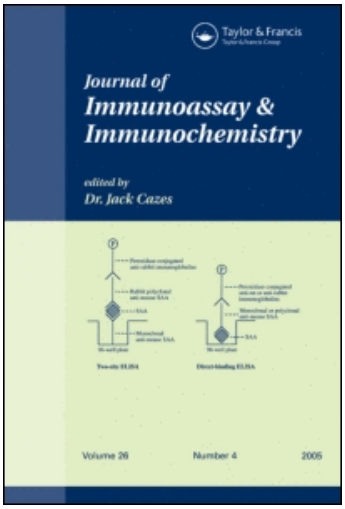

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

EFFECTS OF THE COMPETITOR ON ANTIBODY-HAPTEN BINDING IN IMMUNOASSAYS

Jeongeun Choi[a]; Choonmi Kim[b]; Myung Ja Choi[a]
[a] Bioanalysis & Biotransformation Research Center, Korea Institute of Science and Technology, Seoul, Korea [b] College of Pharmacy, Ewha Womans University, Seoul, Korea

Online publication date: 04 March 2002

To cite this Article Choi, Jeongeun , Kim, Choonmi and Choi, Myung Ja(2002) 'EFFECTS OF THE COMPETITOR ON ANTIBODY-HAPTEN BINDING IN IMMUNOASSAYS', Journal of Immunoassay and Immunochemistry, 23: 1, 69 — 83
To link to this Article: DOI: 10.1081/IAS-120002275
URL: http://dx.doi.org/10.1081/IAS-120002275

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 23(1), 69–83 (2002)

EFFECTS OF THE COMPETITOR ON ANTIBODY-HAPTEN BINDING IN IMMUNOASSAYS

Jeongeun Choi,[1,*] Choonmi Kim,[2] and Myung Ja Choi[1]

[1]Bioanalysis & Biotransformation Research Center, Korea Institute of Science and Technology, P. O. Box 131 Cheongryang, Seoul 130-650, Korea
[2]College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea

ABSTRACT

The effects of competitors on antibody (Ab)-hapten binding in an immunoassay were investigated using a goat anti-methamphetamine (MA) antibody (Ab). An *N*-4-aminobutyl derivative of methamphetamine (4-ABMA) was conjugated with keyhole limpet hemocyanine (KLH) and used as an immunogen. The antiserum was purified by affinity chromatography with various ligands, including 4-ABMA-protein conjugates, free haptens, and protein G. Direct and indirect competitive enzyme-linked immunosorbent assays (ELISA) were conducted with a competitor of 4-ABMA-fluorescein isothiocyanate (4-ABMA-FITC). The results were compared to those of ELISA with a different competing antigen,

*Corresponding author. E-mail: jechoi@physiology.wisc.edu

4-ABMA-ovalbumin (4-ABMA-OVA), in terms of sensitivity and specificity. In both direct and indirect assay formats, the sensitivity was much improved with 4-ABMA-FITC, compared to that with 4-ABMA-OVA, suggesting that different labels on the same haptenic moiety for competitors considerably influence the assay performance. All the purified Abs also showed a distinct feature of strong affinity for benzphetamine with 4-ABMA-FITC, whereas they had their respective binding specificities with 4-ABMA-OVA. Comparing the results to those from other assay systems, we determined that the assay sensitivity was dependent on both the system and the competitor employed, and that the specificity was primarily dependent on the competitor used.

INTRODUCTION

Conventional immunoassay is used as an initial screening method in forensic sciences because of its relative rapidity and simplicity. It requires no sample preparation steps and is able to assay a large number of samples in a short time. In the competitive immunoassay for haptens, the performance of the assay is greatly influenced by various parameters. These variables include the chemical structure of the hapten used in the antibody (Ab) production (1,2), affinity of the elicited Ab (3), the choice of the labeled antigen (4,5), optimization of the assay (6), and the assay format.(7)

The ligand of the affinity column in the Ab purification step has also proven to be a critical factor.(8) Among these parameters, preparation of the competitor or the tracer is usually selected on the basis of sensitivity improvement or convenience of use. Isotope labeling (4) and introduction of the avidin-biotin system (9) are examples of the former, and the use of the enzyme or the fluorescent tracer (10) is that of the latter. The fluorescence polarization immunoassay (FPIA), in which the fluorescent tracer employed has been widely used in the field of drug analysis.(10–12) This popularity is due to its simplicity and rapidity and it is a homogeneous assay which needs no separation of the Ab-bound antigen (Ag) from the free Ag. Its simplicity, however, is often accompanied by some loss of sensitivity. On the contrary, enzyme-linked immunosorbent assay (ELISA) gives quite sensitive results, even though the assay procedure takes extra time and labor.

Various immunoassays for haptenic and protein antigens have been performed in our laboratory. Methamphetamine (MA), which is a potent central nervous stimulant, is an analyte of interest since MA abuse has become a serious concern in Asia. We previously produced anti-MA

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

antibodies against *N*-(4-aminobutyl)methamphetamine (4-ABMA)-protein conjugates.(13) The Abs were characterized using various assay systems: capillary electrophoresis-based immunoassay (14), FPIA with 4-ABMA-fluoresceine isothiocyanate (FITC) (7), and ELISA with 4-ABMA-ovalbumin (OVA).(8,13) The results, however, were somewhat inconsistent with each other. In this paper, we have made close investigation into the effects of the competitor and the format on the assay performance in an attempt to obtain more information on the Ab–Ag interaction.

EXPERIMENTAL

Chemicals and Reagents

d-MA (1*S*-MA), *dl*-MA, benzphetamine, and *dl*-amphetamine were obtained from the Doping Control Center, Korea Institute of Science and Technology. The drugs used for the cross-reactivity study were purchased from Sigma (St. Louis, Mo): *l*-ephedrine ([1*R*,2*S*]-ephedrine), *d*-ephedrine ([1*S*,2*R*]-ephedrine), *d*-pseudoephedrine ([1*S*,2*S*]-ephedrine), *l*-pseudoephedrine ([1*R*,2*R*]-ephedrine), *d*-*N*-methylephedrine ([1*S*,2*R*]-*N*-methylephedrine), *l*-*N*-methylephedrine ([1*R*,2*S*]-*N*-methylephedrine), *d*-norephedrine ([1*S*,2*R*]-phenylpropanolamine), *l*-norephedrine ([1*R*,2*S*]-phenylpropanolamine), and *dl*-epinephrine. *N*-(4-Bromobutyl)phthalimide, hydrazine hydrate, Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), 1-ethyl-3-(3-methyl-aminopropyl)carbodiimide (EDC), ethanolamine, FITC, and *o*-phenylenediamine (OPD) were also purchased from Sigma.

Bovine serum albumin (BSA), keyhole limpet hemocyanine (KLH), and OVA, used to prepare hapten-protein conjugates, were purchased from Pierce (Rockford, IL). CNBr-activated Sepharose 4B and protein G Sepharose 4 Fast Flow were purchased from Pharmacia Biotech (Uppsala, Sweden). Peroxidase conjugated rabbit IgG fraction to goat IgG (whole molecule), peroxidase conjugated goat IgG fraction to rabbit IgG (whole molecule), goat IgG fraction, and mouse IgG fraction were purchased from Cappel (Durham, NC). Rabbit anti-FITC antiserum was obtained from Fitzgerald Industries Int. Inc. (Concord, MA). Rabbit anti-goat IgA-alpha chain specific (affinity purified), rabbit anti-goat IgG-Fc fragment (affinity purified), and rabbit anti-goat IgM-mu chain specific (affinity purified), used to examine the immunoglobulin class distribution of MA-Abs, were purchased from Bethyl Laboratories Inc. (Montgomery, TX). Goat IgG fraction, donkey IgG fraction, and mouse IgG fraction were also purchased from Sigma. All other chemicals used were of analytical grade.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Preparation of MA-Antiserum and Ab Purification

The 4-ABMA-KLH conjugate was prepared as reported previously (8,13), and used as an immunogen. The antiserum produced from a goat was purified using immuno-affinity chromatography and protein G column chromatography as described in previous work.(8,13) The ligands of the immunoaffinity columns were 4-ABMA-protein conjugates, *d*-MA, *d*-4-ABMA and *dl*-amphetamine. The specific Ab in MA-antiserum, which had bound to the ligands in *tris*-HCl containing 0.5 M NaCl (pH 8.3), was eluted with triethylamine (pH 11.5). The Ab bound to the protein G column was also eluted with glycine-HCl (pH 2.7).

Preparation of 4-ABMA-FITC Competitor

The *N*-(4-aminobutyl)methamphetamine was labeled with FITC and used as a competitor in ELISA. Synthesis and purification of the FITC conjugate were conducted as reported previously.(7,10)

ELISA Procedure

Direct Competitive ELISA (MA-Ab Coating)

Microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μL of purified MA-Ab at a concentration of 10 or 2.0 μg/mL and blocked with 3% BSA in phosphate-buffered saline (PBS). Then, aliquots of 100 μL of 4-ABMA-FITC (0.37∼10 nM) were incubated. Rabbit anti-FITC antiserum (1 : 2000 dilution) was added to the wells to measure the amount of 4-ABMA-FITC bound to MA-Abs. After incubation of peroxidase-conjugated anti-rabbit IgG, the OPD substrate and H_2SO_4 were added to determine the amount of enzyme bound to the plate. The optical density (OD) was read at 490 nm using a kinetic microplate reader (Molecular Devices, USA). The Ab concentration required to yield an A_{490} of 2.0 was used as the titer level, described previously.(8)

For the sensitivity and specificity study, MA and other cross-reactants were co-incubated with 4-ABMA-FITC to compete for the Ab binding. Cross-reactivity was estimated by comparing the IC_{50} for MA to that for the cross-reactant.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Indirect Competitive ELISA (4-ABMA-FITC Coating)

4-ABMA-FITC was attached to the plate in indirect ELISA using anti FITC Ab as a capture molecule, and this operation is reversed in direct ELISA. Anti-FITC antiserum (500- or 2000-fold dilution) was immobilized on microtiter wells. After blocking with BSA solution, 4-ABMA-FITC was incubated in the range of 0.2 to 20 nM for two hours. MA-Ab was then added to the wells and the amount of bound MA-Ab was estimated using peroxidase-conjugated anti-goat IgG, OPD substrate, and the H_2SO_4 solution, as described in direct ELISA. Sensitivity and specificity study were also performed as in direct ELISA.

Distribution of the Immunoglobulin Class of MA-Ab

For the immunoglobulin classification of MA-Ab, microtiter wells were coated with 100 μL of MA-Ab at concentration of 0.1 or 0.01 μg/mL and blocked with 150 μL of 3% BSA in PBS. Aliquots of 100 μL of rabbit anti-goat IgA (5 μg/mL), rabbit anti-goat IgM (5 μg/mL) or rabbit anti-goat IgG (1 μg/mL) were then incubated for 2 h. Incubation of peroxidase-conjugated anti-rabbit IgG and the substrate was the same as in direct ELISA. IgG fractions from goat, donkey, and mouse were used as positive/negative controls in checking specificity of Abs to heavy chains of goat imunoglobulins.

RESULTS AND DISCUSSION

Sensitivity

We have raised three kinds of polyclonal antibodies from goats against 4-ABMA-BSA and 4-ABMA-KLH immunogens.(13) When they were screened by FPIA with 4-ABMA-FITC, only antiserum against 4-ABMA-KLH immunogen generated a response.(7,14) The antisera were purified by affinity chromatography using various ligands, and all the purified Abs were characterized using ELISA with 4-ABMA-OVA.(8) When the competitor was 4-ABMA-OVA, purified Abs showed specific sensitivity and specificity patterns depending on ligands of affinity columns.

In this study, all the purified Abs from the three antisera were also tested using the ELISA system with 4-ABMA-FITC to examine the effect of the competitor. The consistent result was obtained with our previous FPIA study. It was only Abs from antiserum III, whose immunogen was

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

4-ABMA-KLH, that produced sensitive dose-response curves for MA (Figure 1), even though Ab III-3 did not produce any specific response in the assay format (Table 1). Ab III-3 showed very high non-specific binding which seemed to obscure any specific binding, resulting in a poor dose-response curve. That might be due to the poor purity of the Ab, since the Ab was purified by protein G chromatography and not by hapten or hapten-conjugate affinity chromatography.

There are some reports on the development of a fluorescence polarization immunoassay (FPIA) for MA using 4-ABMA-FITC.(15,16) The antibodies used in those studies were from ewe and sheep and all were raised against the 4-ABMA-KLH immunogen. Furthermore, when we produced some anti-MA polyclonal antibodies for FPIA from rabbits using BSA, OVA, and KLH as carrier proteins, we were able to obtain certain responses only using anti-4-ABMA-KLH antiserum (data not shown). Considering those results above, as well as previous work in our laboratory (7,14), the 4-ABMA-KLH immunogen might be related to the 4-ABMA-FITC competitor. During an antibody response, the hapten is presented to T cells

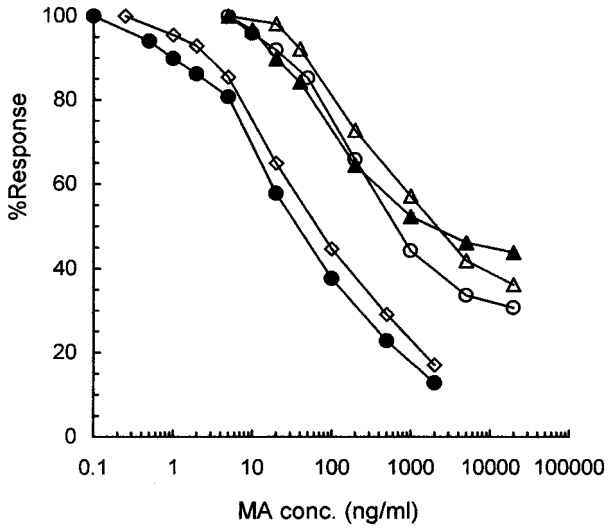

Figure 1. Standard curves for MA by direct ELISA with 4-ABMA-FITC: Ab III-1 (◇), III-2 (●), III-4 (▲), III-5 (△), III-6 (○). The Abs are as in Table 1. MA-Abs were immobilized to the well and 4-ABMA-FITC competed with free MA for the Ab binding. The *y*-axis is the relative response over the maximum response (the OD at 490 nm at the zero concentration of MA); the *x*-axis is the concentration of MA (ng/mL).

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 1. Detection Limits of Methamphetamine Antibodies in Various Assay Conditions

			Detection Limit (ng/ml)[a]				
			ELISA			FPIA	CE-LIF
				4-ABMA-FITC			
Ab	Immunogen	Ligand of the Affinity Column	4-ABMA-OVA	Direct Format	Indirect Format	4-ABMA-FITC	4-ABMA-FITC
Antiserum III	4-ABMA-KLH	Not purified	80			132	19
III-1		4-ABMA-BSA	120	0.90	1.6		
III-2		4-ABMA-OVA	310	0.32	0.24		
III-3	4-ABMA-	Protein G	200	–[b]	–		
III-4	KLH	MA	11	10	–		
III-5		4-ABMA	200	10	–		
III-6		Amphetamine	8.0	8.5	1.3		

[a]The detection limit was defined as the concentration of MA equivalent to the mean OD of eight replicates of the zero concentration plus two standard deviations. Data from ELISA with 4-ABMA-OVA, FPIA and CE-LIF were cited from previous work (7,8,14). Blanks mean that the assays were not performed.
[b]–, not determined because of slow slopes of calibration curves.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

in a conjugated state with part of the carrier protein. There is the possibility that certain side chains of KLH can facilitate the recognition of the FITC competitor.

The standard curves for MA from direct ELISA, which were constructed as described in our previous work (8), are shown in Figure 1. The detection limit, defined as the concentration of MA equivalent to the mean OD of eight replicates of the zero concentration plus two standard deviation, is also presented in Table 1. Some Abs (Ab III-1, III-2, and III-5) showed a great improvement in sensitivity by direct ELISA in comparison with results of ELISA with the OVA conjugate. The possibility exists that the improved detection limit might be due to the difference in formats between direct ELISA in this study and indirect ELISA employed previously. In the direct ELISA in the study, MA-Ab was immobilized on the solid phase and could recognize the free 4-ABMA-FITC conjugate in solution. Therefore, multivalent interactions of antibody are evidently not involved. On the contrary, procedures in previous studies might allow for multivalent interactions since the 4-ABMA-protein conjugate coated on wells was bound to the antibody in solution. Multivalent interactions of an Ab on the coated antigen lead a strong avidity for the Ab-Ag complex and they are apt to result in poor sensitivity, since a competitor with high affinity for the Ab will not compete with free antigen in the sample.(17,18) We designed indirect ELISA with 4-ABMA-FITC, in which the FITC conjugate was attached to the well through the carrier molecule of anti-FITC Ab, in order to investigate whether the multivalent interaction had some effect on the sensitivity or not. Immunoglobulin classification of the Abs was also examined for the same purpose.

Detection limits from indirect ELISA with the FITC competitor were about the same as those from direct ELISA with the competitor, expect for Ab III-4 and III-5 (Table 1). We could not obtain dose-response curves to quantitate MA with the two Abs; the slopes of the curves were too slow. Results of the immunoglobulin classification are shown in Figure 2. Although the affinities of antibodies to goat IgA, IgM, and IgG are not provided in this study, we confirmed that the classes of Abs were mostly IgG and the amount of IgA was negligible. The amount of IgM was far less than that of IgG, and Ab III-3 seemed to have only IgG class. However, specific class patterns related to sensitivity of each Ab with assay formats were not found.

Considering the results of the Ab classification, the gentle slopes of standard curves of Ab III-4 and III-5 in indirect ELISA in the assay did not seem to be caused by the avidity of Ab-Ag interaction. A possible reason could result from non-specific binding influenced by some of the reagents employed. Previous studies have shown that non-specific binding can have a major influence on assay sensitivity.(9,19)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

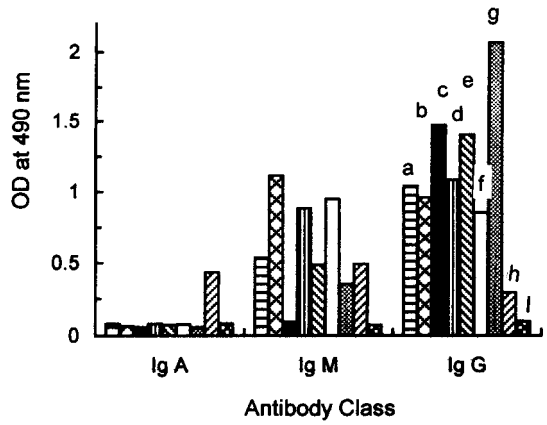

Figure 2. Immunoglobulin class distribution of MA Abs from antiserum III: Ab III-1 (a), III-2 (b), III-3 (c), III-4 (d), III-5 (e), III-6 (f), goat IgG fraction (affinity purified) (g), donkey Ab (affinity purified) (h), mouse monoclonal Ab (protein A purified) (i). The MA Abs are as in Table 1. MA-Abs were coated at the concentration of 0.1 μg/mL for classes of IgA and IgM, and were coated at 0.01 μg/mL for IgG. Rabbit anti-goat IgA, IgM, or IgG was then incubated and followed by peroxidase conjugated anti-rabbit IgG. The *y*-axis is the OD at 490; the *x*-axis is the antibody class.

In the same manner, it was considered that the much improved sensitivity of the assay was caused by the competitor, 4-ABMA-FITC, without the effect of multivalent interaction of Ab. It is a well-established principle that competitors modulate sensitivity and specificity of a particular Ab in an immunoassay.(5,18,20,21) In studies on the effects of competitors, competitors are usually changed in chemical structures by different bridge lengths or positions in the haptenic moiety. The data in Table 1, however, demonstrate that different labels on the same competing hapten for competitors still have great influence on the sensitivity of Ab (Ab III-1, III-2, and III-5). Table 1 also shows the effect of assay system on sensitivity. In both FPIA and capillary electrophoresis-laser induced fluorescence (CE-LIF)-based immunoassay, the competitors were all 4-ABMA-FITC. But the detection limit of CE-LIF was 7 times as low as that of FPIA. ELISA with 4-ABMA-FITC was also better in sensitivity than FPIA and CE-LIF. That could be expected since the heterogeneous immunoassay is considered superior in sensitivity to the homogeneous immunoassay. Antiserum III, however, gave lower detection limit in CE-LIF than in ELISA with 4-ABMA-OVA, indicating that assay sensitivity was affected by both the competitor and the assay system simultaneously.

Specificity

Table 2 shows cross-reactivity results of Abs by ELISA with 4-ABMA-FITC. As discussed previously (8,10), a great affinity of Abs for the tertiary amino group was also demonstrated (Figure 3). Ab binding to benzphetamine and methylephedrine was strong. The inhibitory effect of the hydroxyl group in ephedrines and stereospecificity data were also the same as in the previous work.(8)

Unlike the results of ELISA with 4-ABMA-OVA, all the Abs showed very high affinities for benzphetamine with the 4-ABMA-FITC competitor. Considering that benzphetamine is a drug of abuse, the high affinity for the drug is desirable. The results were consistent with those of FPIA and CE-LIF.(7,14) The specificity patterns were almost the same between direct and indirect ELISA, except that the cross-reactivities to benzphetamine and methylephedrine decreased to a certain extent in indirect ELISA. These data indicate that the specificity of an Ab in an immunoassay is mainly governed by the competitor employed regardless of the assay system used.

An interesting finding could be derived from the cross-reactivity results; 4-ABMA-FITC competitor seems to have close relation to benzphetamine in the Ab binding. A great number of publications deal with Ab-Ag interaction using structure-based analysis, for modulation of Ab specificity or Ab engineering. It was reported that part of antigen was buried in the Ab binding pocket (22) and concave and moderate concave interfaces were presented to the Ab-hapten binding.(23) It was also shown that only a subset of complementarity determining region (CDR) residues in Ab binding site interacts with antigens directly, and that is considered more common for haptenic antigens.(24) There may be Ab populations in antiserum III which contained certain CDR residues that could make some interaction with the large hydrophobic fluorescein group in FITC (Figure 3); hydrophobic residues such as tryptophan and tyrosine are overrepresented in CDR (25) and considered that they play a major role in Ab binding.(26) The result in the study that competitors with different labels on the same haptenic moiety yielded different sensitivity supports this possibility. Some of the CDR residues are also likely to provide Ab with a better accommodation for benzphetamine, which contains two aromatic rings, resulting in high cross-reactivity to the drug.

In summary, we have shown that the fluorescent competitor measured using fluorescence detection can lead to significant improvement of assay performance in the ELISA system. The investigation into structural relationship between the competitor and drugs also suggest that the competitor or the labeled molecule itself could modulate the specificity of Abs.

Table 2. Cross-Reactivity Results of Methamphetamine Antibodies by ELISA Using the 4-ABMA-FITC Competitor

		Cross-Reactivity												
		(A)		(B)	(C)	(D)		(E)		(F)		(G)		(H)
Format	Purified Ab	*d*-A	*dl*-A		*dl*-C	*d*-D	*l*-D	*d*-E	*l*-E	*d*-F	*l*-F	*d*-G	*l*-G	*dl*-H
Direct	III-1	1.0	1.3	27.6	0.09	–[a]	0.31	–	–	0.03	1.1	–	0.03	–
	III-2	1.0	1.4	22.9	0.10	–	0.23	–	–	–	1.0	–	0.02	–
	III-4	1.0	1.1	300	–	–	0.05	–	–	–	1.2	–	–	–
	III-5	1.0	1.1	110	–	–	0.18	–	–	–	1.7	–	–	–
	III-6	1.0	1.1	320	0.04	–	0.15	0.03	–	–	0.80	–	0.01	–
Indirect	III-1	1.0	1.3	14.0	0.13	–	0.13	0.05	0.02	0.02	0.90	–	0.05	–
	III-2	1.0	1.1	10.3	0.10	–	0.10	0.04	0.02	0.03	0.70	–	0.04	–
	III-6	1.0	1.0	50.0	0.03	–	0.11	0.04	–	–	0.57	–	–	–

Note. The cross-reactivity was expressed as the ratio of the IC_{50} for MA to that for the cross-reactant. (A) methamphetamine; (B) benzphetamine; (C) amphetamine; (D) ephedrine; (E) pseudoephedrine; (F) methylephedrine; (G) norephedrine; (H) epinephrine; Abs are as in Table 1.

[a]–, less than 0.01.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

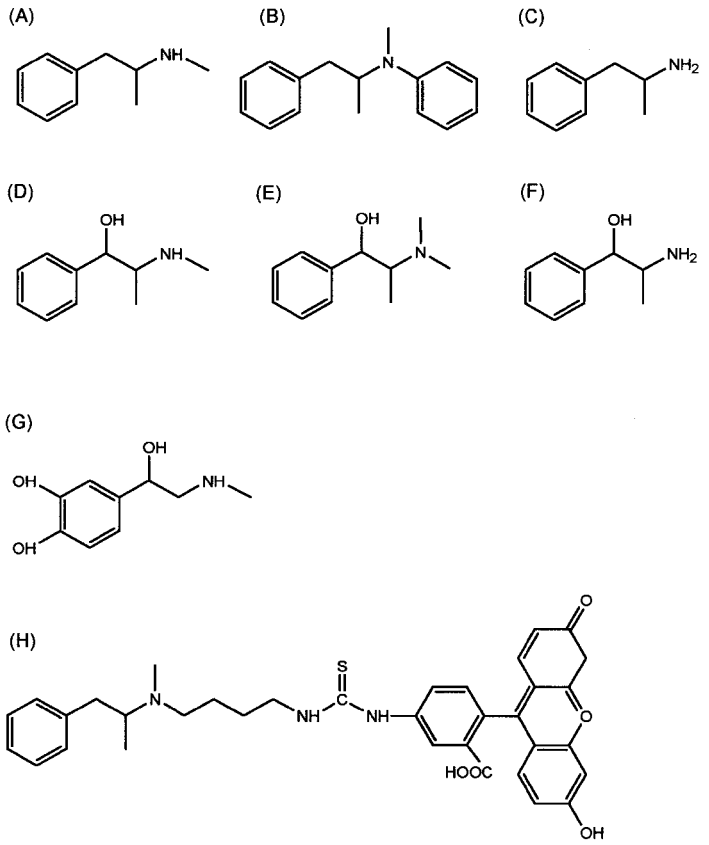

Figure 3. Chemical structures of cross-reactants related to methamphetamine and 4-ABMA-FITC: (A) methamphetamine; (B) benzphetamine; (C) amphetamine; (D) ephedrine and pseudoephedrine; (E) methylephedrine; (F) norephedrine; (G) epinephrine; (H) 4-ABMA-FITC.

The information could be of great value in study of Ab–Ag interaction or design of immunoassays.

ABBREVIATIONS

Ab, antibody; MA, methamphetamine; 4-ABMA, *N*-(4-aminobutyl)-methamphetamine; KLH, keyhole limpet hemocyanine; OVA, ovalbumin; FITC, fluorescein isothiocyanate.

REFERENCES

1. Got, P.A.; Guillaumet, G.; Boursier-Neyret, C.; Scherrmann, J.-M. Production and Characterization of Polyclonal Anti-S 20499 Antibodies: Influence of the Hapten Structure on Stereospecificity. J. Pharm. Sci. **1997**, *86* (6), 654–659.
2. Got, P.A.; Scherrmann, J.-M. Stereoselectivity of Antibodies for the Bioanalysis of Chiral Drugs. Pharm. Res. **1997**, *14* (11), 1516–1523.
3. O'Connor, T.; Gosling, J.P. The Dependence of Radioimmunoassay Detection Limits on Antibody Affinity. J. Immunol. Meth. **1997**, *208* (2), 181–189.
4. Chapman, D.J.; Cross, M.J.; Joel, S.P.; Aherne, G.W. A Specific Radioimmunoassay for the Determination of Morphine-6-glucuronide in Human Plasma. Ann. Clin. Biochem. **1995**, *32* (3), 297–302.
5. Colbert, D.L.; Eremin, S.A.; Landon, J. The Effect of Fluorescein Labels on the Affinity of Antisera to Small Haptens. J. Immunol. Meth. **1991**, *140* (2), 227–233.
6. Junghans, R.P. Optimization of Radioligand Binding and Radioimmuno assays. Anal. Biochem. **1996**, *240* (1), 75–89.
7. Choi, J.; Kim, C.; Choi, M.J. Comparison of Capillary Electrophoresis-Based Immunoassay with Fluorescence Polarization Immunoassay for the Immunodetermination of Methamphetamine Using Various Methamphetamine Antibodies. Electrophoresis **1998**, *19* (16–17), 2950–2955.
8. Choi, J.; Kim, C.; Choi, M.J. Influence of the Antibody Purification Method on Immunoassay Performance: Hapten-Antibody Binding in Accordance with the Structure of the Affinity Column Ligand. Anal. Biochem. **1999**, *274* (1), 118–124.
9. Norton, R.; Heuzenroeder, M.; Marnning, P.A. Non-Specific Serum Binding to Streptavidin in a Biotinylated Peptide Based Enzyme Immunoassay. J. Immunoassay **1996**, *17* (3), 195–204.
10. Choi, M.J.; Choi, J.; Park, J.; Eremin, S.A. Localization of the Epitope in Methamphetamine and its Antibody Use for the Detection of Methamphetamine and Benzphetamine by Polarization Fluoroimmunoassay. J. Immunoassay **1995**, *16* (3), 263–278.
11. Choi, M.J.; Choi, J.; Yoon, D.Y.; Park, J.; Eremin, S.A. Fluorescence Polarization Immunoassay of Progesterone. Biol. Pharm. Bull. **1997**, *20* (4), 309–314.
12. Dasqupta, A.; Datta, P. Rapid Detection of Cardioactive Bufalin Toxicity Using Fluorescence Polarization Immunoassay for Digitoxin. Ther. Drug Monit. **1998**, *20* (1), 104–108.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

13. Choi, J.; Choi, M.J.; Kim, C.; Cho, Y.S.; Chin, J.; Jo, Y.-A. The Optimization of ELISA for Methamphetamine Determination: The Effect of Immunogen, Tracer and Antibody Purification Method on the Sensitivity. Arch. Pharm. Res. **1997**, *20* (1), 46–52.
14. Choi, J.; Kim, C.; Choi, M.J. Immunological Analysis of Methamphetamine Antibody and its Use for the Detection of Methamphetamine by Capillary Electrophoresis with Laser-Induced Fluorescence. J. Chromatogr. B. Biomed. Sci. Appl. **1998**, *705* (2), 277–282.
15. Eremin, S.A.; Schiavetta, D.E.; Lotey, H.; Smith, D.S.; Landon, J. Design and Development of a Single-Reagent Polarization Fluoroimmunoassay for Methamphetamine. Ther. Drug Monit. **1988**, *10* (3), 327–332.
16. Eremin, S.A.; Gallacher, G.; Lotey, H.; Smith, D.S.; Landon, J. Single-Reagent Polarization Fluoroimmunoassay of Methamphetamine in Urine. Clin Chem. **1987**, *33* (10), 1903–1906.
17. Jockers, R.; Bier, F.F.; Schmid, R.D. Enhancement of Immunoassay Sensitivity by Molecular Modification of Competitors. J. Immunol. Meth. **1993**, *163* (2), 161–167.
18. Li, K.; Chen, R.; Zhao, B.; Liu, M.; Karu, A.E.; Roberts, V.A.; Li, Q.X. Monoclonal Antibody-Based ELISAs for Part-per-Billion Determination of Polycyclic Aromatic Hydrocarbons: Effects of Haptens and Formats on Sensitivity and Specificity. Anal. Chem. **1999**, *71* (2), 302–309.
19. Bodmer, D.M.; Tiefenauer, L.X. Treatment of Antibodies to Reduce Non-Specific Binding in Immunoassays Using the Avidin-Biotin Complex. J. Immunoassay **1990**, *11* (2), 139–145.
20. Choi, M.J.; Jo, Y.-A.; Choi, J.; Kang, C.-Y.; Han, C.-T. Production and Characterization of Monoclonal Antibodies Specific to Atrazine Group Compounds: Effects of Coating Ligand Structure on the Variation of Sensitivity and Specificity. J. Immunoassay **1999**, *20* (1–2), 57–77.
21. Tiefenauer, L.X.; Bodmer, D.M.; Frei, W.; Anders, R.Y. Prevention of Bridge Binding in Immunoassays: A General Estradiol Tracer Structure. J. Steroid Biochem. **1989**, *32* (2), 251–257.
22. Jeffrey, P.D.; Schildbach, J.F.; Chang, C.Y.; Kussie, P.H.; Margolies, M.N.; Sheriff, S. Structure and Specificity of the Anti-Digoxin Antibody 40-50. J. Mol. Biol. **1995**, *248* (2), 344–360.
23. MacCallum, R.M.; Martin, A.C.R.; Thornton, J.M. Antibody–Antigen Interactions: Contact Analysis and Binding Site Topography. J. Mol. Biol. **1996**, *262* (5), 732–745.
24. Chen, G.; Dubrawsky, I.; Mendez, P.; Georgiou, G.; Iverson, B.L. In Vitro Scanning Saturation Mutagenesis of all the Specificity

Determining Residues in an Antibody Binding Site. Protein Eng. **1999**, *12* (4), 349–356.
25. Vargas-Madrazo, E.; Lara-Ochoa, F.; Jimenez-Montano, M.A. Skewed Distribution of Amino Acids at Recognition Sites of the Hypervariable Region of Immunoglobulins. J. Mol. Evol. **1994**, *38* (1), 100–104.
26. Padlan, E.A. X-Ray Crystallography of Antibodies. Adv. Protein Chem. **1996**, *49*, 57–133.

Received March 14, 2001
Accepted April 22, 2001
Manuscript 3030

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Request Permission or Order Reprints Instantly!