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# The Optimization of ELISA for Methamphetamine Determination: the Effect of Immunogen, Tracer and Antibody Purification Method on the Sensitivity

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To obtain more sensitive immunoassay for methamphetamine (MA) determination, the optimum condition of enzyme-linked immunosorbent assay (ELISA) was investigated in regard to immunogens, antibody purification methods and coating tracers. Activated MA, N-(4-aminobutyl)methamphetamine (4-ABMA), was conjugated with bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) and used as immunogen. The antibodies were purified by protein G chromatography or various immunoaffinity chromatography-linked MA-protein ligands, such as MA-BSA, MA-KLH or MA-ovalbumin (OVA). Each purified antibody was characterized by means of sensitivity and cross-reactivity using the three MA-protein coating tracers, MA-BSA, MA-KLH and MA-OVA. The best sensitivity of each antibody was acquired with the MA-OVA tracer although the tracer concentration and the antibody titer level at optimum condition were varied. The antibody with high titer level did not always yield good sensitivity. At optimum condition, immunoaffinity chromatography-purified antibodies were better for sensitivity and for specificity than protein G-purified antibodies. The cross-reactivity of the purified antibodies seemed to be affected by immunogen structure and showed somewhat different patterns according to the immunoaffinity ligand utilized. These data show that the antibody purification method as well as choice of coating tracer and immunogen is essential for the sensitivity and specificity of EIA; the optimum condition for assay should be discovered using various methods and combinations.

**Key words** : Methamphetamine, ELISA, Antibody purification method, Coating tracer, Optimization

## INTRODUCTION

Methamphetamine, a potent sympathomimetic drug, has been widely abused. It is excreted rapidly and about 40% of a dose is excreted in its unchanged form (Beckett and Rowland, 1965). The detection of MA in urine was attempted by various chromatographic methods (Lebish *et al.*, 1970; Terada *et al.*, 1982) and immunoassay techniques (Mason *et al.*, 1983; Nam *et al.*, 1993; Choi *et al.*, 1994). EIA has been used as the initial screening method because it is rapid, technically simple and needs no sample treatment procedure.

To yield sensitive and rapid EIA, the antibody character is very important. The sensitivity and cross-reactivity of the antibody may be affected by the structure of the immunogen (Choi *et al.*, 1995). In competitive EIA such as MA, assay sensitivity is especially influenced because of the similarity in antigen and tracer chemical structures (Eremin *et al.*, 1987; Eremin *et al.*, 1988). The antibody has affinity not only to the antigen but also to the tracer itself. Since the antigen and the tracer compete for antibody binding, an antibody with affinity to the tracer that is too strong or too weak will not give good sensitivity.

The characterization of polyclonal antibody is not as simple as monoclonal antibody. This is because polyclonal antibody is a mixture of antibodies whose affinities or specificities are different. If an antibody (or antibodies) with good affinity is obtained from the polyclonal antibody mixture, the sensitivity can be improved greatly.

Hapten should be conjugated with a carrier protein in order to produce antibody. There are several carrier proteins with various conjugation and immuno-

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genicity capacities. Therefore, we prepared two kinds of immunogens, MA-BSA and MA-KLH, to obtain better antibody. To yield more specific antibody, we also prepared three kinds of affinity columns whose ligands are MA-BSA, MA-KLH and MA-OVA respectively. All the purified antibodies were evaluated in terms of sensitivity and specificity using the three MA-protein tracers.

## MATERIALS AND METHODS

### Materials

MA, benzphetamine, amphetamine were obtained from the Korean National Institute of Health. Phenylpropanolamine, ephedrine, methylephedrine, N-(4-bromobutyl) phthalimide, hydrazine hydrate, Freunds complete adjuvant (FCA), Freunds incomplete adjuvant (FIA), 1-ethyl-3-(3-methyl-aminopropyl)carbodiimide HCl (EDC), ethanolamine HCl, o-phenylenediamine (OPD) and BSA were purchased from Sigma Chemical Co. (Mo., USA). BSA, KLH and OVA, used to prepare haptene-protein conjugates, were purchased from Pierce (IL., USA). CNBr-activated Sepharose 4B and Protein G Sepharose 4 Fast Flow were purchased from Pharmacia Biotech (Sweden). Peroxidase-conjugated rabbit IgG fraction to goat IgG (whole molecule) were purchased from Cappel (NC., USA). All other chemicals were of analytical reagent grades, and were used in our laboratory. MicroWell module (maxisorp) were purchased from Nunc (Denmark) and Kinetic Microplate Reader (Molecular Devices, USA) was used to measure the optical density of the result of ELISA.

### Preparation of assay standards

A stock solution of MA·HCl in distilled water (1 mg/ml) was diluted with 1% BSA-PBS in concentrations of 0, 0.5, 5 and 50 μg/ml. The same method and concentrations were applied to prepare cross-reactant standards, benzphetamine, amphetamine, phenylpropanolamine, ephedrine and methylephedrine. (Fig. 1). Standards were stored at 4°C.

### Preparation of N-4-aminobutyl derivative of MA

MA was derivatized to N-4-aminobutyl methamphetamine (4-ABMA) prior to conjugation with the protein BSA, KLH or OVA. MA was first reacted with N-(4-bromobutyl)phthalimide and was then reduced to 4-ABMA by hydrazine hydrate as described in Tamaki *et al.* (1983). The confirmation and purity of 4-ABMA was checked by TLC, UV-spectrum, NMR and GC/MSD, as reported previously (Choi *et al.* 1995).

### Preparation of MA-protein conjugates and antisera

The MA-protein conjugates, MA-BSA, MA-KLH and MA-OVA, were prepared as described in Choi *et al.* (1994). The reaction mixture of 4-ABMA, cross-linking reagent,1-ethyl-3-(3-methyl-aminopropyl)carbodiimide·HCl (EDC) and protein was incubated at pH 6 for four hours at RT and the conjugate was dialyzed against PBS more than four times. The confirmation of MA-protein conjugates and hapten densities in conjugates were checked by a slightly modified method of Goodrow *et al.* (1990). The UV-spectrum of MA-BSA was compared with those of 4-ABMA and BSA standard. The molar ratio of 4-ABMA to BSA in MA-BSA conjugate was estimated by measuring absorbance at 262 and 280 nm (corresponding approximately to the respective peaks of MA and BSA). $A_{280}$ of BSA conjugate was used for BSA molarity. After removing BSA, the resulting $A_{262}$ was used for hapten molarity. The same method was applied to other MA-protein conjugates.

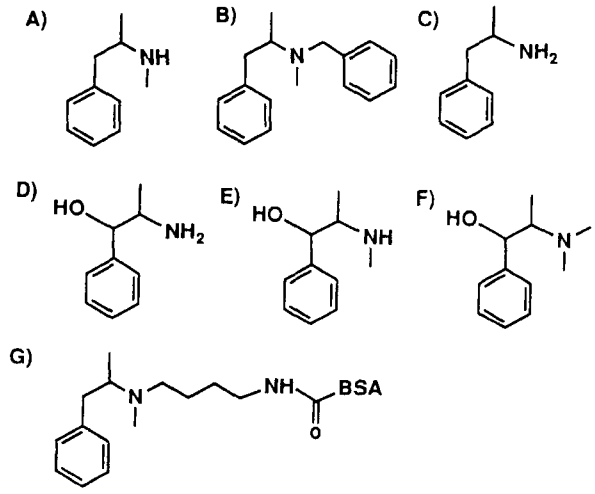


**Fig. 1.** Chemical structures of methamphetamine (A), benzphetamine (B), amphetamine (C), phenylpropanolamine (D), ephedrine (E), methylephedrine (F) and BSA immunogen (G).

MA-BSA and MA-KLH conjugates were used as immunogens. All three protein conjugates were used as ligands of immunoaffinity columns and ELISA coating tracers.

The polyclonal antisera were obtained by immunizing goats as described in Eremine *et al.* (1988).

### Preparation of immunoaffinity columns

2 g of CNBr-activated Sepharose 4B was swollen in 1 mM HCl for 20 minutes at RT and washed with 50 ml of the same solution. The swollen gel was washed with 50 ml of conjugating solution (0.1 M sod. bicarbonate buffer containing 0.5 M NaCl, pH 8.3) and 8 mg of MA-protein ligand in 10 ml conjugating solution was added to the gel. After a two hour incubation at RT, the remaining reactive groups were blocked by leaving the gel in 1 M ethanolamine for

two hours at RT. The coupled gel was washed twice with 0.1 M sod. acetate (containing 0.5 M NaCl, pH 4.0) and washed as many times with reaction buffer (0.1 M tris·HCl containing 0.5 M NaCl, pH 8.3). The gel was then packed into a column (1.5 cm×1.0 cm) and equilibrated with 2-3 column volumes of the reaction buffer.

### Immunoaffinity column chromatography

After adjusting the pH to 8.3 with 1 M tris·HCl buffer, pH 8.3, the MA antiserum was centrifuged at 12, 000 rpm for ten minutes at 4°C to remove precipitate. The treated serum was applied to the immunoaffinity column and incubated for 18 hours at 4°C. After unbound proteins were washed out with the reaction buffer, the specific antibodies were eluted with 0.1 M triethylamine (pH 11.5). 0.5 ml fractions of the eluent solution was collected in tubes containing 25 μl of 1 M phosphate buffer (pH 6.8). Antibody peaks were detected by UV absorbance at 280 nm. The specific antibody fraction was dialyzed against PBS and the purity was checked by SDS-PAGE.

### Protein G column chromatography

2 ml of protein G Sepharose 4 fast flow (Pharmacia, Sweden) was washed with 20 mM phosphate buffer (pH 7.0) and then was packed into a column. The column was equilibrated with 2-3 bed volumes of start buffer (20 mM phosphate buffer, pH 7.0) at a flow rate of 0.6 ml/min. The MA antiserum was diluted with 1/10 antiserum volume of start buffer and filtered through a 0.45 μm filter. The serum was then applied to the column and unbound proteins were washed off using start buffer, until no protein was detected in the effluent. The antibody fractions were eluted with glycine·HCl buffer (pH 2.7) and 0.5 ml of fractions were collected in tubes containing 15 μl of 2 M trisma. The purity of immunoglobulins was also checked by SDS-PAGE.

### Titration level assessment of the purified antibodies by ELISA

The ELISA procedure employed was the indirect competitive method. 96 well microtiter plates were coated with 200 μl of MA-protein conjugate in 50 mM carbonate buffer (pH 9.6) by a overnight incubation at 4°C. Blocked with 200 μl of 3% BSA in PBS for two hours at RT, the wells were incubated with 200 μl of serially diluted specific antibobies for another two hours at RT. 200 μl of HRP conjugated rabbit anti-goat IgG (1:4000 dilution) was added to the wells and incubated for two hours at RT. The amount of enzyme bound, as indicated by the conversion of colorless substrate to colored product, directly related to the amount of purified antibody bound to the immobilized antigen. 200 μl of o-phenylenediamine (OPD) substrate solution was added and incubated for ten minutes at RT. The color reaction was stopped with 50 μl of 2 N-$H_2SO_4$ and the optical density was read at 490 nm using a Kinetic Microplate Reader.

The antibody concentration yielding a response of OD 2.0 at 490 nm was used as the titer level.

### Sensitivity and specificity of the purified antibody by ELISA

For sensitivity and specificity study, the antibody at the titer level incubated with serially diluted free MA or other chemically related drugs for the competition with immobilized tracer. The standard curve was constructed by plotting the relative responses (%) to the response at MA zero concentration against the concentrations of MA.

## RESULTS AND DISCUSSION

The confirmation of 4-ABMA was checked by various methods as reported previously (Choi *et al.*, 1995). Using GC/MSD, purity was estimated to be more than 95%.

Two kinds of MA-BSA conjugates, having low (39) and high (81) molar ratios (MA/BSA) respectively, were obtained. The molar ratio (MA/OVA) of the MA-OVA conjugate was estimated at 32. Unfortunately, the molar ratio of the KLH conjugate was not calculated due to the broad molecular weight of KLH (4. $5\times10^5$ to $1.3\times10^7$).

Three kinds of goat anti-MA antisera were obtained by immunizing two types of MA-BSA conjugates which have low and high hapten/protein molar ratios, and one type of MA-KLH conjugate. All MA antisera were purified by immunoaffinity columns and a protein G column. To eliminate non-specific antibodies, which recognize the carrier proteins or the hapten-protein bridge without MA, MA-KLH and MA-OVA immunoaffinity columns were applied to the antisera that were obtained from the MA-BSA immunogen. In the same way, the antiserum from MA-KLH immunogen was purified by MA-BSA and MA-OVA immunoaffinity columns.

The amount of purified antibodies is summarized in Table I. It is no wonder that the yield of protein G column was much higher than that of the immunoaffinity column. (Protein G purified antibody contains not only specific MA antibody but also all non-specific antibodies). The quantity of MA-KLH affinity column-purified antibodies (#1 or #4) from MA-BSA immunogen antisera (I or II) was greater than the quantity of MA-OVA affinity column-purified antibo-

**Table I.** List of purified anti-methamphetamine antibody

| MA antisera | # of antibody | Immunogen | Molar ratio (MA/protein) | Immunoaffinity column ligand | Purified antibody (mg antibody/ml serum) | Yield (mg antibody/ml serum) |
|---|---|---|---|---|---|---|
| I | 1 | MA-BSA | Low (39) | MA-KLH | 42.8/41 | 0.96 |
| | 2 | | | MA-OVA | 18.0/38 | 0.47 |
| | 3 | | | Protein G | 48.2/3 | 16.07 |
| II | 4 | MA-BSA | High (81) | MA-KLH | 52.4/30 | 1.71 |
| | 5 | | | MA-OVA | 22.2/30 | 0.74 |
| | 6 | | | Protein G | 52.1/3 | 17.37 |
| III | 7 | MA-KLH | | MA-BSA | 29.0/42 | 0.69 |
| | 8 | | | MA-OVA | 34.2/41 | 0.83 |
| | 9 | | | Protein G | | 22.63 |

dies (#2 or #5) respectively. Even though the molar ratio (MA/protein) in MA-KLH conjugate was not estimated exactly, this ratio is probably high (KLH has a large molecular mass and many amino acids available for conjugation). Therefore, MA-KLH affinity column is considered to be more capable of purifying antibodies compared to MA-OVA affinity column. Since the purified antibodies yielded two bands (one at about 50,000 and one at about 25,000 on SDS-PAGE; data not shown), it was thought that no other proteins except immunoglobulins were present in the purified antibodies.

Titer level of each purified antibody was determined by ELISA. To investigate the effects of coating tracers and their concentrations, the microplate wells were coated with MA-BSA (high molar ratio at 0.01, 0.1, 1 μg/ml concentration), MA-KLH (at the same concentration as MA-BSA), or MA-OVA (at 0.2, 1, 5 μg/ml concentration). The titer level (antibody concentration yielding OD 2.0 at 490 nm) was varied from antibody to antibody in accordance with the types of coating tracers and coating concentrations applied. The higher the concentration of tracer used, the less the amount of antibody required. Fig. 2 shows the typical titration curves of MA antibodies with MA-BSA tracer (0.01 μg/ml). As expected, the titer level of protein-G purified antibodies was inferior to the titer level of its affinity purified counterparts. The titration curve shifted to the left according to increasing levels of coating concentration (Fig. 3, A). Interestingly, the titration curves of high titer antibodies (#7 and #8) were unchanged when coating concentration increased by even a 100-fold (Fig. 3, B). This tendency manifested with MA-KLH and MA-OVA coating tracers, as well.

We constructed the standard curves for MA, with a range of 0, 0.5, 5, 50 μg/ml, using the three tracers at all three coating concentrations to discover optimum conditions. Fig. 4 shows the dose response curves of purified antibody #1 with various coating tracers. Among the three conjugates, MA-OVA was the best tracer for antibody #1 and the best sensitivity was

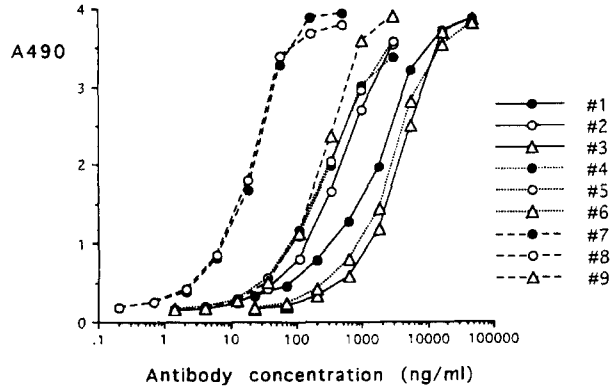


**Fig. 2.** Typical titration curves of purified anti-methamphetamine antibodies using MA-BSA tracer at 0.01 μg/ml coating concentration.

achieved with a 5 μg/ml concentration (Fig. 4, A). For the other eight purified antibodies, MA-OVA was again found to be the best tracer. In other words, more sensitive assay was acquired using MA-OVA than when using MA-BSA or MA-KLH conjugates. Furthermore, the optimum coating concentration for each conjugate varied, depending upon the antibodies. This suggestes that OVA conjugate is suitable as the tracer for EIA. For immunogen, it is better to use a high molar ratio hapten-protein conjugate. However, for the assay tracer, the molar ratio doesn't have to be large; rather, in this case a small molar ratio conjugate will give more sensitive results. As a result, the OVA conjugate is advantageous as a tracer with respect to other protein conjugates as OVA has fewer amino acids available for conjugation. Our data supports this finding.

Table II shows the titer level and sensitivity of each purified antibody at its respective optimum condition. Sensitivity was defined as the MA concentration which yielded 70% of the maximum response (at MA zero concentration) on the dose-response curve. It is certain that the protein G-purified antibodies (#3, #6, #9) were not as highly sensitive as the affinity-purified antibodies. Unexpectedly the sensitivities of #5

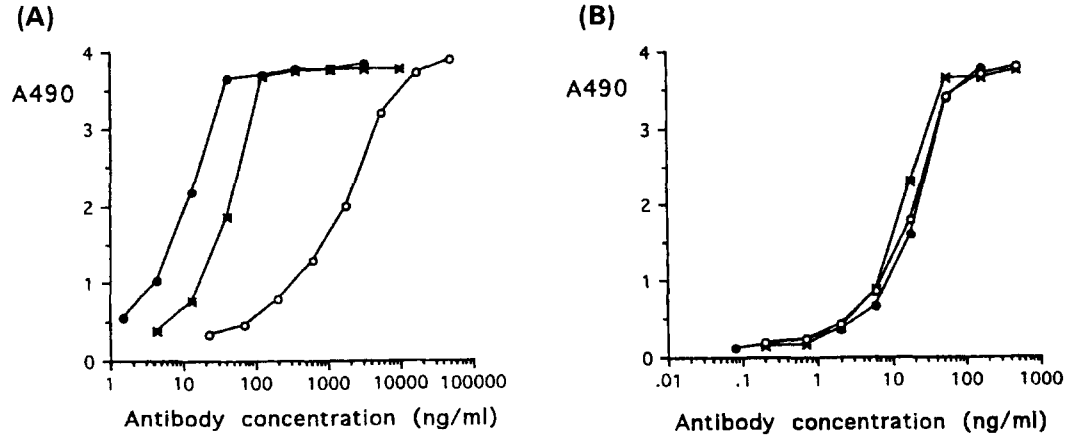


**Fig. 3.** The effect of coating concentration on the titration curves of antibody #1 (A) and antibody #7 (B). MA-BSA was coated at 1 (●—●), 0.1 (*—*) and 0.01 (○—○) μg/ml concentration. The titration curve of the antibody #1 shifted to the left according to changes in coating concentration, whereas the titration curve of antibody #7 did not shift even when coating concentration increased by 100-fold.

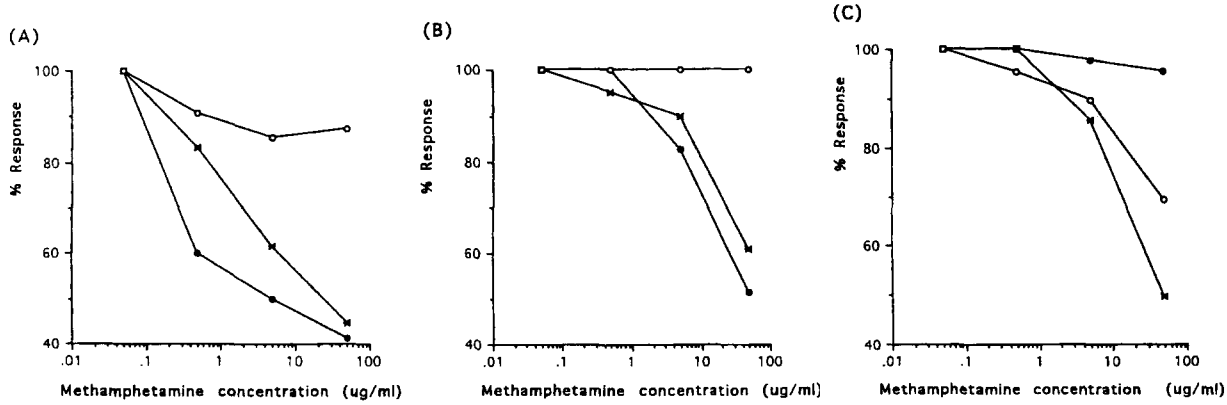


**Fig. 4.** Dose response curves of antibody #1 with various coating tracers. (A): MA-OVA coating at 5 (●—●), 1 (*—*), 0.2 (○—○) μg/ml concentration. (B): MA-KLH coating at 1 (●—●), 0.1 (*—*), 0.01 (○—○) μg/ml concentration. (C): MA-BSA coating at 1 (●—●), 0.1 (*—*), 0.01 (○—○) μg/ml concentration. The best sensitivity was obtained with MA-OVA at 5 μg/ml concentration.

**Table II.** Titer levels and sensitivities of purified anti-methamphetamine antibodies by ELISA at their optimum conditions, respectively

| # of antibody | Coating tracer | Coating concentration (μg/ml) | Titer (μg/ml) | Sensitivity (μg/ml) |
|---|---|---|---|---|
| 1 | MA-OVA | 5 | 3 | 0.2 |
| 2 | | 5 | 0.9 | 9 |
| 3 | | 1 | 8 | 8 |
| 4 | MA-OVA | 5 | 1 | 2.5 |
| 5 | | 5 | 0.7 | 0.3 |
| 6 | | 1 | 6 | 10 |
| 7 | MA-OVA | 0.2 | 1.5 | 15 |
| 8 | | 1 | 0.03 | 11 |
| 9 | | | 4.5 | 13 |

and #6 antibodies were as poor as antibody #9 in spite of their high titer levels. It is suspected that the poor sensitivity of the high titer antibodies was caused either by the high affinities of the tracer itself or by the affinities of the bridge of hapten-protein conjugate. If an antibody has a very strong affinity for the tracer, good sensitivity of an assay cannot be obtained (because free antigen cannot be bound to the antibody easily in competition with the tracer). Colbert *et al.* (1991) also reported that increased sensitivity was achieved by reducing the effective antibody affinity of the tracer.

Table III shows cross-reactivities of purified antibodies with chemically-related drugs at optimum condition. All antibodies showed some cross-reactivity with benzphetamine (Fig. 1, B) and methylephedrine (Fig. 1, F), but the extent of cross-reactivity was different from antibody to antibody. As reported previously (Choi *et al.*, 1995; Tamaki *et al.*, 1983), the antibody affinity is affected by immunogen structure, especially by modified immunogen structure. The MA immunogen (Fig. 1, G), like benzphetamine and methylephedrine, has a tertiary amine group. Antibody #4 seemed to recognize the tertiary

**Table III.** Cross-reactivities of purified anti-methamphetamin antibodies with chemically-related drugs

| # of antibody | Relative cross-reactivity (%) | | | | | |
|---|---|---|---|---|---|---|
| | Methamphetamine | Benzphetamine | Amphetamine | Phenylpropanolamine | Ephedrine | Methylephedrine |
| 1 | 100 | 2.5 | n/d | n/d | n/d | 3.8 |
| 2 | 100 | 10 | n/d | n/d | n/d | 45 |
| 3 | 100 | 36 | n/d | n/d | n/d | 47 |
| 4 | 100 | 500 | 14 | n/d | n/d | 3 |
| 5 | 100 | 25 | 10 | n/d | 1.7 | 2 |
| 6 | 100 | 15 | 15 | n/d | 15 | 60 |
| 7 | 100 | n/d | n/d | n/d | n/d | 67 |
| 8 | 100 | 12 | n/d | n/d | n/d | 30 |
| 9 | 100 | 5 | n/d | n/d | n/d | 55 |

amine group even though the benzyl group was attached to the tertiary amine group. Antibody #4 interfered with the hydroxyl group on the beta-carbon because it has great affinity for benzphetamine and negligible affinity for methylephedrine. Antibody #4 also showed low or no cross-reactivities with amphetamine, phenylpropanolamine and ephedrine, indicating that neither a primary amine group nor a secondary amine group containing hydroxyl group could be an epitope of antibody #4. Antibody #5 showed the different cross-reactivity pattern from antibody #4 even if both types of antibodies originated form the same antiserum. Antibody #5 had very low affinity for benzphetamine as well as for methylephedrine. This results suggest that the binding of antibody #5 is affected by the hydroxyl group on the beta-carbon and also affected by the benzyl group attached to the tertiary amine group. The amine group of 4-ABMA is conjugated with a carrier protein via aspartic acid or glutamic acid. This suggests that the binding of antibody, immunized from 4-ABMA conjugate, can be obstructed by the benzyl group attached to the amine group due to steric hinderance. This suggestion may be correct in the case of antibody #1; but, the benzyl group seemed to have larger influences than the hydroxyl group on antibodies #7, #8, and #9, resulting low cross-reactivity with benzphetamine and high cross-reactivity with methylephedrine.

Our data shows that the characterization of the antibody in terms of sensitivity and specificity should be investigated by various methods. The optimum condition should be selected carefully trying various tracers and tracer concentrations. Among the things influencing antibody character, the antibody purification method, tracer and immunogen are critical. Immunoaffinity purification is superior to the protein G purification method. Using appropriate affinity ligands, a good antibody can be selected from antiserum (that contains various antibodies) and highly sensitive immunoassay can be obtained.

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