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Immunoassays of Amphetamines: Immunogen Structure *vs* Antibody Specificity

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SUMMARY **Various immunoassays have been developed for the detection of amphetamines. These have varying degrees of cross-reactivity to other drug and food components. Information on the immunogen structures used, and the specificities of the antibodies obtained, have allowed formulation of a "structure-specificity" pattern delineated on the basis of immunochemistry and stereochemistry. The 'structure-specificity' relationship should be useful to future developments of these immunoassays. Specifically, immunoassays intended to detect either amphetamine or methamphetamine with minimal cross-reaction, should employ immunogens with amphetamine (or methamphetamine) derivatized via the para position of the phenyl ring. Such assays should show minimal cross-reaction with other secondary (or tertiary) amines but should strongly cross-react with phenyl ring substituted analogs. On the other hand, assays intended for detection of both amphetamine and methamphetamine should employ amphetamine (rather than methamphetamine) derivatized via its amino group as an immunogen. Such assays should show minimal cross-reaction with other tertiary amines and phenyl-substituted amphetamine/methamphetamine.**

Amphetamine use and abuse

Amphetamines are potent CNS stimulants used and abused for various purposes. d-Amphetamine is used clinically in the treatment of hyperkinetic children,[1,2] narcolepsy,[3] obesity[4] and diabetic neuropathy.[5] The drug also produces a psychosis that has been a useful model for the study of schizophrenia.[6,7] Amphetamine and methamphetamine are widely abused as CNS stimulants. They are used by students, factory workers and truck drivers for wakefulness, for alertness and to decrease the sense of fatigue. They are known to increase confidence and elevate moods to the extent of elation and euphoria. The initial drug-induced increase in mental and physical activity is followed later by fatigue and often depression. With truck drivers, these pharmacological actions can have devastating consequences for themselves and for other innocent accident victims. A recent study showed that tests for amphetamines were positive in 82% of the night-shift, ten-wheel truck drivers surveyed in Thailand.[8] The data also suggested that some of the drivers not only used the drugs while driving, but habitually, and therefore might be addicted. Such wide-spread abuse of these dangerous drugs undoubtedly contributes significantly to the high incidence of traffic accidents which are currently the country's number one killer.

Assays of amphetamines

Various assays of amphetamines have been developed for the purpose of a) identification or diagnosis of drug abusers, 2) identification of the drug in case of overdose or poisoning and c) monitoring the drug plasma level in clinical situations. These assays can be classified into 2 main types depending on the basic principles of the assay. First are those assays based on the physico-chemical properties of amphetamines. These are color tests,[9] thin layer chromatography,[10] gas chromatography,[11,12]

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gas chromatography/mass spectrometry,[13,14] high performance liquid chromatography,[15-17] ultraviolet spectroscopy,[18] spectrophotofluorometry[19,20] and enzymatic assay.[21] Another type of assay is based on immunochemical properties (i.e. antigen-antibody reactions) of amphetamines. These assays employ antibodies raised against drug derivatives, and thus they exhibit the inherent characteristic of a high degree of specificity. The antigen-antibody reactions can be amplified by various means: radioactivity (radioimmunoassay or RIA),[22] enzymatic activity (enzyme-linked immunosorbent assay or ELISA, enzyme-multiplied immunoassay technique or EMIT),[23] fluorescence (e.g., polarization fluoroimmunoassay),[24-26] and carrier particles (e.g., the latex agglutination inhibition reaction test or LAIRT,[27-29] and hemagglutination).[30] These various immunoassays, apart from their specificity, also have other individually advantageous characteristics. The latex agglutination tests for example, are very rapid, economic and simple, and they do not require expensive equipment/expertise. They are rather qualitative but are suitable for on-the-spot screening of drug abusers. By contrast, RIA which uses radioisotopes and a radioactive counter, requires expensive equipment but is very sensitive and accurate.

Immunogen structure and antibody specificity

Immunoassays have been developed for the purpose of detecting amphetamines and/or their metabolites of interest. Tests are available (e.g. Colbert *et al.*[26] Abuscreen^{R} ONTRAK^{TM}) which detect amphetamine exclusively and do not cross-react with methamphetamine. Tests which preferentially detect methamphetamine have also been studied.[23,27] In Thailand where both amphetamine and methamphetamine are widely abused, a test has been developed to detect both.[29] Needless to say, care must be taken by investigators to

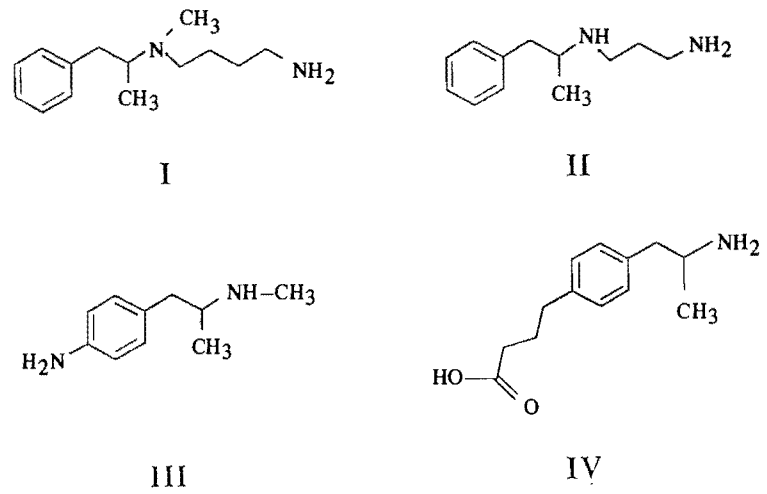


Fig. 1 Structures of amphetamine/methamphetamine derivatives used in the synthesis of immunogens.

Table 1. Specificity of antibody produced against methamphetamine derivatized via the amino group[28].

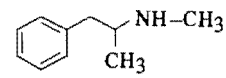
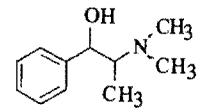
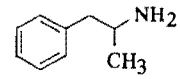
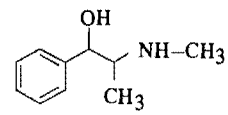
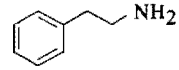
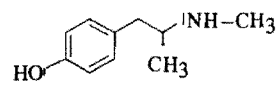
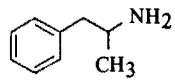
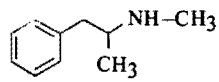
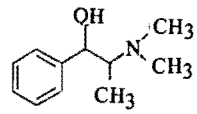
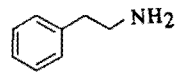
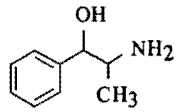
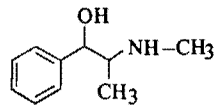
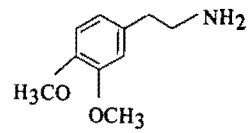
Compound	Structure	Detection limit μg/ml urine
Methamphetamine		0.4
Methylephedrine		2.0
Amphetamine		8.0
Ephedrine		22.0
β–phenylethylamine		40.0
p–OH–Methamphetamine		52.0

Table 2. Specificity of antibody produced against amphetamine derivatized via the amino group[29].

Compound	Structure	Detection limit μg/ml urine
Amphetamine		0.6
Methamphetamine		4.0
Methylephedrine		>800
β–phenylethylamine		>800
Phenylpropanolamine		>2450
Ephedrine		>2450
3,4–dimethoxy–phenylethylamine		>2450

avoid any troublesome cross-reaction with compounds commonly used in proprietary medicines or locally used foods.

The degree of specificity in immunoassays is attributable to the specificity of the antibodies used. This is determined in turn almost entirely by the chemical structures of the immunogens used to raise the antibodies. From a review of information on structures of various amphetamine immunogens used and the specificities of the corresponding antibodies obtained, certain patterns of a "structure-specificity" relationship emerged. This article reviews that information and outlines the "structure-specificity" relationship.

A. Specificities of antibodies raised against immunogens with amphetamine/methamphetamine derivatized via the amino group.

Cheng *et al*[22] synthesized N (4-aminobutyl) methamphetamine (Derivative I) in which the isopropylamine moiety was a tertiary amino group. This derivative was conjugated with bovine serum albumin (BSA) using glutaraldehyde and sodium borohydride.[28] The conjugate was used in rabbits to raise an antibody that exhibited the specificity shown in Table 1 as assayed by LAIRT.[28]

It is apparent from the table that the antibody is highly specific to methamphetamine which is the haptenic group of the immunogen. The antibody cross-reacted very well (20%) with methylephedrine, a tertiary amine in proprietary medicines for the common cold. The antibody bound less well (5%) to amphetamine. The reactivity ratio of methamphetamine/amphetamine as assayed by LAIRT using this antibody was 20. Itoh *et al* in similar study found the ratio to be 50.[27] Methamphetamine analogs with a para-hydroxy group bound extremely poorly to the antibody.

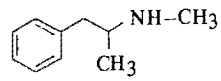
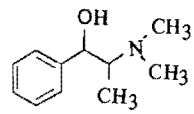
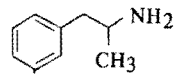
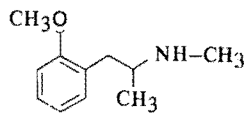
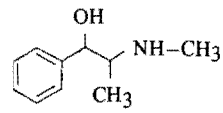
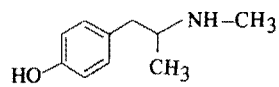
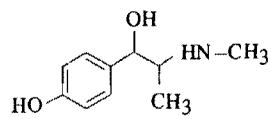
The antibody raised against Derivative I was also extensively studied by Faraj *et al* using 45 structurally related amphetamine analogs.[31] From the results obtained with rigid and semi-rigid systems, it was shown that the prefered conformation for binding the antibody was amphetamines where the amino group and the phenyl ring were in a trans conformation.

Mongkolsirichaikul *et al* synthesized a novel amphetamine derivative N-(3-aminopropyl) amphetamine (Derivative II).[29] This derivative was conjugated with BSA using carbodiimide as the coupling reagent. The rabbit antibody raised against this immunogen exhibited the specificity shown in Table 2 as assayed by LAIRT.[29] The antibody reacted best with amphetamine and slightly less well with methamphetamine. The reactivity ratio of amphetamine to methamphetamine was 6.6. The antibody did not react with ephedrine (a secondary amine) or the tertiary amine methylephedrine, which is a component of common cold medicines.

Thus, we may hypothesize that when the immunogen contains am-

phetamine/methamphetamine derivatized via the amino group, the phenyl ring protrudes from the immunogen surface and becomes immunodominant. Antibodies raised against such immunogens do not appear to accept any substitution on the aromatic ring and so immunoassays employing them do not detect such substituted analogs. Less specific interactions are expected on the isopropylamine part of amphetamines, although conversion of the amine to amide completely abolishes antibody binding.[29] As the degree of N-alkyl substitution increases in the immunogen (i.e., from the primary amine of amphetamine to the secondary amine in Derivative II, and from the secondary amine of methamphetamine to the tertiary amine of Derivative I) the antibodies produced generally recognized both amphetamine and methamphetamine. From the structure of Derivative II, the antibody produced against N-(3-aminopropyl) amphetamine should bind both amphetamine and methamphetamine. This is, in fact, observed. In recent experiments, we have found a reactivity ratio of amphetamine/methamphetamine of 1.6 (unpublished). These antibodies, in contrast to those produced against N-substituted methamphetamine, minimally cross-react with other tertiary amines.

Table 3. Specificity of antibody produced against methamphetamine derivatized via the phenyl ring.

Compound	Structure	Detection limit μg/ml urine
Methamphetamine		0.1
Methylephedrine		1000
Amphetamine		100
O–methoxyphenamine		4.1
Ephedrine		6.6
p–OH–methamphetamine		0.06
p–OH–ephedrine		3.4

B. Specificities of antibodies raised against immunogens with amphetamine/methamphetamine derivatized via the phenyl ring.

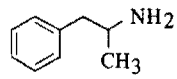
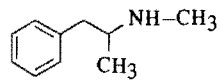
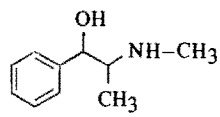
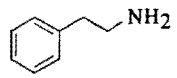
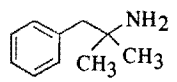
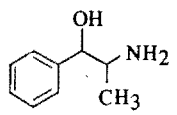
Because of troublesome cross-reactions of their antibody with the tertiary amine methylephedrine (Table I), Aoki *et al* synthesized new immunogens based on methamphetamine derivatized via the para or ortho positions of the phenyl ring.[23] They used para (or ortho) aminomethamphetamine (Derivative III) to conjugate to BSA using glutaraldehyde as a cross-linker. The resulting antibodies produced in rabbits were used to develop immunoassays of amphetamines via enzyme immunoassay, ELISA and LAIRT. The specificity of the antibodies (against the para aminoamphetamine derivative) are shown in Table 3 as studied by LAIRT.

The antibody bound very well with methamphetamine, which was the haptenic group in the immunogen. The antibody failed to bind the tertiary amine, methylephedrine. Amphetamine, a primary amine, also reacted very poorly. The reactivity ratio of methamphetamine/amphetamine was 1000. The antibody, however, bound extremely well with ortho or para hydroxy derivatives of methamphetamine or ephedrine.

Colbert *et al* synthesized a p-carboxy propylamphetamine derivative (Derivative IV) and conjugated its activated ester to keyhole limpet hemocyain to raise antibody in sheep.[26] By using polarization fluoroimmunoassay, the anti-amphetamine antibody obtained exhibited the specificity shown in Table 4.

The anti-amphetamine antibody reacted very well with amphetamine but failed to bind the secondary amine, methamphetamine. Ephedrine and phenylpropanolamine, each with an extra hydroxyl group, did not react with the antibody.

Table 4. Specificity of antibody produced against amphetamine derivatized via the phenyl ring[26].

Compound	Structure	Detection limit μg/ml urine
Amphetamine		0.2
Methamphetamine		>125
Ephedrine		>500
β–phenylethylamine		32
Phentermine		32
Phenylpropanolamine		>250

We may hypothesize that when the immunogen contains amphetamine/methamphetamine derivatized via the phenyl ring, the isopropylamine side chain extends further out on the surface of the immunogen molecule and becomes 'immunodominant'. The resultant antibody is therefore highly specific to this part of the molecule and less specific to substitutions on the phenyl ring. The antibody produced by Colbert *et al* failed completely to bind methamphetamine; the antigen-binding site of the antibody simply did not have room for the extra methyl group. In the case of Aoki *et al*, where a para-substituted methamphetamine was used as an immunogen,[23] the corresponding antibody was again very specific to methamphetamine and bound 1000x less well with amphetamine. In this case, the lower reactivity of amphetamine as compared to methamphetamine was not due to steric effects but rather to a difference of about 500 cal/mole of binding energy contributed by the N-methyl group.[35] It should be noted that these antibodies interact well with para-substituted amphetamine, as expected from the structure of immunogen used. Some of these para-substituted analogs may be found in human urine under certain conditions.[33]

C. Specificities of antibodies in commercially available immunoassays.

A few immunoassays of amphetamines are commercially available. These include, EMIT[R] d.a.u.[TM] produced by Syva Co., Abuscreen[R] ONTRAK[TM] and Roche Amphetamine Radioimmunoassay produced by Roche Diagnostic Systems. The structures of the amphetamine immunogens used to produce the antibodies for these immunoassays are generally not known. However, the specificity of the assay, supplied by the manufacturer and/or reported by investigators, usually provides some information on the structure of the immunogen used. For example, Budd *et al*, used 62 amines to determine the specificity of the EMIT[R] d.a.u.[TM] amphetamine assay.[32] They concluded that the antibody was most likely raised against an immunogen with amphetamine derivatized via its amino group. On the other hand, information supplied by Roche Diagnostic Systems, and published reports[33,34] suggest that the immunogens used to produce antibodies of ONTRAK[TM] and Roche Amphetamine RIA were amphetamines substituted via the para position of the phenyl ring.

Guidelines for future developments of amphetamine immunoassays

From the above discussion, the following guidelines on immunogen structures are proposed for use in the development of specific immunoassays for amphetamines.

1. Immunoassay designed to detect either amphetamine or methamphetamine with minimal cross-reaction should employ immunogens with amphetamine (or methamphetamine) derivatized via the para-position of the phenyl ring. The resulting assay would not cross-react with other tertiary amines but would be prone to cross-react with para-substituted analogs.

2. Immunoassays intended to detect both amphetamine and methamphetamine should employ immunogens with amphetamine (not methamphetamine) derivatized via its amino group. The assay would show minimal cross-reaction with other tertiary amines and phenyl substituted amphetamine analogs.

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