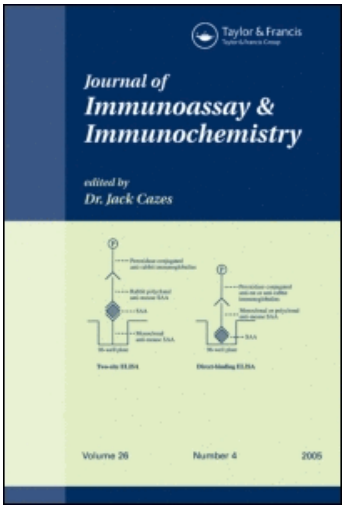

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Localization of the Epitope in Methamphetamine and Its Antibody Use for the Detection of Methamphetamine and Benzphetamine by Polarization Fluoroimmunoassay

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LOCALIZATION OF THE EPITOPE IN METHAMPHETAMINE AND ITS ANTIBODY USE FOR THE DETECTION OF METHAMPHETAMINE AND BENZPHETAMINE BY POLARIZATION FLUOROIMMUNOASSAY

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ABSTRACT

An antibody was prepared, using a four carbon-bridged methamphetamine molecule as an immunogen in order to develop a polarization fluoroimmunoassay for urine screening of methamphetamine and benzphetamine. Also, it s binding characteristics were investigated to locate epitope sites of methamphetamine. The study showed that the antibody was highly capable of eliciting a polarization fluoroimmunoassay response. However, the detection limit was much greater for benzphetamine(0.05ppm) than for methamphetamine(0.2ppm) and weakly antibody binding was found with methamphetamine. This difference in sensitivity may reflect the similarity of benzphetamine to the immunogen used to produce the antibody. Both benzphetamine and the immunogen have a tertiary amine attached to a carbon bridges whereas methamphetamine has only a scondary amine and amphetamine has a primary amine group. The difference of cross-reactivity data between phenylethylamine drugs and beta-hydroxyl phenylethylamine drugs indicates that the beta-carbon position have a major influence on the antibody interaction. Thus, the substitution of hydroxyl group on beta-carbon resulted in virtually no antibody affinity, even if a tertiary amine or secondary amine group was present in the molecule. This suggests that the beta-carbon chain plays a primary role as the epitope site with cooperative binding site of tertiary amine or secondary amine in alpha-carbon position. A hydroxyl group at the beta-carbon position plays an important inhibitory role to the antibody binding. (KEY WORDS: methamphetamine, benzphetamine, epitope site, polarization fluoroimmunoassay, abused drugs, urine screening)

INTRODUCTION

Phenylethylamine derivatives such as amphetamine, methamphetamine and benzphetamine are widely abused, potent sympathomimetic amines. Its presence in urine has been measured by various chromatographic methods(1) and immunoassay techniques (2,3,8). In immunoassay, a high affinity antibody is essential to prepare a sensitive and rapid immunoassay. The immunogenicity of an immunogen used to produce the desired antibodies is determined by the intrinsic chemical structure of the injected molecule, and requires for both an epitope and a class II T-cell receptor binding site on the immunogen molecule. Hapten molecules or molecules less than 3KDa generally lack immunogenicity and need to be conjugate with a carrier protein to provide the missing class II T-cell receptor binding sites. Derivatization of a four carbon-bridged methamphetamine molecule is the most popular process used to prepare methamphetamine immunogen. An *N*-4-aminobutyl-derivatized molecule to which a BSA molecule has been attached leads to a change in the chemical structure of methamphetamine. It becomes a tertiary amine structure which is similar to benzphetamine. It is possible that the binding affinity of an antibody produced using a four carbon-bridged methamphetamine immunogen, may be affected by the epitope of the analyte drug, resulting in various degrees of cross-reactivities, and sensitivities of the assay. Thus the antibody was characterized for its specificity and sensitivity to phenylethylamine drugs, and possible epitope sites of the drug were investigated using a polarization fluoroimmunoassay (PFIA). PFIA using an antibody is one of the best available methods to quickly analyze phenylethylamine drug levels in urine. The FITC tracer can be applied using the photo-check mode of a TDx analyzer (Abbott Labs., TX, USA) which

is equipped with a polarized light detector with an excitation wavelength of 481nm-489nm and an emission wavelength of 525nm-550nm. PFIA, as a competitive homogeneous immunoassay, requires no separation step. Recently, a sensitive PFIA was developed to measure amphetamine(4) and methamphetamine (5). These methods resemble the standard TDx assay procedure using the automatic mode of TDx analyzer (6).

Thus, we evaluated the antibody specificity and affinity of the drugs that were structurally related to the derivatized methamphetamine immunogen used to produce the antibody. The epitope of the drugs was also investigated using PFIA. In addition, PFIA was developed for routine doping control tests to screen for methamphetamine and benzphetamine in urineusing a TDx analyzer in the photo-check mode. Any other polarization fluorometer can be used for this test.

MATERIALS AND METHODS

Materials

Methamphetamine, benzphetamine, DL-amphetamine and other controlled drugs were obtained from the Korean National Institute of Health. *N*-(4-bromobutyl) phthalimide and hydrazine hydrate, used to prepare the *N*-4-aminobutyl derivative of methamphetamine, were purchased from Sigma Chemical Co. (Mo., USA). Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), bovine serum albumin (BSA, Fraction V), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide HCl (EDC), and fluorescein isothiocyanate (FITC) were also pur-chased from Sigma Chemical Co. Precoated preparative TLC plates (PLC plate silica gel 60 F_{254}, glass backed) were purchased from Merck Co.(Germany). All other inorganic chemicals and organic solvents were of analytical reagent grades, and were used in our laboratory. A

TDx Analyzer (Abbott, TX., USA) in photo-check mode, was used to measure the fluorescence polarization resulting from antibody binding and tracer displacement in the polarization fluoroimmunoassay.

Preparation of assay standards

A stock solution of methamphetamine hydrochloride in distilled water (1 mg/ml) was diluted in human urine to give final concentrations of 0, 0.5, 2.0, 10.0 and 50.0 ppm. The same method was used to prepare the benzphetamine standards of 0, 0.1, 0.2, 0.5 and 1.0 ppm, and amphetamine standards of 1, 10, 20, 50 and 100 ppm. Standards were stored at 4 ℃.

Preparation of N-4-aminobutyl derivative of methamphetamine

Methamphetamine(FW:149) was derivatized to *N*-4-aminobutyl methamphetamine(4-ABMA, FW:220) prior to attaching BSA or FITC for its use as an immunogen or tracer. 4-ABMA was prepared using *N*-(4-bromobutyl) phthalimide and hydrazine hydrate as described in Tamaki et al.(7).
The purity of 4-ABMA was checked by TLC, UV-spectrum and NMR. The ^{1}H NMR spectra was measured on a Varian Gemini-300 300MHz spectrometer using tetramethylsilane (TMS) as an internal standard. Gas chromatography/mass selective detector (GC/MSD) confirmation was performed using an HP5890A GC/HP 5970B MSD (Hewlet-Packard, CA, USA) under the same operating conditions as reported previously (8).

Preparation of immunogen and antiserum

The immunogen was prepared as described in Choi et al.(8) and purified by column chromatography with Sephacryl S-200 in 0.01 M PBS. The polyclonal

antiserum was obtained by immunizing a rabbit as described in Eremin et al.(10). The antiserum(PAb-7) used for the assay was obtained from the third bleeding of rabbit #7.

Preparation of fluorescein-labeled methamphetamine tracer

Fluorecein-labeled tracer was prepared by using a method previously reported (5), but slightly modified. A *N*-4-aminobutyl derivatives of methamphetamine(16mg, 70 umol) was dissolved in a mixture of 1 ml of methanol and 100 ul of triethylamine. FITC(19mg, 50umol) was then added. The reaction mixture was stirred overnight at room temperature and the solvents were evaporated. The product was dissolved in a small volume of methanol and small portions of this solution were separated on a TLC plate, using chloroform/ methanol (5/1 by volume) as the developer. Three fluorescent bands appeared on the plate. The major band at R_f 0.5 was scraped, extracted with methanol, and stored at 4℃ in the dark.

The concentration of fluorescein-labeled methamphetamine was estimated spectrophotometrically, measuring an absorption maximum at 492nm in 50 mM sodium carbonate buffer, pH 9.0, using an extinction coefficient of 8.78×10^4 L. mol^{-1}. cm^{-1} (9). FITC-labeled 4-ABMA was confirmed using NMR (deuterated dimethyl sulfoxide) spectrum.

Assessment of antiserum by polarization fluorescence technique

4-ABMA-tracer was diluted to 10 nmol/L with a resulting fluorescence intensity of approximately 6,000 arbitary units per milliliter in 2.5mM borate buffer, pH 9.2, containing 0.1% sodium azide. An aliquot of tracer (0.5ml) was added to 500 ul of serially diluted antiserum (20, 40, ...10,240 fold dilution) in

the same buffer. The fluorescence polarization was measured without incubation using the TDx analyzer in the photo-check mode. The antibody titration curve was constructed by plotting the fluorescence polarization in mP units against the antiserum dilution.

Polarization fluoroimmunoassay method

Ten microliters of the standards, 0.5 ml of the tracer solution (10 nmol/L), and 0.5mL of the diluted antiserum were mixed in a test tube. The dilution of the antiserum was chosen at a level which would result in approximately 70% of maximum binding. The reaction mixture required no incubation, and the fluorescence polarization was measured directly using the TDx analyzer in the photo-check mode. The standard curve was constructed by plotting the fluorescence polarization in mP units against the concentrations of the standards.

RESULTS

Reagents

The immunogen and tracer were synthesized from *N*-(4-aminobutyl) methamphetamine(4-ABMA) derivatized from methamphetamine. 672mg of 4-ABMA was obtained from 1.0g of methamphetamine hydrochloride.

The purity of 4-ABMA was checked by TLC(R_f 0.1 in chloroform/methanol (5/1 by volume)). The maximum absorption in UV spectrum was 220nm. The structure of this compound was confirmed by NMR and GC/MSD spectrometry. In a ^1H NMR(deuterated dimethylsulfoxide) spectrum using a Varian Gemini-300MHz, peaks were seen at 0.85 [d, 3H, CH_3-CH], 1.25-1.60 [m, 4H, -CH_2CH_2-], 2.20 [s. 3H, N-CH3], 2.25-2.60 [m, 5H, N-CH, N-CH_2, NH_2-$\underline{CH_2}$], 2.75-2.90 [m, 2H, C_6H_5-$\underline{CH_2}$-CH], and 7.10-7.30[m, 5H, aromatic H]. 4-ABMA

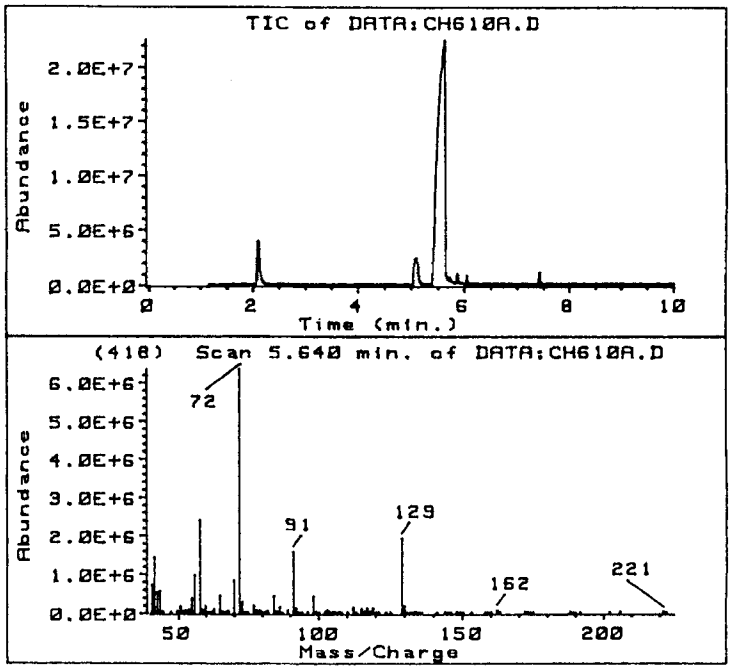

FIGURE 1. GC/MSD spectrum of N-4-aminobutyl derivative of methamphetamine. A fused silica capillary (SE54, 12m length x 0.2mm i.d.x 0.33um FT) was used. *N*-4-aminobutyl methamphetamine was eluted as a main peak at a retension time of 5.6 min under the experimental conditions.

was further verified by GC/MSD using an HP5890A GC/HP 5970B MSD. 4-ABMA was eluted as a main peak at a retention time of 5.5min under the experimental conditions. In the GC/MSD spectrum, the characteristic mass fragments appeared at m/z 221[MH^+], 72[$(CH_2)_4NH_2$] >58[72-CH_2] > 129[M-$C_6H_5CH_2$] > 91[$C_6H_5CH_2$] > 42[$NCHCH_3$] in abundance (Fig. 1).

The chemical structure of the FITC tracer was confirmed by ^1H NMR. The spectrum showed peaks at 0.85 [d, 3H, $\underline{CH_3}$-CH], 1.35-1.60 [m, 4H, -CH_2CH_2-], 2.20 [s, 3H, CH_3-N], 2.36-2.45 [m, 5H, CH, CH_2], 2.80-2.90 [m, 2H, C_6H_5-CH_2-

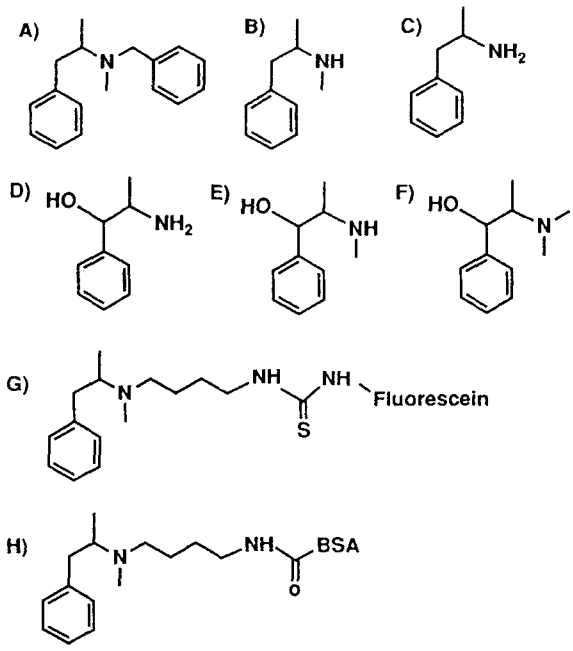

FIGURE 2. Chemical structures of benzphetamine (A), methamphetamine (B), amphetamine (C), phenylpropanolamine(D), ephedrine(E), methylephedrine(F), FITC tracer (G) and BSA immunogen (H).

CH0], 6.38-6.55 [m, 4H, aromatic H], 6.65 [d, 2H, aromatic H], 7.03-7.30[m, 7H, aromatic H], 7.7 [d, 1H, aromatic H], 8.20 [s, 1H, NH], and 8.35-8.57 [m, 1H, NH]. We were not able to obtain the mass spectrum for the tracer.

The chemical structures of the phenylethylamine derivatives (methamphetamine, benzphetamine, amphetamine), the beta hydroxy phenylethyl amines (ephedrine, methylephedrine, phenylpropanolamine), the FITC tracer and the BSA immunogen are shown in figure 2, enabling a comparison of the different antibody binding sites.

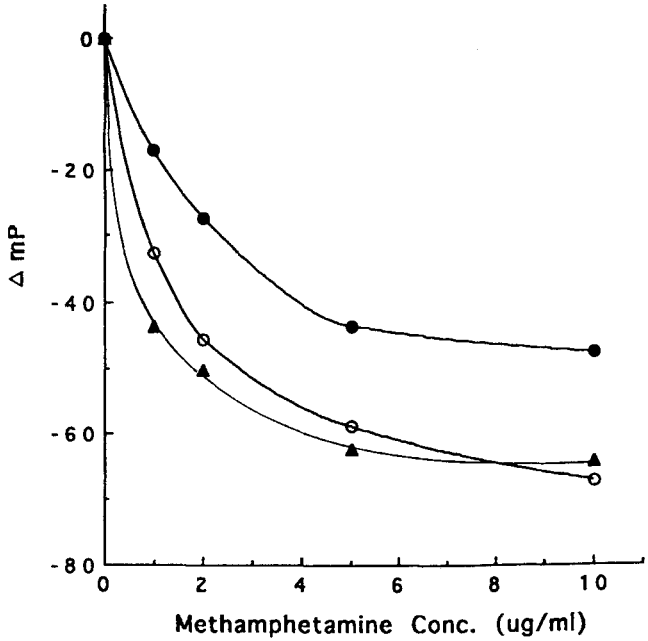

FIGURE 3. PFIA standard curves for methamphetamine at different concentrations of tracer (○-○ 20 nmol/L; ▲-▲ 10 nmol/L and ●-● 5 nmol/L). The y-axis is reduction in fluorescence polarization in arbitrary units (mP); the x-axis is concentration of methamphetamine(ppm).

Titration curve

For any immunoassay method, an antibody with high affinity and specificity needs to be developed. For this reason, a simple and quick test to screen for the appropriate antibody is very important. The polarization fluorescence technique is a recommended method to determine the titer level for an antibody because it takes only a few minutes to evaluate the titer levels of several antibodies simultaneously. After characterizing the antibodies in terms of titer level and antigen-antibody response, we developed a specific assay for both methamphetamine and benzphetamine using antiserum PAb-7.

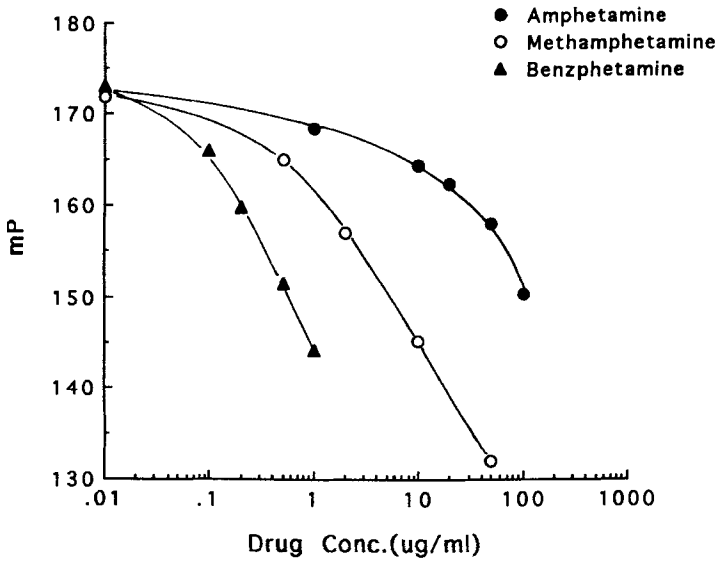

FIGURE 4. Typical PFIA standard curve for benzphetamine (▲-▲), methamphetamine (○-○) and amphetamine (●-●). The y-axis is in arbitrary units of fluorescence polarization (mP); the x-axis is concentration of analyte (ppm).

PFIA standard curve

Figure 3 shows the dose-response curves at different concentrations of the tracer. The optimal antiserum dilution level was found to be 1/30, which gives approximately 70% of maximum binding with the tracer. The optimal concentration of the tracer is approximately 10nmol/L. We also investigated the optimal incubation time for PFIA of methamphetamine and found that no incubation time is required. Figure 4 shows the PFIA standard curves for methamphetamine, benzphetamine and amphetamine at optimal conditions of the assay using optimal antisera dilution and tracer concentrations. This competitive displacement data of drug-antibody binding with 4-ABMA-FITC tracer indicates that benzphetamine had a better affinity to the antibody than methamphetamine; furthermore, amphetamine had almost no affinity to the antibody.

Performance characteristics

The minimum detectable concentration of benzphetamine and methamphetamine at the 95% confidence level was measured to be 0.05 ppm and 0.2 ppm respectively in 10ul urine. In order to measure assay precision, PFIA was tested on human urine specimens spiked with methamphetamine. Three different levels were used with 5 replicates for each assay. The mean results were 0.6, 3.6, and 23.6 ppm, within assay CVs of 9.3%, 3.3%, and 18.2% respectively. Measurement of the same set of samples at 3 different times gave between-assay CVs of 9.2%, 2.4%, and 4.5% respectively. The cross-reactivity of methamphetamine at a 1ppm level with amphetamine, ephedrine, methylephedrine, and phenylpropanolamine was 5.0%, 0.5%, 1.8% and 1.1% respectively.

No significant cross-reaction (less than 0.5%) was observed with other widely abused drugs such as morphines (ethylmorphine, heroin, levorphanol, hydrocodone, oxycodone, methadone, codeine), norpseudoephedrine, benzodiazepines (diazepam, chlordiazepoxide) and phenobarbital.

DISCUSSION

A hapten, lacking a class II T-cell receptor binding site, is unable to induce the production of a specific antibody. Thus, it needs be derivatized with a space arm in order to be coupled with a carrier protein molecule which provides the missing class II T-cell recepter binding site. A previous report (10) indicates that using an antibody produced with a 4-ABMA-KLH immunogen led to an enhanced polarization signal. An optimal dose-response binding curve was also obtained when the antibody interacted with a four or five carbon-bridged FITC tracer.

We derivatized the methamphetamine molecule at the site of the amine group to prepare it for its use as an immunogen and tracer. The secondary amine group of methamphetamine was changed a tertiary amine structure where the four carbon bridged amine was attached. BSA and FITC were coupled respectively to the aminobutyl bridge of the derivatized methamphetamine.

As expected in this PFIA system, the antibody showed an excellent polarization signal when using the 4-ABMA-FITC tracer. The polarization signal also decreased as methamphetamine concentrations increased in the sample. However, we found that the depolarization signal was more enhanced when detecting benzphetamine rather than methamphetamine or amphetamine. This finding may be explained by the hypothesis that the antibody more readily recognizes the tertiary amine group attached to the carbon bridge, as in benzphetamine, than it does the secondary amine group, as in methamphetamine. In this sense, the benzphetamine is structurally similar to the modified methamphetamine used to prepare the immunogen and tracer. It is general publicized knowledge for the antibody production that a portion of antibody not only recognizes the hapten and carrier protein, but also the bridge when any particular hapten-bridge-protein conjugate is used as an immunogen.

Another possible reasoning is that the 4-ABMA-FITC tracer sharing the same bridge as the 4-ABMA-protein immunogen has a higher affinity for the antibody than free methamphetamine. An appropriate binding affinity of a tracer to an antibody is essential in developing an immunoassay. A high binding affinity between the FITC tracer and the antibody leads to less binding of the free hapten in a competitive immunoassay model which results in a less sensitive assay. We found that the dose-response displacement only occurred at higher concentrations of free methamphetamine (0.2ppm sensitivity) compared with free benzphetamine (0.05ppm senstivity) which is structurally similar to the four-carbon bridged

TABLE 1

Cross-Reactivity of Methamphetamine with Phenylethylamine Drugs

Drugs	Relative Cross-Reactivity (%)
Benzphetamine	1000
Methamphetamine	100
Amphetamine	5.0
Phenylpropanolamine	1.1
Ephedrine	0.5
Methylephdrine	1.8

tracer used in this system. The same conclusion was found in the previous report (10) where the FITC-labeled methamphetamine, without the bridge, negligibly bound to the antibody produced using a 4-ABMA immunogen, whereas it bound to the four-carbon bridged methamphetamine tracer (4-ABMA) with a high affinity, presumably because of the structural modification of the methamphetamine amine group to resemble to immunogen.

In addition, Table 1 showed that the antibody cannot interact with phenylethylamine drugs having the functional hydroxyl group at the position of the beta-carbon chain. The antibody binding affinity of methamphetamine was compared with those of beta-hydroxy phenlethylamine derivatives such as phenylpropanolamine and ephedrine, which have corresponding structures to amphetamine and methamphetamine respectively. A negligible depolarization signal was obtained with the beta-hydroxy derivatives. The same result was obtained for methylephedrine, which has a tertiary amine group like benzphetamine, even though benzphetamine binds very strongly to the antibody resulting in a sensitive depolarization curve.

This result suggests that the hydroxyl group on the beta-carbon chain of phenylethylamine drugs cannot fit into the binding site pocket of the antibody due

to geometrical or steric hindrance of the hydroxyl group, indicating that the beta-carbon chain is an essential antigen determinant for antibody recognition. Consequently, strong antibody affinities were observed for benzphetamine and methamphetamine. However, amphetamine, which has only a primary amine group, was found to bind weakly to the antibody, despite its beta-carbon chain. This finding suggests that part of the epitope lies in the amine group of methamphetamine. From the above data, we conclude that the antigen determinant of phenylethylamine drugs involve the beta-carbon chain with either a tertiary amine or a secondary amine group at the alpha-carbon position.

Based on the antibody produced, we developed the methamphetamine and benzphetamine assay utilizing the PFIA system. In a positive sample for PFIA, the unlabeled drug and the FITC-labeled drug tracer complete for the binding site on the antibody, resulting in a lower polarization signal than that given by the tracer alone. No separation step is required. The total time for an assay of 10 samples is only 7 minutes. The antibody was found to recognize benzphetamine more effectively than recognizing methamphetamine. The detection limits of benzphetamine and methamphetamine in 10 ul of urine were 0.05 ppm and 0.2 ppm respectively. The precision and the recovery of different amounts of drug added to the urine were satisfactory (93-105%), and no significant cross-reactivities were seen with morphines, benzodiazephines and phenobarbital. The discrepency of the antibody binding affinity between structurally related drugs appears mostly as cross-reactivities resulting in differences in the assay sensitivity and detection range. We found that the detection limit and detection range of benzphetamine was approximately one order better than that of methamphetamine, which in turn was one order better than that of amphetamine.

In conclusion, the PFIA system described is an easy and useful method to estimate antibody titer levels and also enables rapid and reliable screening of

urine samples for benzphetamine and methamphetamine in routine doping control tests. We also demonstrate that the beta-carbon chain, buried in the binding pocket of antibody, is a critical part of the epitope site, in addition to the presence of a tertiary amine or a secondary amine group at the alpha-carbon position. The finding also suggest that the hydroxyl group at the position of beta-carbon of phenylethylamines plays an important inhibitory role to the antibody binding even if a tertiary amine or secondary amine group was present in the alpha-position.

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Abbreviations used; PFIA, polarization fluoroimmunoassay; 4-ABMA, 4-amino-butyl methamphetamine; PBS, phosphate buffered saline; BSA, bovine serum albumin; KLH, keyhole limpet hemacyanin; FITC, fluorecein isothiocyanate; TLC, thin-layer chromatography; CV, coefficient of variation; PAb, polyclonal antibody

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