A Visual Membrane Immunoassay for the Detection of Methamphetamine Using an Enzyme-Labeled Tracer Derived from Methamphetamine and Amphetamine

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A visual membrane enzyme immunoassay is described for the measurement of methamphetamine in urine. To increase assay sensitivity, tracers with chemically similar structures were cross-checked with the antibodies to determine their influence on the antibody binding. Tracers of horseradish peroxidase-labeled methamphetamine (MA–HRP) and amphetamine (A–HRP) derivatives were prepared for this purpose. Significant differences in antibody specificity were found between the two tracers. Based on the results of this study, a pair of an antibody and a tracer was selected and a membrane enzyme immunoassay (EIA) was developed utilizing the competitive binding between methamphetamine and the drug-HRP tracer. UltraBind membrane (0.45 μm) was used as the solid matrix to which the antibody was attached. Using diaminobenzidine substrate with Co^{2+} ion, a stable grey color appeared on the surface of membrane for MA-negative urine samples. No color appeared for MA-positive urine with a cut-off level of 0.8 ppm.

Keywords methamphetamine; amphetamine; immunogen; tracer influence; visual membrane immunoassay

The development of a rapid and sensitive on-site detection method is a main concern in controlled drug screening for methamphetamine and its metabolites. The immunoassay method has been used to test for the presence of drugs and a small drug molecule should be modified to label enzyme or fluorescence for use as a tracer. The binding relationship between a modified tracer and an antibody often determines the sensitivity and specificity of the immunoassay. Eremin *et al.* reported that fluorescence-labeled amphetamine was a better tracer than labeled methamphetamine in fluorescence polarization immunoassay for the detection of methamphetamine.[2)] To upgrade the sensitivity of methamphetamine in membrane enzyme immunoassay, the affinity and specificity of antibodies were investigated with tracers prepared from 4-aminobutyl methamphetamine and amphetamine derivatives.

On-site screening tests require a simple and rapid, instrument-independent procedure. These "express" tests detect drug levels qualitatively and semi-quantitatively and utilize reagents immobilized on the membranes of porous carriers.[3-5)] They are also usually ready-to-use analytical devices containing all the necessary reagents. The tests allow analyte determination by comparing the color intensity of the active zone with a reference color detected either visually or by using a handy reflectance spectrometer. Even though the homogeneous immunoassay of methamphetamine[2,6)] is more rapid than the solid phase immunoassay,[7-10)] it is not appropriate in a field test because it requires an expensive and complicated instrument.

The purpose of our methamphetamine detection method is to overcome such limitations and provide on-site screening. This method needs no instrument and the resulting color differences between positive and negative urine samples can be recognized with the naked eye with a 0.8 ppm cut-off level of methamphetamine.

MATERIALS AND METHODS

Methamphetamine was obtained from the Korean National Institute of Health. *N*-(4-Bromobutyl)phthalimide and hydrazine hydrate, used to prepare the 4-aminobutyl derivative of methamphetamine, were purchased from Aldrich (WI., U.S.A.). Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), bovine serum albumin (BSA, fraction V), horseradish peroxidase (EC 1.11.1.7, specific activity 288 U/mg solid, RZ=3.1), sodium *m*-periodate, *o*-phenylenediamine (OPD), diaminobenzidine (DAB), glutaraldehyde (GAD), L-lysine and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide HCl (EDC) were purchased from Sigma Chemical Co. (MO., U.S.A.). UltraBind membrane (0.45 μm) was purchased from Gelman Sciences Inc. (MI., U.S.A.). All other inorganic chemicals and organic solvents were from our laboratory and of analytical reagent grades. The methamphetamine antibody (PAb_{DCC}) was prepared at the Genetic Engineering Research Institute (Daedok, Korea). A set of immunoreagents, a donation from Dr. Eremin of Moscow State University (Moscow, Russia), was obtained from the Russian Academy of Sciences (Moscow, Russia). Included were a methamphetamine antibody (PAb_{MSU}) which was prepared by immunizing *N*-(4-aminobutyl)methamphetamine–BSA immunogen, *N*-(4-aminobutyl)amphetamine, and *N*-(4-aminobutyl) amphetamine–HRP tracer.

Confirmation of *N*-(4-Aminobutyl)methamphetamine and Amphetamine Derivatives by GC/MSD *N*-(4-Aminobutyl)methamphetamine was prepared according to the method of Tamaki *et al.*[10)] The gas chromatographic separation of *N*-(4-aminobutyl)methamphetamine (4-ABMA) and *N*-(4-aminobutyl)amphetamine (4-ABA) was

performed using an HP5890A GC interfaced to an HP5970B series mass selective detector and an HP5970B mass chemstation including an HP 7946 disc derive and HP 2934A printer. 4-ABMA and 4-ABA were dissolved in chloroform and injected directly. The operating conditions of GC/MSD were as follows: column, fused silica capillary cross-linked 5% phenylmethyl silicon (SE54, 12 m length × 0.2 mm i.d. × 0.32 μm F.T.); detector temp., 300 °C; injector temp., 280 °C; oven temp., starting from 100 °C ending at 300 °C increasing at the rate of 20 °C/min; flow rate, 0.8 ml/min He; injection mode, split (1 : 10); data acquision, scan mode.

Preparation of Methamphetamine Immunogen and Horseradish Peroxidase Tracer The immunogen, *N*-(4-aminobutyl)methamphetamine–bovine serum albumin (4-ABMA–BSA), was prepared by the modified method of Tamaki *et al.*[10] Twelve milligrams of 4-ABMA and 4.8 mg of BSA were coupled using 72.9 mg of EDC in 0.68 ml H_2O. After 3 h of stirring at room temperature (RT), the reaction mixture was dialyzed against 10 mM phosphate buffered saline (PBS), pH 7.4. We used Sephacryl S-200sf chromatography (column size: 1.0 × 9.5 cm, flow rate: 0.4 ml/min, fraction size: 0.4 ml/fraction) to remove polymerized 4-ABMA–BSA using 10 mM PBS, pH 7.4.

Methamphetamine-horseradish peroxidase tracer (4-ABMA–HRP) was prepared by coupling 15 mg 4-ABMA and 24 mg HRP using 120 μl of 1% glutaraldehyde. The mixture was stirred for 2 h at RT, and 2 ml of 2 M lysine was added to terminate the reaction. After stirring for 2 h at 4 °C, the reaction mixture was dialyzed against 10 mM PBS, pH 7.4, and the precipitate was removed by centrifugation. We used Sephacryl S-200sf chromatography (column size: 1.5 × 44 cm, flow rate: 0.8 ml/min, fraction size: 1 ml/fraction) to remove polymerized 4-ABMA–HRP. We determined the molar ratio of MA to HRP by evaluating their spectral characteristics at 260, 280, and 403 nm.

Characterization of Antibody MA antiserum (PAb_{DCC}) was obtained from an Albino rabbit (female, 1.5 kg body weight). The primary immunization with an emulsified mixture of 300 μg antigen in 0.5 ml saline and 0.5 ml FCA was administered intramuscularly at two sites behind each shoulder blade. One or two booster injections were given at biweekly intervals using the same concentration of immunogen and FIA. Antiserum of PAb_{DCC} was evaluated for its specificity to both methamphetamine and amphetamine. For this purpose, a microtiter plate well was coated with 200 μl of appropriately diluted antibody in 50 mM carbonate buffer, pH 9.6, and stored overnight at 4 °C. Twenty microliters of 4-ABMA–HRP tracer in PBS (0.3 μg/ml) and 200 μl of serially diluted methamphetamine or amphetamine (0—500 μg/ml) was added to the well and incubated for 2 h at RT. After washing three times with 10 mM PBS containing 0.05% Tween 20 (PBST), 200 μl of OPD substrate (2.5 mg of *o*-phenylenediamine 2HCl and 0.6 μl of H_2O_2 per ml of 0.1 M phosphate-citrate buffer, pH 5.3) was added and the absorbance was measured at 450 nm. Antiserum of PAb_{MSU} was evaluated for its specificity using the same method as PAb_{DCC}, but an ABA–HRP tracer was used instead of the ABMA–HRP tracer.

Choice of Membrane Matrix Six different types of porous membranes were examined: 1) pure nitrocellulose membrane AE98 (pore size 5.0 μm, S&S), 2) pure nitrocellulose membrane BA85 (0.45 μm, S&S), 3) pure unmodified nylon 66 membrane (Biodyne A: 3.0 μm, Pall Biosupport), 4) pure unmodified nylon 66 membrane (Biodyne A: 5.0 μm, Pall Biosupport), 5) immunoaffinity membrane which has a derivatized functional group on its surface (Immunodyne: 5 μm, Pall Biosupport), and 6) UltraBind membrane (0.45 μm, Gelman Sciences Inc.) which possesses an aldehyde functional group on its surface. The membranes were examined for thier staining capability by immersing them in the HRP substrate. After incubating for 30 min at RT, the color intensity at 600 nm was measured by reflectance spectrometer (Hunter Lab. Ultrascan, VA., U.S.A.).

Procedure of Membrane Enzyme Immunoassay Ultrabind membrane (US-450) was cut to proper size (0.7 cm × 5 cm) and soaked in antibody solution (PAb_{MUS}: 0.1 mg/ml, PAb_{DCC}: 1 mg/ml). After overnight incubation at 4 °C (or 2 h at RT), the membrane was washed with PBS. The membrane was incubated in 3% BSA–PBS for 1 h at RT to block the non-specific binding sites. After washing with a PBS solution, each membrane was cut into pieces (0.7 cm × 0.7 cm). The squares were put into 4 wells of a 24-well microtiter plate containing 200 μl methamphetamine standard (0, 0.8, 4, 20 μg/ml, respectively) and 10 μl HRP tracer (4-ABMA: 0.3 μg/ml, or 4-ABA: 0.5 μg/ml) in a PBST solution. After incubation for 10 min at RT, the membrane was washed three times with PBST. The membrane was then incubated for 3 min with HRP substrate (5 mg of 3,3-diaminobenzidine, 0.2 ml of 1% $CoCl_2$ and 5 μl of 35% H_2O_2 in 10 ml PBS) and washed 3—4 times with water. The color intensity of the membrane surface was measured at 600 nm using a reflectance spectrometer. We also visually compared the color intensity of the membrane surface between MA-negative (zero) and MA-positive calibrators prepared in urine matrix.

RESULTS AND DISCUSSION

Confirmation of *N*-(4-Aminobutyl)methamphetamine and Amphetamine Derivatives by GC/MSD Methamphetamine (M.W. = 149) was activated to 4-ABMA prior to attaching BSA or HRP for its immunogen and tracer use. 4-ABA derived from amphetamine (M.W. = 135) was obtained from Moscow. The GC/MSD chromatograms of both 4-ABMA and 4-ABA are shown in Fig. 1.

4-ABMA (F.W. = 220) was eluted as a main peak at a retention time of 5.9 min under the experimental conditions with the characteristic mass fragments of *m/z* 72 $[(CH_2)_4NH_2] > 58[72\text{-}CH_2] > 129[220\text{-}C_6H_5CH_2] > 91[C_6H_5CH_2] > 42[NCHCH_3]$ in abundance (Fig. 1A). 4-ABA (F.W. = 206) was eluted as a main peak at 5.5 min. The chracteristic mass fragments appeared in abundance at *m/z* $98[(CH_2)_4NCHCH_3] > 72[(CH_2)_4\text{-}NH_2] > 115[NH_2(CH_2)_4NHCHCH_3] > 91[C_6H_5CH_2]\text{-} > 56[(CH_2)_4]$ (Fig. 1B). These mass fragmentations confirmed that the major peaks of 4-ABMA and 4-ABA

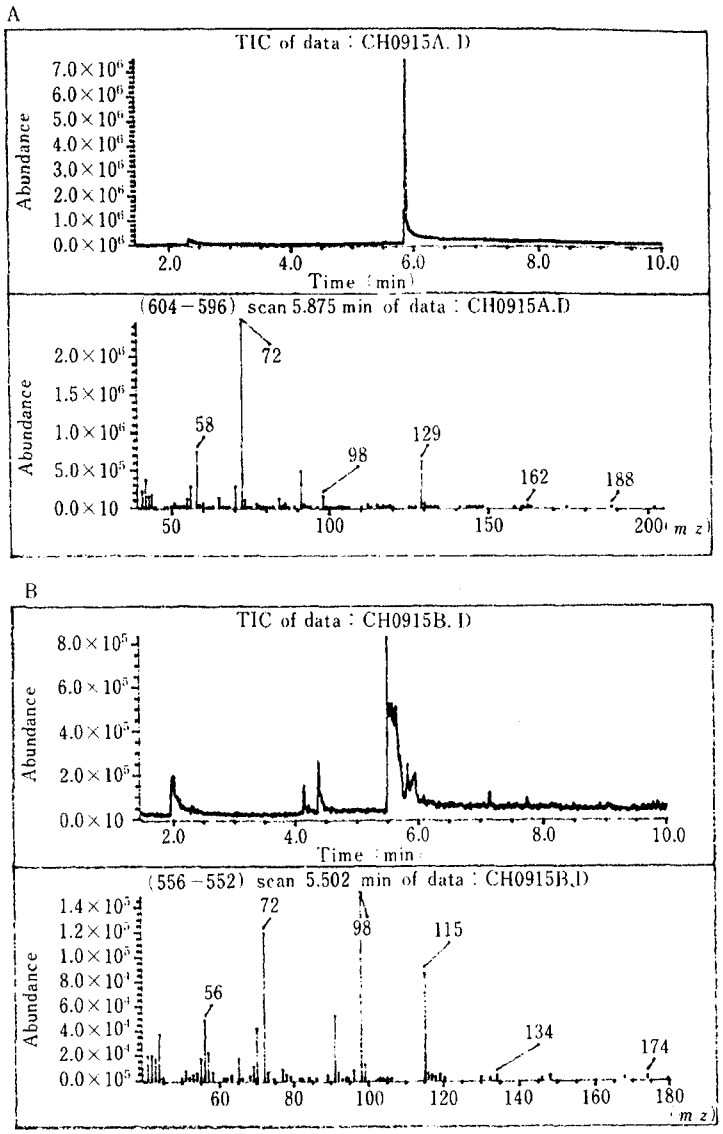

Fig. 1. Confirmation of *N*-(4-Aminobutyl)methamphetamine and Amphetamine Derivatives by Gas Chromatography/Mass Selective Detector

A fused silica capillary (SE54, 12 m length × 0.2 mm i.d. × 0.33 μm FT) was used. The operating conditions are described in the text. 4-Aminobutyl methamphetamine was eluted as a main peak at a retention time of 5.9 min (A) and 4-aminobutylamphetamine at 5.5 min (B) under the experimental conditions.

were synthesized as expected.

Characterization of 4-ABMA-HRP Tracer The 4-ABMA-HRP tracer was prepared using the glutaraldehyde method. The molar ratio was calculated based on the spectral characteristics of MA, HRP and MA-HRP. MA, with absorbance peaks at 260 nm, exhibited good linearity in the dose-response curve at a concentration range of 1—400 μg/ml. HRP has the absorbance characteristics at 403 nm. The absorbance ratio of A_{403}/A_{260} was found to be 3.07 for HRP itself and 1.97 for MA-HRP. The lower absorbance ratio in MA-HRP than in HRP was a result of the higher absorbance of MA at 260 nm in MA-HRP tracer compared to absorbance of HRP itself at 260 nm. We plotted the calibration curve of MA with absorbance at 260 nm, and calibration curve of HRP at 260, 403 nm, and the ratio of A_{403}/A_{260}. After the MA and HRP concentrations were calculated from the calibration curves of MA and HRP, the molar ratio of MA/HRP in MA-HRP tracer was found to be 23.

Binding Characteristics between Antibodies and Tracers The crucial components of a competitive binding immunoassay are the antibody, the unknown antigen and the labeled antigen tracer. The assay sensitivity depends on the competition between the labeled antigen tracer and the unknown antigen for binding sites on the antibody. In order to obtain a sensitive calibration curve, the antigen tracer bound to be antibody must produce a high signal response. Furthermore, the binding affinity of the antigen tracer to the antibody needs to be nearly the same as the binding affinity of the antigen to the antibody in order to obtain a reliable replacement curve.

The four-carbon bridged 4-ABMA-HRP tracer is quite different from the methamphetamine molecule itself. There was also a report that fluorescein isothiocyanate labeled 4-ABA (4-ABA-FITC) was a better tracer than 4-ABMA-FITC in a fluorescence polarization immu-

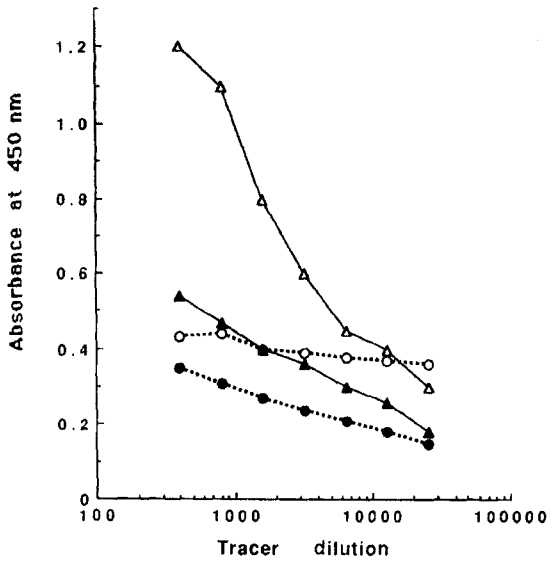

Fig. 2. Binding Characteristics between Antibodies and Tracers

The microplate wells were coated with 200 μl of the antibodies, PAb_{DCC} and PAb_{MSU} (10 μg/ml in carbonate buffer, pH 9.6), at 4°C overnight. The non-specific binding sites were blocked by incubating with 3% BSA in PBS for 1 h at room temperature (RT) and plates were washed with PBST. Two hundred microliter aliquots of serially diluted tracers (4-ABMA–HRP and 4-ABA HRP) were incubated for 1 h at RT. After washing 3 times with PBST, 200 μl of OPD substrate was added and incubated for 20 min. The absorbance was measured at 450 nm using a microplate reader. The binding characteristics between antibodies and tracers were expressed as follows: antibody PAb_{MSU} and tracer 4-ABA HRP (···△···), antibody PAb_{MSU} and tracer 4-ABMA–HRP (—▲—), antibody PAb_{DCC} and tracer 4-ABA–HRP (---○---), antibody PAb_{DCC} and tracer 4-ABMA HRP (---●---).

noassay of methamphetamine assay due to its more effective depolarization.[2] Based on the above report, we then prepared HRP tracers from 4-aminobutyl methamphetamine and amphetamine derivatives and used them to cross-check two different MA antibodies, PAb_{DCC} and PAb_{MSU}. PAb_{MSU} has a higher binding affinity to the 4-ABA–HRP tracer than to 4-ABMA–HRP; however, the binding characteristics of PAb_{DCC} are the opposite (Fig. 2). These results indicate that the tracer should be selected according to the varying binding affinities of individual antibodies. Thus the following pairs of antibody and tracer were chosen for membrane EIA: PAb_{DCC} and the 4-ABMA–HRP tracer, and PAb_{MUS} and the 4-ABA–HRP tracer.

The antibody specificities were studied using the selected pairs of antibody and tracer. Both antibodies, PAb_{DCC} and PAb_{MUS}, were characterized for their affinity and specificity to methamphetamine and amphetamine. The results show that PAb_{MSU} had a higher affinity to amphetamine than to methamphetamine (Fig. 3), and PAb_{DCC} had a better affinity to methamphetamine than to amphetamine (Fig. 4). However, PAb_{MUS} exhibited an affinity to methamphetamine that was 10 times higher than PAb_{DCC}. PAb_{MSU} had a 50% displacement of methamphetamine at a concentration of 8 ng/ml, while that of PAb_{DCC} occurred at 120 μg/ml. Therefore, we found that PAb_{MSU} was better than PAb_{DCC} as the antibody for the MA assay despite its higher affinity to amphetamine.

Choice of Membrane Matrix When preparing any type of membrane immunoassay, it is very important to choose a porous matrix. It should be a neutral polymer which has minimum interactions with the ligand and all

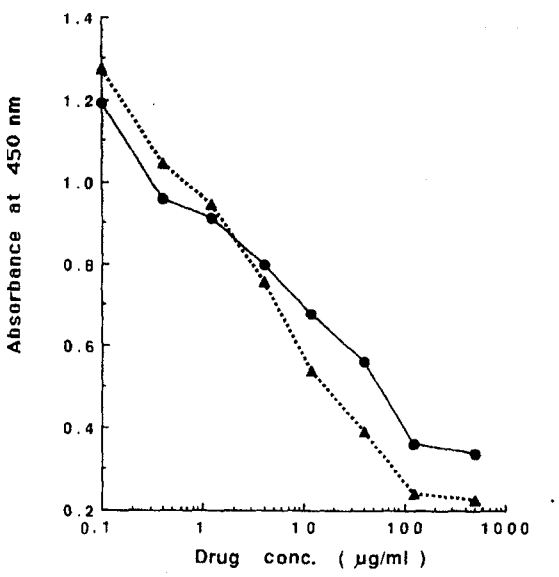

Fig. 3. Dose Response Curve of Methamphetamine and Amphetamine to the PAb_{MSU} Coated Microplate

A microplate was coated with 200 μl of PAb_{MSU} (20 μg/ml carbonate buffer, pH 9.6) at 4°C overnight and the BSA blocking step was performed. After washing with PBST, 20 μl of ABA–HRP (0.3 μg/ml in PBST) and 200 μl of methamphetamine or amphetamine was added in a concentration range between 0 and 500 μg/ml. After incubating 2 h at RT and washing, 200 μl of OPD substrate was added, and the absorbance was measured at 450 nm. Dose response curve of methamphetamine (●), and amphetamine (---▲---).

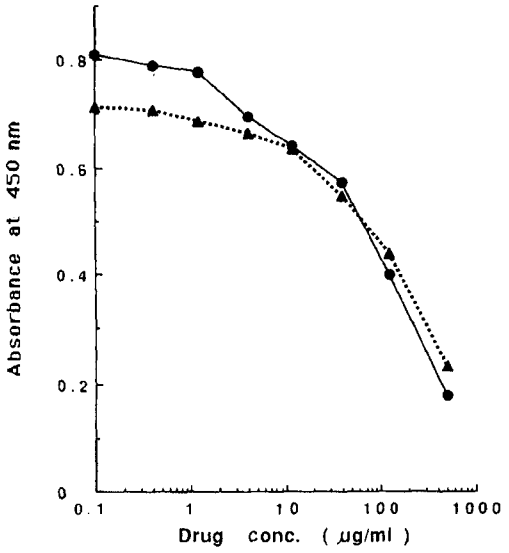

Fig. 4. Dose–Response Curve of Methamphetamine and Amphetamine to the PAb_{DCC} Coated Microplate

The microplate was coated with 200 μl of PAb_{DCC} (40 μg/ml carbonate buffer, pH 9.6) at 4°C overnight. The remaining procedure was the same as described in Fig. 3, except the tracer. Twenty microliters of ABMA–HRP tracer (0.3 μg/ml in PBST) was used instead of ABA–HRP. Dose–response curve of methamphetamine (—●), and amphetamine (---▲---).

components of the staining solution. It should also quantitatively adsorb the final insoluble product of the enzyme reaction. Other requirements include mechanical strength and chemical stability during immobilization and the assay procedure. In our work, several types of membranes were tested and the best one selected in terms of its low non-specific binding to the substrate solution

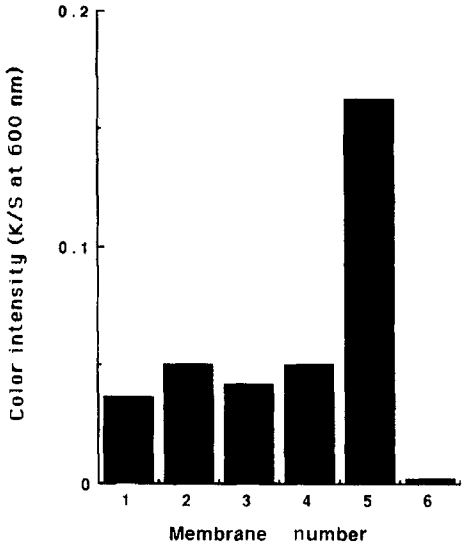

Fig. 5. Membrane Staining Characteristics of Diaminobenzidine/Co^{2+} Enzyme Substrate Solution

The membrane was incubated in diaminobenzidine/Co^{2+} substrate solution for 30 min at RT. The color intensity was measured at 600 nm using a reflectance spectrometer. 1) Pure nitrocellulose membrane AE98 (5 μm), 2) pure nitrocellulose membrane BA 85 (0.45 μm), 3) Biodyne membrane P/N (0.3 μm), 4) Biodyne A membrane (5 μm), 5) Immunodyne membrane P/N (5 μm), 6) UltraBind US-450 membrane (0.45 μm).

of HRP (Fig. 5). The nitrocellulose membrane, Biodyne nylon membrane, and Immunodyne nylon membrane changed their color during the first 5 min of substrate incubation. The most non-specific binding occurred with the Immunodyne membrane. However, the UltraBind membrane showed almost no non-specific binding with the HRP substrate and was thus chosen for the EIA.

Visual Membrane EIA The color change indicating the presence of methamphetamine must be readily visible in the membrane, whereas that due to non-specific binding must be kept at a minimum. Thus, we did a comparison study of the various membranes to determine which was most suitable for our membrane EIA.

The specific antibody was attached to the membranes for 16 h at 4 °C. After the BSA blocking step was carried out for each membrane, they were cut into pieces (0.7 × 0.7 cm) and placed in a mixture of methamphetamine and MA–HRP conjugate. The preliminary study was performed using a high concentration of methamphetamine (up to 1000 μg/ml).

The results indicated that the nitrocellulose membrane (5 μm) and UltraBind membrane (0.45 μm) were most useful in this type of assay. The nitrocellulose membrane (5 μm), however, had a low color intensity and consequently a low sensitivity showing little difference in color intensity between zero and 1000 μg/ml methamphetamine. In the other nitrocellulose membrane (0.45 μm), the color intensity was even lower and no color difference appeared between zero and 1000 μg/ml methamphetamine. This membrane therefore seemed to have no antibody binding capability. On the other hand, the Biodyne and Immunodyne membranes had a very high level of color intensity but also showed no difference between zero and high concentration of MA. These membranes had high non-specific binding activity as

TABLE I. Optimum Conditions for the Membrane Immunoassay to Detect Methamphetamine

PAb	Ab conc. (mg/ml)	HRP tracer (μg/ml)	Incubation time (min)	Substrate Rxn time (min)	Sensitivity (μg/ml)
PAb_{DCC}	1.0	4-ABMA (0.3)	10	3	Approx. 4.0
PAb_{MSU}	0.1	4-ABA (0.5)	5	3	Approx. 0.8

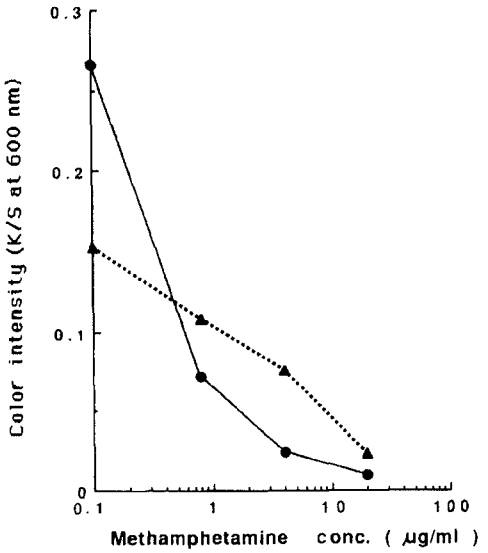

Fig. 6. The Color Response Curve of Methamphetamine by Visual Membrane Enzyme Immunoassay

The Ultrabind US-450 membrane was treated with an antibody solution, PAb_{MSU} (1.0 mg/ml). After blocking with 3% BSA PBS, 200 μl of MA in a concentration range between 0 and 20 μg/ml, and 10 μl of tracer were added. The solution was incubated for 10 min at RT. After washing with PBST 3 times, the membrane was icubated with diaminobenzidine/Co^{2+} substrate for 3 min and washed with water. The color intensity of the membrane was measured at 600 nm using a reflectance spectrometer. Methamphetamine response with PAb_{MSU} treated membrane and 4-ABA HRP tracer (0.3 μg/ml) (●), and with PAb_{DCC} treated membrane and 4-ABMA HRP tracer (0.5 μg/ml) (---▲---).

well as a non-uniformity of surface color.

We then compared the UltraBind membrane (so far the best candidate) with a photosensitized membrane which had been reported to be an excellent solid matrix for membrane immunoassay having azide groups on its surface.[3] Under UV irradiation, the photoactive azide groups were reacted covalently with the antibody solution to immobilize antibody on the membrane surface. However, the data showed that the binding affinity of the analyte to the antibody was very poor for the photo-immobilized antibody membrane compared to the UltraBind membrane. As a result of the comparison study, the UltraBind membrane was found to be the choice matrix for our visual membrane EIA.

Optimum Conditions for Visual Membrane EIA A common disadvantage of all types of solid phase enzyme immunoassays is that they are time-consuming (1.5—2 h in general). Thus, we studied ways to simplify the analytical procedure and to decrease its assay time, as well as to increase its sensitivity. The optimum concentration of antibody to be immobilized on the membrane was determined by varying the specific antibody concentration and was found to be 1000 μg/ml for PAb_{DCC}

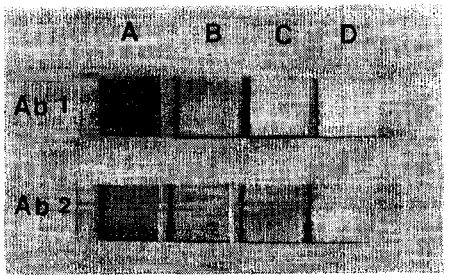

Fig. 7. Visual Color Differences in the Membrane Response to Different Methamphetamine Concentrations

The results were read at 600 nm by a reflectance spectrometer as in Fig. 5 and the visual differences are shown here. Ab 1: PAb_{MSU} treated membrane and 4-ABA–HRP tracer (0.3 μg/ml), Ab 2: PAb_{DCC} treated membrane and 4-ABMA HRP tracer (0.5 μg/ml). Methamphetamine concentrations are: A, 0 μg/ml; B, 0.8 μg/ml; C, 4 μg/ml; D, 20 μg/ml.

and 100 μg/ml for PAb_{MUS}. Drug–HRP concentration was also studied to ensure the best surface color response on the membrane. The optimum concentrations were 0.3 μg/ml and 0.5 μg/ml for PAb_{DCC} and PAb_{MUS}, respectively.

The color response in the membrane assay system (mixture of MA and MA–HRP) was examined for various assay times of 5 to 20 min. When testing the substrate solution of HRP tracer, we found that an MA cut-off level of 0.8 μg/ml could be achieved after a 3 min incubation with a DAB/$CoCl_2$ substrate by visual comparison with zero control in the membrane. The same degree of color response was obtained with a DAB/$NiCl_2$ substrate after incubating 10 min. Thus the color produced by the DAB substrate is better enhanced by Co^{2+} than Ni^{2+}. Table I is a summary of the optimum conditions for the visual membrane immunoassay.

Figure 6 shows the standard curve response for methamphetamine with the color intensity (K/S value) measured at 600 nm. Figure 7 allows a visual comparison of membrane color at different methamphetamine concentrations. The resulting color difference could be easily recognized with an MA cut-off level of 0.8 ppm in urine.

Accuracies of the assay procedure were studied at two levels of control urine for every assay using a reflectance spectrometer. Within-run coefficient of variations ($n=7$) were 5.6% and 7.2% for urine controls containing methamphetamine at 0.8 ppm and 4 ppm. Coefficient of variations of the run-to-run assay ($n=5$) were 6.4% and 8.5% for the same urine controls as used in the within-run assay. The color difference on the membrane was visually distinct between negative (zero ppm) and 0.8 ppm of MA in urine samples.

To evaluate the visual membrane EIA method, we screened 25 urine samples for the MA detection. Urine samples were obtained from athletes for the drug doping test and from suspected drug abusers at the police office. All samples were screened by the membrane EIA and fluorescence polarization immunoassay (FPIA) using TDx reagent packs (TDx analyzer, Abbott Labs.), and confirmed by GC/MSD. Four cases out of 25 urine samples were MA positive by the visual membrane immunoassay. The results of comparison study indicated that there was full agreement for the MA-positive samples screened by the visual membrane EIA and those confirmed by the two other methods with a negative/positive cut-off level of 0.8 ppm.

In conclusion, each individual antibody has a characteristic binding affinity with different tracers. Even though the antibodies were produced using the same immunogen, *N*-(4-aminobutyl)methamphetamine, the binding specificities of antibodies varied from one rabbit to another. This shows that the pairing of the antibody and drug tracer is the key to developing a highly sensitive immunoassay.

The visual membrane immunoassay described here, using the antibody and tracer pairs PAb_{MSU} and 4-ABA–HRP, and PAB_{DCC} and 4-ABMA–HRP, provides a reliable and sensitive on-site test for methamphetamine in urine with a 0.8 ppm cut-off level. The method does not require the use of a particular instrument.

REFERENCES AND NOTES

1) Present address: *A. N. Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky Pr., 33, 117071 Moscow, Russia.*
2) S. A. Eremin, G. Gallacher, H. Lotey, D. S. Smith, J. Landon, *Clin. Chem.*, **33**, 1903 (1987).
3) B. M. Gorovits, A. P. Osipov, V. V. Doseeva, A. M. Egorov, *Anal. Lett.*, **24**, 1937 (1991).
4) B. M. Gorovits, A. P. Osipov, A. M. Egorov, *J. Immunol. Methods*, **157**, 11 (1993).
5) G. Osikowicz, M. Beggs, P. Brrokhart, D. Caplan, S. Sampedro, D. Stimpson, F. Walsworth, *Clin. Chem.*, **36**, 1586 (1990).
6) D. L. Colbert, G. Gallacher, R. W. Mainwaring-Burton, *Clin. Chem.*, **31**, 1193 (1985).
7) K. Aoki, Y. Kuroiwa, *J. Pharmacobio-Dyn.*, **6**, 33 (1983).
8) M. Iwasaki, *Nippon Hoigaku Zasshi*, **41**, 217 (1987).
9) T. Inoue, Y. Kanda, T. Kishi, T. Sakai, S. Suzuki, T. Niwaguchi, H. Hori, S. Inayama, *Chem. Pharm. Bull.*, **32**, 344 (1984).
10) Y. Tamaki, M. Fukuda, T. Kishida, N. Takahashi, *Nippon Hoigaku Zasshi*, **37**, 417 (1983).