Tetsukichi Niwaguchi,[1] Ph.D.; Takako Inoue,[1] B.Sc.;
Tohru Kishi,[1] Ph.D.; Yukio Kanda,[1] M.Sc.;
Takeshi Niwase,[1] Ph.D.; Teruo Nakadate,[2] M.Sc.; and
Seiichi Inayama,[2] Ph.D.

Hemagglutination-Inhibition Test for Methamphetamine Excreted in Human Urine

Recently radioimmunoassay (RIA) methods have been developed for the determination of nanogram levels of methamphetamine in urine [*1-3*]. These methods are suitable for rapid screening but require expensive instrumentation. This communication describes a rapid and simple hemagglutination-inhibition (HI) test that might be adaptable to the mass screening of urine samples without special equipment.

Materials and Methods

Preparation of Antigen

To a solution of methamphetamine (500 mg) in benzene (15 ml), ethyl γ-bromobutyrate (2530 mg) was added by drops in the presence of anhydrous sodium carbonate (2120 mg) and the mixture was refluxed overnight. The resulting solution was poured into diluted hydrochloric acid. The aqueous layer was washed with benzene, alkalinized with 1*N* sodium hydroxide solution, and extracted with benzene. The organic layer was evaporated to give *N*-(3-ethoxycarbonylpropyl) methamphetamine (I) (700 mg) as a colorless oil, boiling point 140 to 142°C (133 Pa or 1 mm Hg), $C_{16}H_{25}NO_2$. Infrared analysis with a KBr disk showed maximum frequency at 1735 cm^{-1} (C = 0). Nuclear magnetic resonance analysis with deuterochloroform as the solvent gave the following information: δ = 0.90 (d, 3H; $\mathcal{J}$ = 6.0; $\gtrless$CHC$\underline{H}_3$); 1.24 (t, 3H; $\mathcal{J}$ = 7.0; —$CO_2CH_2C\underline{H}_3$); 1.55 (m, 2H; $\gtrless NCH_2C\underline{H}_2CH_2CO_2$—); 2.26 (s, 3H; $\gtrless NC\underline{H}_3$); 2.2 to 2.6 (m, 5H; $\gtrless C\underline{H}CH_3$ and $\gtrless NC\underline{H}_2CH_2C\underline{H}_2CO_2$—); 2.90 (m, 2H; $C_6H_5C\underline{H}_2$—); 4.13 (q, 2H; —$CO_2C\underline{H}_2CH_3$); 7.20 (m, 5H; aromatic $\underline{H}$), where δ is the chemical shift in parts per million relative to the internal reference, s is singlet, d is doublet, t is triplet, q is quartet, m is multiplet, $\mathcal{J}$ is a coupling constant in hertz, and the δ value is assigned to the underlined H. Chemical ionization mass spectrometry using methane as a reagent gas gave *m/e* 264 (quasimolecular ion [QM^+]).

A solution of I (700 mg) in 5% potassium hydroxide/methanol was stirred for 24 h at

Received for publication 27 July 1978; revised manuscript received 11 Oct. 1978; accepted for publication 13 Oct. 1978.

[1]Chief, Forensic Science Division; forensic chemist, First Chemical Laboratory; forensic chemist, Second Chemical Laboratory; principal chemist, Second Chemical Laboratory; and chief, First Chemical Laboratory, respectively; National Research Institute of Police Science, Sanban-cho, Chiyoda-ku, Tokyo, Japan.

[2]Assistant, Department of Pharmacology; and professor, Pharmaceutical Institute, respectively; School of Medicine, Keio University, Shinanomachi, Shinjuku-ku, Tokyo, Japan.

room temperature. Gaseous hydrochloric acid was blown into the reaction mixture to adjust to pH 3. The residue obtained by evaporation of the solvent was dissolved in ethanol and chromatographed on alumina. Elution with methanol and water gave a colorless sticky oil in the latter eluate, which was repeatedly chromatographed on SP-Sephadex C-25. Elution with 0.5*N* aqueous ammonia gave colorless needles, which were recrystallized from acetone to give *N*-(3-carboxypropyl) methamphetamine (II) (494 mg) as colorless needles, melting point 57 to 59°C, $C_{14}H_{21}NO_2$. Infrared analysis with a KBr disk showed maximum frequency at 1560 cm^{-1} (C = O). Nuclear magnetic resonance analysis with deuterochloroform as the solvent gave the following data: δ = 1.07 (d, 3H; $\mathcal{J}$ = 6.0; >CHC$\underline{H}_3$); 2.55 (s, 3H; >NC$\underline{H}_3$); 2.4 to 2.7 (m, 3H; >C$\underline{H}$NCH$_2$CH$_2$C$\underline{H}_2$CO$_2$H); 2.90 (t, 2H; $\mathcal{J}$ = 6.0; > NC$\underline{H}_2$CH$_2$CH$_2$CO$_2$H); 3.22 (m, 2H; $C_6H_5CH_2$—); 7.25 (m, 5H; aromatic $\underline{H}$). Chemical ionization mass spectrometry using methane as a reagent gas gave *m/e* 236 (QM^+).

Compound II was coupled to bovine serum albumin (BSA) by the mixed anhydride method [*4–6*]. About 10 moles of the hapten were found to bind to a mole of BSA by the fluorometric determination (excitation wavelength 400 nm, emission wavelength 465 nm) in the presence of Marquis reagent [*7*].

Preparation of Antisera

The antigen (II-BSA) (2 mg) was dissolved in 1 ml of saline and emulsified with an equal volume of Freund's complete adjuvant. This emulsion was injected in male albino rabbits subcutaneously at multiple sites along the back. A booster injection was given twice every fortnight and then twice every four weeks. The animals were bled 16 weeks after the initial injection, and the sera harvested were stored at −20°C. Before being used in the HI test, the sera were inactivated at 56°C for 30 min and absorbed with BSA which was polymerized by the mixed anhydride method. The sera treated were diluted with 0.15*M* phosphate-buffered saline (pH 7.2) prepared by mixing 100 ml of saline, 28 ml of 0.15*M* monobasic potassium phosphate, and 72 ml of 0.15*M* dibasic sodium phosphate containing 1% normal rabbit serum. The normal rabbit serum used in this experiment was inactivated at 56°C for 30 min.

Fixation of Rabbit Red Blood Cells with Glutaraldehyde

The rabbit red blood cells were washed three times with saline and 0.25 ml of the washed red blood cells were suspended in 5 ml of the phosphate-buffered saline. To this suspension, 0.15 ml of 0.25% glutaraldehyde solution was added and the mixture was incubated for 15 min at room temperature [*8*]. To the incubation mixture, 5 ml of the phosphate-buffered saline and 10 ml of 0.005% tannic acid in saline were added, and the mixture was incubated for 10 min at 37°C. The cells were collected by centrifugation, washed with 10 ml of the phosphate-buffered saline, and resuspended in 10 ml of saline [*9*].

Sensitization of Tannic Acid Cells

Forty millilitres of 0.15*M* phosphate-buffered saline (pH 6.4), made by mixing 100 ml of saline, 67.7 ml of 0.15*M* monobasic potassium phosphate and 32.3 ml of 0.15*M* dibasic sodium phosphate; 2 mg of II-BSA in 10 ml of saline; and 10 ml of the suspension of tannic acid cells were mixed in this order, and the mixture was incubated for 10 min at room temperature. The cells were centrifuged, washed with 20 ml of 1% normal rabbit serum, and resuspended in 10 ml of 1% normal rabbit serum [*9*].

Test Procedure

The HI test was performed in U-shaped cups of microtiter plates (Cooke Engineering Co., U.S.A.). In a cup 20 μl each of 0.15*M* phosphate-buffered saline (pH 7.2), 1% normal rabbit serum, sample solution, and the antiserum were mixed by using a micromixer (Tomy Seiko Co., Ltd., Japan) for 1 min, and the mixture was incubated for 30 min at room temperature. To this mixture 20 μl of a suspension of the sensitized tannic acid cells was added and mixed with a micromixer for 1 min. The results were observed after an additional 60 to 120 min of incubation at room temperature.

Results and Discussion

It was verified by the ring test and Ouchterlony technique that the antibody to methamphetamine was present in the serum obtained from the immunized rabbit. When this serum, which had an hemagglutination titer of 1:6400, was used for HI test in dilutions of 1:1600, 1:3200, and 1:6400, the minimal amounts of methamphetamine required for the inhibition were 10, 1, and 0.2 ng, respectively.

The specificity of the antiserum directed toward methamphetamine was examined with principal metabolites and some homologs of methamphetamine, and the results are shown in Table 1.

It was verified that the antiserum obtained was specific for methamphetamine; it showed no significant inhibition with principal metabolites and homologs of methamphetamine.

For the detection of methamphetamine excreted in human urine, the urine specimens must be diluted tenfold with water because they show nonspecific inhibition without being diluted. Human urine specimens from 30 individuals suspected of taking methamphetamine were examined by the HI test. Of these, 25 specimens gave a positive reaction. These results agreed with those obtained by gas chromatography and thin-layer chromatography. It was established that this HI test using antiserum obtained was useful as a screening test for methamphetamine excreted in human urine.

Summary

Antibodies reactive with methamphetamine were prepared by immunizing rabbit with *N*-(3-carboxypropyl) methamphetamine-BSA conjugate. A hemagglutination-inhibition

TABLE 1—*Sensitivity and specificity of the HI test for methamphetamine.*[a]

Compound	Minimal Amount Required for Inhibition, ng
Methamphetamine	1
Amphetamine	100
Ephedrine	50
Methylephedrine	50
Chloroephedrine (1-phenyl-1-chloro-2-methylaminopropane)	200
Norephedrine	1 000
Chloronorephedrine (1-phenyl-1-chloro-2-aminopropane)	200
p-Hydroxymethamphetamine (pholedrine)	200
p-Hydroxyephedrine [1-(*p*-hydroxyphenyl)-1-hydroxy-2-methylaminopropane]	10 000
Methoxyphenamine	200

[a] The antiserum was used in a dilution of 1:3200.

test for methamphetamine excreted in urine was investigated. More than 1 ng of methamphetamine in 20 μl of urine diluted ten times with water has been detected by the use of sensitized rabbit red cells prepared by adsorption of *N*-(3-carboxypropyl) methamphetamine-BSA on tannic acid-treated rabbit red blood cells. Principal metabolites and several homologs of methamphetamine have shown no significant interference in this test.

References

[1] Cheng, L. T., Kim, S. Y., Chung, A., and Castro, A., "Amphetamines: New Radioimmunoassay," *FEBS Letters*, Vol. 36, No. 3, 1973, pp. 339-342.

[2] Faraj, B. A., Israili, Z. H., and Kight, N. E., "Specificity of an Antibody Directed Against *d*-Methamphetamine. Studies with Rigid and Nonrigid Analogs," *Journal of Medicinal Chemistry*, Vol. 19, No. 1, 1976, pp. 20-25.

[3] Inayama, S., Tokunaga, Y., Hosoya, E., Nakadate, T., and Niwaguchi, T., "The Radioimmunoassay for Methamphetamine," *Chemical and Pharmaceutical Bulletin*, Vol. 25, No. 4, 1977, pp. 840-842.

[4] Vaughan, J. R., Jr. and Osato, R. L., "The Preparation of Peptides Using Mixed Carbonic-Carboxylic Acid Anhydrides," *Journal of the American Chemical Society*, Vol. 74, No. 3, 1952, pp. 676-678.

[5] Erlanger, B. F., Borek, F., Beiser, S. M., and Lieberman, S., "Steroid-Protein Conjugates. I. Preparation and Characterization of Conjugates of Bovine Serum Albumin with Testosterone and with Cortisone," *The Journal of Biological Chemistry*, Vol. 228, No. 2, 1957, pp. 713-727.

[6] Wainer, B. H., Fitch, F. W., Rothberg, R. M., and Fried, J., "Morphine-3-Succinyl-Bovine Serum Albumin: An Immunogenic Hapten-Protein Conjugate," *Science*, Vol. 176, No. 4039, 1972, pp. 1143-1145.

[7] Kozuka, H. and Kawase, S., "Fluorometric Analysis of Drugs. (I)—Fluorescence of Drugs by Marquis Reaction," *Reports of the National Research Institute of Police Science*, Vol. 20, No. 4, 1967, pp. 252-256.

[8] Suzuki, T., Tanaka, S., and Kawanishi, Y., "An Improved Method for Preparation of Stable Antigen Coupled Erythrocytes for Passive Hemolysis," *Immunochemistry*, Vol. 11, No. 8, 1974, pp. 391-394.

[9] Stavitsky, A. B., "Micromethods for the Study of Proteins and Antibodies. I. Procedure and General Applications of Hemagglutination and Hemagglutination-Inhibition Reactions with Tannic Acid and Protein-Treated Red Blood Cells," *The Journal of Immunology*, Vol. 72, No. 5, 1954, pp. 360-367.

Address requests for reprints or additional information to
T. Niwaguchi, Ph.D.
Chief, First Forensic Science Division
National Research Institute of Police Science
6. Sanban-Cho, Chiyoda-Ku
Tokyo, Japan