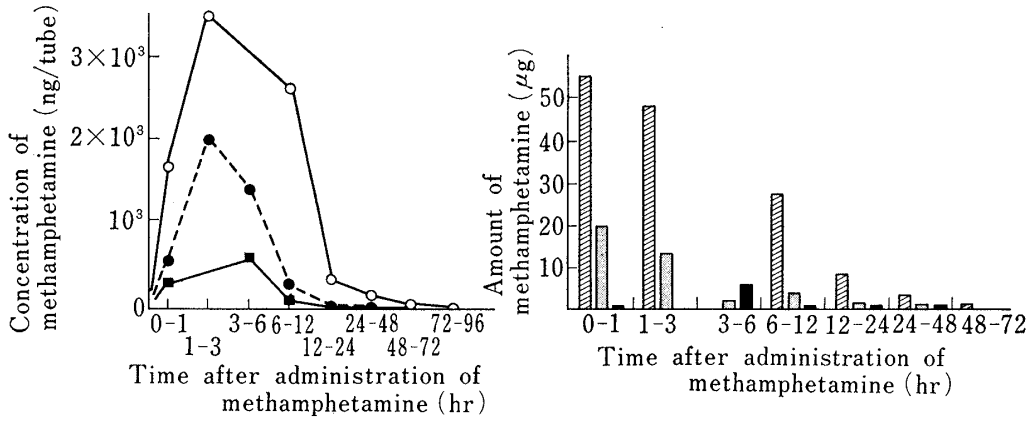


Fig. 2 (a) Fig. 2 (b)

Fig. 2. Urinary Excretion of Methamphetamine in Rats

The con centration (a) and amount (b) of methamphetamine in samples of rat urine excreted at the designated intervals were determined by the present radioimmunoassay.

Caldwell *et al.*[9] reported that about one-tenth of the dose of MA was excreted unchanged in rat urine, together with *ca.* 3% of amphetamine, and that the predominant metabolites excreted with 24 hr were phenols such as *p*-hydroxymethamphetamine (31%), including their conjugates, mainly with glucuronic acid. The total MA in the urine of rats detected by the present method was estimated to be approximately 10% of the dose. These results indicate that our RIA is highly specific for unchanged MA, and that the antibody is able to distinguish MA from its metabolites, especially the phenols and their glucuronides. In the case of man, the most abundant metabolite in urine is also unchanged MA, amounting to 23% of the dose over 24 hr, together with small amounts of metabolites such as *p*-hydroxymethamphetamine (15%), hippuric acid (5%), amphetamine (2—3%), *etc.*[9] Therefore, the detection of unchanged MA is probably the most useful guide to the intake of MA.

However, there might still be some risk of false results in the case of humans taking very large quantities of ephedrine (exceeding the therapeutic dose), because the antibody has some, though very low, cross-reactivity against ephedrine. In such cases, MA could be separated from ephedrine before the RIA procedure.

In conclusion, our RIA procedure possesses several advantages as a primary screening test for MA in urine for forensic and clinical purposes: (1) high sensitivity, (2) simple technique, (3) rapid procedure, (4) no pretreatment, and (5) micro-scale amounts of samples. The use of this RIA approach for the screening for human urine will be reported in a subsequent paper.

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9) J. Caldwell, L.G. Dring, and R.T. Williams, *Biochem. J.*, **129**, 11 (1972).