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DEVELOPMENT AND APPLICATION OF POLYCLONAL AND MONOCLONAL-
ANTIBODY BASED ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE
ANALYSIS OF NEONICOTINOID INSECTICIDES IMIDACLOPRID AND
THIAMETHOXAM

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THIS DESSERTATION IS DEDICATED TO MY PARENT, JAE-CHUL KIM AND
PIL-JA HA AND WIFE, SOO-HEE SON.

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ABSTRACT

The sensitivity of previously developed enzyme-linked immunosorbent assay (ELISA) for imidacloprid was considerably improved with a purified polyclonal antibody and a direct competitive assay format (dcELISA). Under the optimized conditions, the half-maximal inhibition concentration (I_{50}) and the limit of detection (LOD) were approximately 1 $\mu\text{g/L}$ and 0.06 $\mu\text{g/L}$, respectively. Computational analysis suggests that the antibody specificity primarily relate to the dihedral angles, steric hindrance and electrostatic charges on the imidazolidinyl ring. The ELISA analysis of water samples fortified with imidacloprid showed a satisfactory correlation with the fortified levels.

Two ELISAs were developed for thiamethoxam and imidacloprid with an antiserum and monoclonal antibody (MAb), respectively. Three antisera against thiamethoxam were raised from rabbits immunized with the hapten-KLH conjugate. Three imidacloprid specific MAbs were obtained from mice immunized with the hapten II-ovalbumin conjugate. Antisera and MAbs were characterized with indirect competitive ELISA (icELISA). Antiserum I with the lowest I_{50} value was selected for an ELISA for thiamethoxam and MAb E6F3 for imidacloprid. The antiserum I was specific for thiamethoxam and tolerant of up to 5% acetonitrile and 5% acetone. MAb E6F3 can tolerate up to 15% (v/v) acetone or 20% (v/v) methanol. Assay sensitivities of both ELISAs were not influenced at tested range of pHs and ionic strengths. Under the optimized conditions, I_{50} and limit of detection (LOD) were approximately 9.0 and 0.1 $\mu\text{g/L}$ of thiamethoxam. I_{50} and the limit of detection were approximately 0.8 and 0.1 $\mu\text{g/L}$ of imidacloprid in icELISA, and 0.3 and 0.03 $\mu\text{g/L}$ in direct competition ELISA

(dcELISA), respectively. Antiserum I and MAb E6F3 were specific to thiamethoxam and imidacloprid, respectively. The ELISA analysis of water and cucumber samples showed good correlation between the level determined and levels fortified.

Affinity ($1/K_d$) of E6F3 determined with kinetic exclusion immunoassay (KinExA) for acetamiprid and clothianidin were similar, but 50-fold weaker than that of imidacloprid. MAb E6F3 had no measurable affinity for other neonicotinoids and analogs.

Thiamethoxam specific MAbs were produced with the same hapten used for antisera for thiamethoxam. MAbs showed low I_{50} values and little cross reactivities to other neonicotinoids.

TABLE OF CONTENTS

Acknowledgements.....	iv
Abstract.....	v
List of tables.....	xi
List of figures.....	xii
Chapter 1. Introduction.....	1
1.1. Overview.....	1
1.2. Development of an ELISA.....	4
1.2.1. Hapten design and synthesis.....	4
1.2.2. Covalent binding to carrier molecules.....	7
1.2.3. Antibody production.....	8
1.2.4. Immunoassay formats.....	10
1.2.5. Optimization of an assay conditions.....	11
1.2.6. Real sample analysis.....	11
1.3. Kinetic Exclusion Assay (KinExA).....	12
1.4. Neonicotinoids.....	15
1.4.1. Overview.....	15
1.4.2. Degradation of imidacloprid and thiamethoxam.....	18
1.4.3. Mode of action.....	20
1.4.4. Analysis of neonicotinoids.....	22
1.4.5. Neonicotinoid immunoassay.....	25
1.5. Working hypothesis and objective.....	30
1.6. Literature cited.....	30
Chapter 2. Improved enzyme-linked immunosorbent assay for the insecticide imidacloprid.....	39
Abstract.....	39
2.1. Introduction.....	40
2.2. Materials and methods.....	40

2.2.1. Reagents.....	40
2.2.2. Synthesis of 1-[(6-chloro-3-pyridinyl)methyl]-3-carboxypentyl-N-nitro-2-imidazolidinimine (hapten I).....	41
2.2.3. Synthesis of 1-[(6-carboxylethylthio-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine (hapten II).	42
2.2.4. Direct competitive ELISA.....	43
2.2.5. Determination of cross-reactivity.....	43
2.2.6. Molecular modeling.....	43
2.2.7. Fortification of imidacloprid in water.....	45
2.3. Results and discussion.....	45
2.3.1. Effect of assay buffer ionic strength.....	46
2.3.2. pH effect.....	46
2.3.3. Effect of Tween 20.....	47
2.3.4. Typical inhibition curve.....	49
2.3.5. Solvent effect.....	49
2.3.6. Cross-reactivity.....	50
2.3.7. Molecular modeling of imidacloprid analogs.....	53
2.3.8. Analysis of imidacloprid in water.....	56
2.4. Conclusion.....	56
2.5. Literature cited.....	58

Chapter 3. Development of an Enzyme-Linked Immunosorbent Assay for the Insecticide Thiamethoxam.....	61
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Abstract.....	61
3.1. Introduction.....	62
3.2. Materials and methods.....	64
3.2.1. Reagents.....	64
3.2.2. Spectroscopy and chromatography.....	64
3.2.3. Molecular modeling	65
3.2.4. Safety precaution.....	65
3.2.5. Synthesis of hapten (Figure 3.1).....	65

3.2.5.1. Preparation of 3-methyl-4-nitroimino-1,3,5-oxadiazinane (I)	66
3.2.5.2. Preparation of 2-chloro-5-methylthiazole (II).....	65
3.2.5.3. Preparation of 2-chloro-5-bromomethylthiazole (III).....	66
3.2.5.4. Preparation of thiamethoxam (IV).....	67
3.2.5.5. Preparation of 3-(2-(2-carboxyethylthio)-5-ylmethyl)-5-methyl-4-nitroimino-1,3,5-oxadiazinane (V).....	67
3.2.6. Preparation of Protein-Hapten Conjugates.....	69
3.2.6.1 Hapten-KLH and BSA conjugation.....	69
3.2.6.2. Preparation of enzyme tracer.....	69
3.2.7. Immunization.....	69
3.2.8. Direct competitive ELISA (dcELISA).....	70
3.2.9. Fortification of thiamethoxam in water.....	70
3.3. Results and discussion.....	71
3.3.1. Hapten design and synthesis.....	71
3.3.2. Immunization and characterization of antisera.....	76
3.3.3. Competitive inhibition.....	77
3.3.4. Cross-reactivity.....	77
3.3.5. Chemical effects on assay performance.....	80
3.3.6. Solvent effect.....	82
3.3.7. Effect of pH and ionic strength.....	82
3.3.8. Thiamethoxam-fortified water samples.....	85
3.4. Summary and conclusions.....	87
3.5. Literature cited.....	87

Chapter 4. A Monoclonal Antibody-Based ELISA for the Insecticide Imidacloprid.....	92
4.1. Introduction.....	92
4.2. Experimental.....	93
4.2.1 Materials and reagents.....	93
4.2.2. Preparation of protein-hapten conjugates.....	94
4.2.3. Monoclonal antibody generation and characterization.....	96

4.2.4. Assay optimization.....	97
4.2.5. icELISA.....	97
4.2.6. dcELISA.....	98
4.2.7. Measurements of binding kinetics.....	98
4.2.8. Fortification of imidacloprid in water and cucumber.....	99
4.3. Results and discussion.....	100
4.3.1. Characterization of MAbs.....	100
4.3.2. Competitive inhibition.....	102
4.3.3. Physicochemical effects on assay performance.....	104
4.3.4. Kinetics of MAb binding and cross reactivity.....	110
4.3.5. Imidacloprid-fortified water and cucumber samples.....	111
4.4. Conclusion.....	114
4.5. Literature cited.....	114
Chapter 5. Production and characterization of thiamethoxam specific MAbs.....	118
5.1. Introduction.....	118
5.2. Experimental.....	119
5.3. Results and discussion.....	119
Chapter 6. Summary, potential application of assays, and future work.....	123
6.1. Summary.....	123
6.2. Potential application and future work.....	126
Appendix I.....	129

LIST OF TABLES

Table 1.1. Analysis of imidacloprid with conventional methods.....	26
Table 1.2. ELISAs for the neonicotinoid insecticides imidacloprid, acetamiprid and thiamethoxam.....	29
Table 2.1 Comparison of assay cross-reactivity and length and dihedral angles of imidacloprid and its structural analogs.....	52
Table 3.1. RMS errors of the superimposition on thiamethoxam and molecular dimensions of global energy minimum structures.....	75
Table 3.2. Titer and apparent binding affinity of antisera.....	76
Table 3.3. Cross-reactivity of Ab-I to thiamethoxam and its structural analogues.....	79
Table 4.1. Competitive binding of MAbs to imidacloprid haptens.....	101
Table 4.2. Binding characteristics of MAb E6F3 to neonicotinoid insecticides and 6-chloronicotinic acid.....	112
Table 4.3. Percentage recovery of imidacloprid fortified to water and cucumber samples by the icELISA.....	113
Table 5.2. Summary of icELISA.....	122
Table 5.3. Cross-reactivity of MAb G2 I to four neonicotinoids.....	122
Table 6.1. Performance of ELISAs in this dissertation.....	128

LIST OF FIGURES

Figure 1.1. Structures of the neonicotinoid insecticides and the discovery companies.....	17
Figure 2.1. Length and dihedral angles of imidacloprid and its metabolites.....	44
Figure 2.2. Effects of assay buffer concentration (A), pH (B) and Tween 20 (C) on the assay, and typical imidacloprid inhibition curves of direct and indirect cELISAs (D) at the optimized condition.....	48
Figure 2.3. Effects of methanol (A), acetone (B), acetonitrile (C) and DMSO (D) on the assay.....	51
Figure 2.4. Structures and electrostatic potentials mapped on electron density isosurfaces of imidacloprid and its analogs.....	55
Figure 2.5. Correlation between concentrations of imidacloprid determined by ELISA and those fortified in tap water.....	57
Figure 3.1. Synthetic scheme of thiamethoxam hapten.....	68
Figure 3.2. Structures and dihedral angles of thiamethoxam and hapten candidates.....	73
Figure 3.3. Electron density isosurfaces colored by electrostatic potential.....	74
Figure 3.4. Standard inhibition curves of thiamethoxam in dcELISA and icELISA formats.....	78
Figure 3.5. Effects of acetone (A), acetonitrile (B), DMSO (C), and methanol (MeOH) on the assay.....	81
Figure 3.6. pH effect on assay sensitivity.....	83
Figure 3.7. Effect of ionic strength on assay sensitivity.....	84
Figure 3.8. Correlation between thiamethoxam concentrations determined by ELISA and those fortified in tap water and stream water samples.....	86
Figure 4.1. Structures of imidacloprid haptens, imidacloprid, its major metabolite 6-chloronicotinic acid and other neonicotinoids.....	95
Figure 4.2. Standard inhibition curve of imidacloprid in dcELISA and icELISA formats.....	103
Figure 4.3. Effect of assay buffer pH.....	106
Figure 4.4. Effect of ionic strength on assay performance.....	107

Figure 4.5. Effect of incubation time on assay sensitivity.....	108
Figure 4.6. Effects of DMSO (A), acetone (B), acetonitrile (C), and MeOH (D) on the assay.....	109
Figure 5.1. Titer of six MAbs.....	121
Figure A.1. Determination of K_d for MAb E6F3 against imidacloprid.....	131
Figure A.2. Determination of active binding sites of MAb E6F3.....	132
Figure A.3. Determination of on-rate for MAb E6F3.....	133

Chapter I

INTRODUCTION

1.1. OVERVIEW

Immunoassay is an analytical method that relies on the specific binding of an antibody to an antigen. Since Yalow and Berson [1] developed the first immunoassay to detect insulin in 1960, immunoassay has been a routine practice for at-home pregnancy tests, drug screening programs, AIDS testing, and detection of disease-related proteins in clinics. In early 1980's, immunoassay was introduced to environmental analysis. For the last two decades, there has been a significant progress in the development of immunoassay formats, and improvement of assay sensitivity [2-5] and a variety of immunoassays have been developed for various pesticides, industrial chemicals, and microbial toxins.

Immunochemical approach to environmental analysis was virtually driven to overcome the shortcomings of conventional analytical methods such as gas chromatography (GC), GC-mass spectrophotometry (GC-MS), high performance liquid chromatography (HPLC), or LC-MS. Although these methods have provided accurate quantification and are prevalent for pesticide detection, use of these instruments inevitably involves high analytical cost per sample and time consuming procedures such as sample extraction and partition with large solvent consumption, purification of sample with chromatographic techniques, and concentration to achieve detection limit. Besides, determination of residues requires expensive instruments equipped with sophisticated

monitoring the fate of pesticides in environment and concern about contamination of food or drinking water and human exposure to hazardous chemicals led to the development of new analytical methods that enable the researches to carry out rapid, cost-effective, sensitive, and high throughput analysis. Immunoassay has satisfied these requirements and proven as the alternative to conventional methods [6-11]. The United States Environmental protection Agency (EPA) has established immunoassay guidelines and approved immunoassays for the detection of several chemicals as official methods. Van Emon and Gerlach, reviewed regulatory issues, technical advances, and communication methods in relation to the use of immunoassays for environmental monitoring and human exposure studies [12, 13].

For some chemicals that give a rise to concerns of human exposure and environmental contamination, immunoassay kits are commercially available from several companies around the world. Several kits for polycyclic aromatic hydrocarbons (PAHs), diazinon, triazin, dioxin and furan, and polychlorinated biphenyls (PCBs) recently have been validated by several laboratories comparing accuracy and reproducibility of these kits with those of analytical instruments and were shown to be useful particularly for semi-quantitative screening of pesticides [14-18].

Although various types of immunoassay have been developed and frequently used for the detection of a variety of substances [19-24], enzyme-linked immunosorbent assay (ELISA) is the most widely accepted format in environmental immunoassays. An ELISA is simple in procedures involving coating of a plate (usually on 96-well microplate) with coating antigen or antibody, blocking of unoccupied site, competition between antibody or enzyme tracer and target compound, and quantification of competition by reading

color intensity. Radioimmunoassay which uses radio isotope as a label was once used due to its high sensitivity but concerns associated with handling and disposal of radioactive material led to development of safer methods.

Development of an ELISA is largely dependent on the availability of polyclonal antibody (PAb) or monoclonal antibody (MAb) against target molecules. Thus, production of antibodies with desired affinities and specificities is the key requirement for an assay development. Antibody able to bind to target compound or a structurally-related family of compounds determines types of ELISAs, categorized as a compound or class specific ELISA. Production of desired antibodies can be achieved through judicious design of hapten that refers to the structural mimic of compound interested, considering all parameters that possibly influence on antibody-analyte interaction. Several steps involved in ELISA development will be discussed in the following section.

Neonicotinoid insecticides belong to a new class of pesticides with new mode of action and have been widely used to control sucking and chewing insects such as aphid, whitefly, leafhopper, termites and some beetles. Neonicotinoids act as an agonist at the nicotinic acetylcholine receptor (nAChRs), effecting on insect mobility and feeding behavior finally causing death. Excellent systemic activities of neonicotinoids allow foliar, seed, and soil treatment. Several conventional methods have been reported to determine neonicotinoid residues in various matrices. However, use of conventional instruments is not suitable for the analysis of neonicotinoids due to thermolability and polarity and labor intensive and time consuming procedures. Moreover, high persistence in soil, good solubility in water, and high toxicity to some aquatic organisms give a rise

to concern about leaching of neonicotinoids into surface and ground water by run-off or wind drift.

Imidacloprid and thiamethoxam represent two generations of neonicotinoid insecticides. Imidacloprid and thiamethoxam are metabolized into several compounds, some of which show nearly equal or even higher toxicity than that of imidacloprid. Thus, development of an ELISA for imidacloprid as well as its metabolites and thiamethoxam would be useful tool for monitoring of those compounds in environment and human exposure.

1.2. DEVELOPMENT OF AN ELISA

There are several steps to the development and application of an ELISA. The steps in ELISA include design and synthesis of hapten depending on type of ELISA, covalent binding of hapten to carrier proteins for immunogen, coating antigen, or enzyme tracer, immunization, purification and characterization of antibody, development and optimization of assay format, and validation of assay.

1.2.1. Hapten design and synthesis

Pesticides with small molecular weight (< 1,000 dalton) are not capable of triggering immune response in an animal so that these molecules must first be covalently linked to a larger protein called a carrier protein such as keyhole lipet hemocynine (KLH) to make them immunogenic. Covalent coupling of pesticides to a carrier protein requires derivatization of a parent compound and introduction of functional group to a selected site. The most common functional groups for hapten synthesis include $-NH_2$, $-COOH$, -

OH, -SH which are usually attached to 3 to 6 carbon chains. If a functional group is present on a molecule, it can be used for conjugation to a protein. However, use of the functional group should be avoided if possible, because resulting haptens may show a significant dissimilarity to target compound and the mask of key antigenic determinant by a large bulky carrier protein. The most important consideration in hapten design is that haptens must contain as much of the geometric structure, size, and electronic properties of the target compound as possible plus characteristic moiety of target molecule to facilitate the recognition by antibody and a linker should not act as an antigenic determinant [25-27].

Four important factors to be considered in hapten design are: 1) position of a linker to maximize the exposure of unique portions of the molecule; 2) length of the linker; 3) polarity of the linker and; 4) functional group variations for ease of attachment to protein carriers and enhanced hapten density.

The position of the linker on the target molecule has a profound influence on the selectivity and sensitivity of an assay. A characteristic group of a molecule should be maximally exposed for antibody recognition. Therefore, it is essential to attach the linker to the site remote from an antibody binding moiety. Presence of a functional group on a linker may produce an antibody with recognition to the linker or carrier protein so that alkyl chains are preferable to minimize linker binding. However, in general, the length of linker has less effect on antibody affinity and specificity [28, 29].

The different approaches to hapten design should be used, depending on whether a single compound within a class is targeted or the whole class. Synthesis of haptens with an unique structure of the individual target is required for a compound specific assay, but

for a class specific assay, the hapten would consist of those features common to all the structures within the group to be targeted [30, 31].

Goodrow and Hammock used the concept of size-exclusion for the synthesis of herbicide simazin hapten to produced simazine specific PAb with no cross recognition to structurally related compounds. PAb derived with the smallest hapten but contain the characteristic group of simiazin showed exclusive recognition of analytes larger than the target molecules [32]. This concept was demonstrated by Bruun *et al.* showing antibody specificity is inversely proportional to the size of hapten side chain used for immunization [33]. Mercader and Montoya produced azinphos-methyl specific MAb with haptens that contain only a fragment of the target analyte. For the design of haptens to produce compound specific antibodies, preservation of physicochemical properties of the unique functional and immunodeterminant chemical groups of analyte is important [34].

There is a need to increase sample throughput and to screen samples for more than one analyte per test. This requires antibodies with the ability to bind to families of compound. For a class specific assay, it is important that the linker of a hapten is located at a position which renders the common feature exposed to the immune system. Antibody with class specificity makes it possible to monitor human exposure to a mixture of various xenobiotics by targeting similar urinary metabolites or to detect compounds that belong to the same group as well as metabolites. Preservation of structural similarities between the major functional groups that characterize the pesticide family and the immunizing hapten has resulted in the development of "class specific" assay.

These assays exhibited cross reactivities for related compounds and showed to be suitable for class-specific screening [35-37].

Molecular modeling technique is another approach to hapten design to produce the antibodies with the desired specificity. Molecular modeling enables one to predict aspects of three-dimensional shape of a molecule such as length, depth, dihedral angle, and superimposition of a hapten with the target compound, various bonds involved in antibody-antigen interaction, electron density distribution and can contribute greatly to the understanding of recognition. As a consequence, more informed decisions can be made regarding hapten design and immunoassay development [35, 38-40].

1.2.2. Covalent binding to carrier molecules

Once the hapten is synthesized, it should be linked to an appropriate protein. Conjugation can be achieved through covalent bonding between functional groups of hapten and side chain of amino acid including the amino groups of the N-terminal and lysine residues, the carboxylic groups of the C-terminal and aspartic and glutamic residues, the imidazo and phenolic functions of histidine and the tyrosine residues respectively and sulfhydryl groups of cystein residues. Several different proteins including KLH, ovalbumin (OVA), bovine serum albumin (BSA), Mollusk hemocyanines, thyroglobulin, and fetuin have successfully been used as a carrier protein [41], however, KLH is the most common carrier protein due to its good immunogenicity and large size. The selection of coupling method is dependent on the functional group of the hapten. Reaction of functional group-containing hapten with a cross-linker reagent selected based on conjugation method in water-miscible solvent converts the functional

group to an active form which then reacts with protein. Centrifugation effectively removes precipitated byproducts. Drop-wise addition of the activated hapten to protein dissolved in aqueous buffer allows the formation of covalent bonding between activated form of the hapten and amino group of protein. Then, the conjugate can be purified with gel filtration or dialysis.

Widely used methods to conjugate carboxyl group-containing hapten to protein are the mixed anhydride [42] and NHS ester method. In the mixed anhydride method, carboxyl group is converted to an active acid anhydride by a reaction with tri-n-butylamine and isobutyl chloroformate and active ester is formed as a consequence of reaction with NHS and carbodiimides in NHS ester method. Hapten containing amine groups can be conjugated through a simple diazotization in which the hapten becomes activated to diazonium salts by reaction with sodium nitrite in acidic condition [41, 43].

1.2.3. Antibody production

Antibodies can be produced from all immunized vertebrates. To date, antibodies have been obtained from a variety of animals including goats, sheep, rabbits, mice and rat. Immunization involves three or four sequential injections (subcutaneously or intramuscularly) of immunogen emulsified with an adjuvant which stimulate nonspecific immune response. The most commonly used adjuvant for antibody production is Freund's adjuvant. In general, stimulation of immune response is initiated by injecting immunogen mixed with the complete Freund's adjuvant, followed by subsequent 2 or 3 boost injections which consists immunogen emulsified with incomplete Freund's adjuvant at 3-4 week intervals, which generates predominantly IgG subtype of antibodies.

Rabbits are the most popular species used to generate antisera while mice are the most frequently used for the generation of MAbs. An antiserum for a particular immunogen is a mixture of antibodies produced by various clones of B cells, so that antiserum is heterogeneous with various affinities and different analyte recognition

Limited amount of obtainable antiserum from individual animal and variation of antiserum from one animal to another eventually led to development of hybridoma technology which makes it possible to constantly supply identical antibodies. This technique for the production of MAbs was first developed in 1975 by Köhler and Milstein. In general, hybridoma technology involves the immunization of mice, fusion of B cells with myeloma cells for the B cell immortalization, selection of antibody producing hybridoma clone in selection medium and scale-up antibody production. In contrast to PAb production, MAb production is more difficult, laborious, time-consuming and expensive.

Conduction of checkerboard titration is useful to estimate the titer of antibody or to determine the optimum dilution of antibody /antigen for coating or competition in ELISA performance. Careful selection of antibody/antigen dilution significantly reduces background signal improving assay sensitivity. Checkerboard titration is simply performed on 96 well microplates immobilized with a serial dilution of hapten-protein conjugate. Addition of another serial dilution of primary antibodies allows them to differentially recognize immobilized conjugate. Bound antibodies are detected by adding constant dilution of secondary Ab conjugated with enzyme such as HRP to wells in terms of the strength of color reading.

1.2.4. Immunoassay formats

Direct competitive ELISA (dcELISA) and indirect competitive ELISA (icELISA) are the most prevalent types of ELISA for the analysis of small molecules. It has been reported that sensitivity of dcELISA is better than that of icELISA. However, activity of enzyme conjugated to hapten (enzyme tracer) is easily affected by solvents, which significantly deteriorate assay performance. Currently, dcELISA is preferred for the development of commercial ELISA kits due to its sensitivity and simple procedure. 96-well microplate is the most widely used for ELISA performance even though various solid-supports have been used for the immobilization.

The dcELISA is the simplest format where a constant amount of antibody is immobilized on the microplate usually by overnight incubation at 4°C or at room temperature. The analytes serially diluted in solution compete with the fixed amount of hapten labeled with an enzyme (HRP or AP) for binding to the immobilized antibody. Precoating of plates with protein A or excessive amount of anti primary antibody followed by immobilization of antibody specific to analyte allows binding sites of an antibody to be directed for better competition. Binding of enzyme tracer to antibody depends on the concentration of analytes involved in competition reaction. Once a chromogenic substrate is added, the color change caused by enzyme is measured with a microplate reader. A standard curve can be constructed by plotting absorbance in the function of analyte concentration. The intensity color is inversely proportional to the concentrations of analytes in the solution.

The indirect ELISA consists of immobilizing a hapten-protein conjugate on a microplate. For the competition step, a fixed amount of antibody solution is mixed with

various concentrations of analyte solution. As in the direct assay the analyte competes for binding to the antibodies with the coating conjugate immobilized on the plate. The amount of antibody bound to the coating conjugate is dependent on the amount of free analyte. Occupancy of primary antibody is measured by adding primary antibody specific secondary antibody conjugated with enzyme. A chromogenic substrate is added and the color change is measured spectrophotometrically. The increase in absorbance is again inversely proportional to the amount of herbicide in solution.

1.2.5. Optimization of assay conditions

The assay is optimized after a suitable antibody is identified for the hapten of interest. The user decides whether antigen or antibodies are immobilized on the plates. The 96 –well microtiter plates are suited for analyzing large numbers of samples and analytical procedures involving plates can easily be automated for use in robotic systems.

There are several physicochemical factors which influence assay sensitivity. Parameters that need to be considered for assay optimization include concentration of antigen or antibody for coating which is usually determined by performing checkerboard titration, time and temperature of incubation steps. Sample preparation is important and consideration must be given to the solvent used to extract the compounds of interest. In general, immunoassays give the best results when polar, water-miscible solvents are used. However, immunoassays are affected by the concentration solvent, buffer strength and pH [44-49].

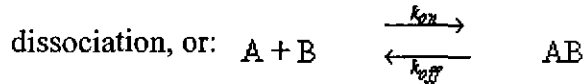
1.2.6. Real sample analysis

The major advantage of immunoassay is simple clean up of sample which is not applicable to conventional analysis. Extraction of sample with water-miscible solvent such as acetone, acetonitrile, or methanol and simple dilution of extract with appropriate buffer is routine procedure for sample preparation [50, 51]. Numerous assays have successfully analyzed a variety of compounds from various matrices. However, since unwanted interactions between co-extract and antibody cause false negative or positive effect which is called matrix effect, sometimes a simple additional cleanup step such as a solid-phase or solvent extraction step is required

1.3. KINETIC EXCLUSION ASSAY (KinExA)

The kinExA instrument (Sapidyne Instrument, Boise, ID), a fully automated flow fluorescent immunoassay system allows one to measure kinetic interactions between antigens and their antibodies. This instrument was designed to measure the amount of free antibody in a solution that contains antibody, antigen, and antibody-antigen complex in equilibrium state giving information about association rate constant (k_{on}) or dissociation rate constant (k_{off}). This can be achieved by quick transfer of a mixture of molecules to the flow cell in which beads coated with hapten-protein conjugate are packed. Minimization of time for transferring reaction mixture does not allow antibody-antigen complex dissociated. As a mixture is passing through the flow cell, antibodies with unoccupied binding site are bound to the immobilized hapten while antibody-antigen complex and analytes are not trapped. The test antibody attached to the beads exposed to excess fluorescent-labeled goat anti-mouse secondary antibody and then

unbound secondary antibody is washed away, and the fluorescence that remains on the beads is measured. When these steps are completed, the beads are automatically pumped out and replaced with a new aliquot for the next measurement. The instrument's fluorescence signal was related to antibody and ligand concentrations by classical binding equation. At equilibrium, the rate of AB complex formation is equal to the rate of



Where: k_{on} = forward rate constant and k_{off} = reverse rate constant

$[B]$ = receptor (antibody) binding site concentration

$[A]$ = ligand (antigen) binding site concentration

$[AB]$ = concentration of complex

At equilibrium,

$$(1) k_{on}[B][A] = k_{off}[AB]$$

The equilibrium dissociation constant, K_d , is defined as k_{off}/k_{on} , therefore:

$$(2) [AB] = \frac{1}{K_d} [B][A]$$

Where: $K_d = k_{off}/k_{on}$. From conservation of mass:

$$(3) [A_0] = [A] + [AB] \text{ Where: } [A_0] = \text{Total ligand (antigen) binding site concentration}$$

$$(4) [B_0] = [B] + [AB] \text{ Where: } [B_0] = \text{Total receptor (antibody) binding site concentration}$$

Using equations (3) and (4) to express equation (2) in terms of $[B_0]$, $[B]$, K_d , and $[A_0]$, and rearranging yields:

$$(5) \frac{1}{K_d} [B]^2 + \left(1 + \frac{[A_0]}{K_d} - \frac{[B_0]}{K_d} \right) [B] - [B_0] = 0$$

The physically realizable solution to the quadratic equation is the positive root:

$$(6) \quad [B] = \frac{-\left(1 + \frac{[A_0]}{K_d} - \frac{[B_0]}{K_d}\right) + \sqrt{\left(1 + \frac{[A_0]}{K_d} - \frac{[B_0]}{K_d}\right)^2 - 4\left(\frac{1}{K_d}\right)(-[B_0])}}{2\left(\frac{1}{K_d}\right)}$$

Which simplifies to:

(7)

$$[B] = \frac{1}{2} \left\{ ([B_0] - K_d - [A_0]) + \sqrt{[B_0]^2 + 2[B_0]K_d - 2[B_0][A_0] + K_d^2 + 2[A_0]K_d + [A_0]^2} \right\}$$

The instrument signal is linearly related to [B] and therefore follows equation (8):

$$(8) \text{ Where: } \text{Signal} = \left(\text{Sig}_{100\%} - \text{Sig}_{0\%} \right) \frac{[B]}{[B_0]} + \text{Sig}_{0\%}$$

Signal = instrument signal for [B]

Sig100% = signal from [B0]

Sig0% = signal from [B] = 0

(non-specific binding or NSB)

Solving equation (8) for [B], substituting [B] into equation (7), and solving for signal

gives:

(9)

$$\text{Signal} = \frac{\text{Sig}_{100\%} - \text{Sig}_{0\%}}{2[B_0]} \left\{ ([B_0] - K_d - [A_0]) + \sqrt{[B_0]^2 + 2[B_0]K_d - 2[B_0][A_0] + K_d^2 + 2[A_0]K_d + [A_0]^2} \right\} + \text{Sig}_{0\%}$$

Equation (9) gives the instrument signal as a function of [A0], [B0], Kd, Sig100%, Sig0%,

and is the basic equation used in the equilibrium analysis files.

Experimental data are in the form of instrument signals measured at various [A0]'s. The KinExA data analysis package uses equation (9) to determine theoretical signals for each [A0], and then calculates the error between the theoretical signal and the corresponding instrument signal. An iterative least squares approach is used to find an optimal solution for the unknowns, [B0], Kd, Sig0%, and Sig100%. The optimal solution values are displayed and used to create a theoretical signal vs. [A0] curve which is plotted with the experimental data.

The 95% confidence interval for the calculated Kd value is determined by varying the Kd parameter and re-optimizing the remaining variables at each point. The 95% confidence interval for [B0] is determined by varying the [B0] value and re-optimizing the remaining variables at each point.

This assay has been used to determine kinetic interaction of MAbs to PCB coplanar congeners [52] and metal-chelate complexes [53] and to detect benzoylecgonine in untreated urine [54], metal ions [55, 56], and 2,4-dinitrophenol [57]. Ohmura et al. modified KinExA procedures to extend dynamic range and detect multiple analytes [58].

1.4. NEONICOTINOIDS

1.4.1. Overview

In the world-wide insecticide market, three chemical classes, organophosphates, carbamates and synthetic pyrethroids, have been dominated for the last several decades. In the last few years a number of insecticides of new chemistries have entered the market place. Among these, the neonicotinoids have established themselves world-wide as key

components in insect control programs because of their unique chemical and biological properties, such as broad-spectrum insecticidal activity, low application rates, excellent uptake and translocation in plants, new mode of action and favorable safety profile [59]. The neonicotinoids are a new class of insecticides acting as agonists of the postsynaptic nAChRs. Unlike organophosphate and carbamates which inhibit unselectively acetylcholine esterases the neonicotinoids have low mammalian toxicity due to their 100-fold or more selectivity for insect nAChRs than for vertebrate nAChRs. They are derived synthetically from nithiazine and the natural nicotine. Seven neonicotinoid insecticides have been developed and commercialized by several companies (Figure 1.1). Among them, imidacloprid, acetamiprid and thiamethoxam are representatives of two classes of neonicotinoid insecticides. Imidacloprid and acetamiprid are chloropyridinyl derivatives, and thiamethoxam is a chlorothiazolyl derivative.

Imidacloprid is the first generation of neonicotinoids developed by Bayer in 1991 and registered as an insecticide in U.S in 1994. Thiamethoxam is a second-generation neonicotinoid discovered and developed by Syngenta Crop Protection and has been marketed under several trademarks. Thiamethoxam has showed the similar toxic effects to imidacloprid. Imidacloprid and thiamethoxam have been widely used as soil, seed and foliar application for control of sucking and chewing insects including rice hopper, aphid, thrips, whiteflies, termites, turf insects, soil insects and some beetles [60-62]. Imidacloprid and thiamethoxam are especially systemic when used as a seed or soil treatment. Control of viruliferous insects such as aphid or whitefly effectively prevents transmission of viruses including barley yellow dwarf virus, tomato spotted wilt

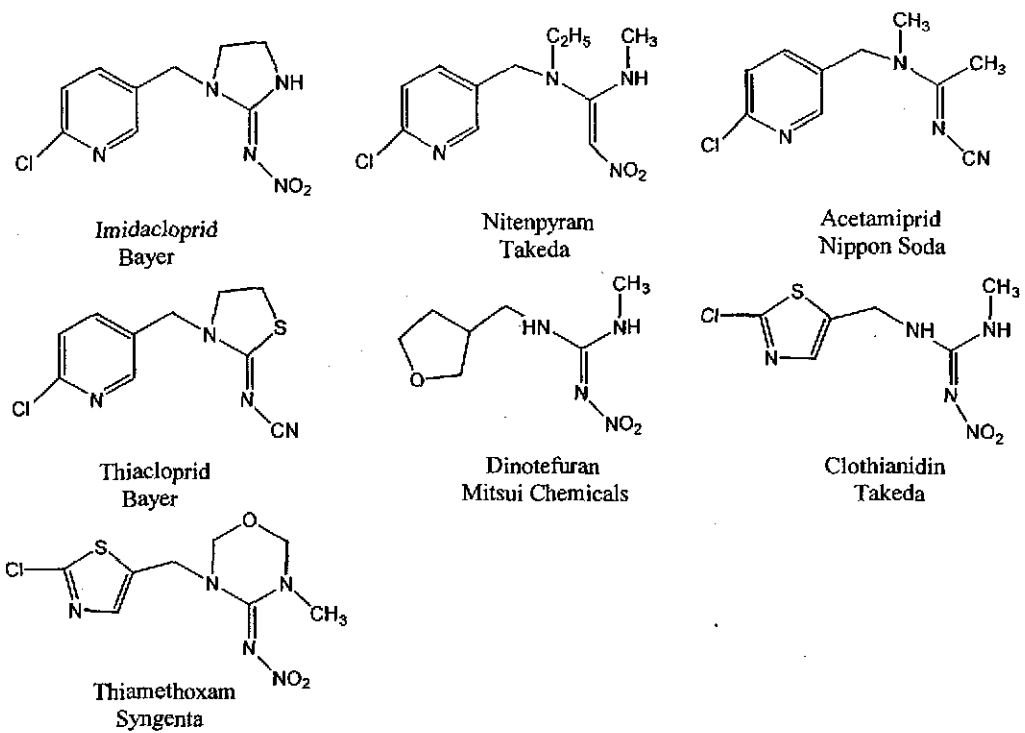


Figure 1.1. Structures of the neonicotinoid insecticides and the discovery companies.

tospovirus, or tomato yellow leaf curl geminivirus by potent insecticidal activity [63-66]. However, seed treatment may cause phytotoxic effect. It has been reported that imidacloprid inhibits insect feeding and impairs insect mobility. Nauen suggested that the death of insects is resulted from anti-feedant effect of imidacloprid based on the observation that apterous aphids placed on cabbage leaves systemically treated with low concentrations of imidacloprid showed nearly the same decrease in weight as untreated starving aphids [67]. Antifeedant effect and impaired larval mobility was also observed from 1st instars of *Diaprepes abbreviatus* L, termite, and flea beetle when they were treated with imidacloprid at sublethal level. However, imidacloprid did not cause antifeedant effect in fourth-insta larvae of tobacco budworm [68, 69]

Imidacloprid and other insecticides in the neonicotinoid chemical family are similar and modeled after the natural nicotine. Because of their molecular shape, size, and charge, nicotine and neonicotinoids fit into receptor molecules in the nervous system that normally receive the molecule acetylcholine which carries nerve impulses from one nerve cell to another or from a nerve cell to the tissue that a nerve controls. Imidacloprid and other neonicotinoids irreversibly block acetylcholine receptors.

1.4.2. Degradation of imidacloprid and thiamethoxam

Systemic effect of seed and soil treatment is well known properties of neonicotinoid insecticides. When sugar beet seeds were treated with [¹⁴C] imidacloprid, 45% and 5% of total applied radio activity, corresponding to metabolites and parent compound, respectively, was detected 97 days after planting while 0.1 % and 27% of total radio activity was detected from root and soil respectively, suggesting imidacloprid parent

compound and its metabolites systemically move toward leaves as plants grow [70]. Half-life and metabolism of imidacloprid in applied plants are dependent on type of treatment and plant species. Imidacloprid shows half-life of 3-5 days in eggplant, cabbage and mustard, and imidacloprid urea and 6-chloronicotinic acid were detected as major metabolites in all three plants. Some of imidacloprid metabolites are as toxic as imidacloprid. One of major metabolites of imidacloprid is imidacloprid olefin which has 16 times higher toxicity to aphid and whitefly than its parent compound. The imidazoline derivative of imidacloprid shows potent aphicidal activity and two hydroxyl metabolites are highly toxic to whitefly. The toxicity of imidacloprid metabolites is different from one insect to another. Thus, long-lasting effect of imidacloprid in plant can be explained from the fact that imidacloprid and its some metabolites have continuous toxicity as plants grow [71-73].

The principal degradative pathways of thiamethoxam are photodegradation in water with half life of 2.3-3.1 days, hydrolysis under alkaline condition with half life of 4.2-8.4 days and microbial degradation under aerobic aquatic conditions with half life of 8.3-16.2 days [74].

Imidacloprid and thiamethoxam are stable in acidic and neutral pH (half-life, > 1 year at room temperature). However they are rapidly hydrolyzed in alkaline condition. For imidacloprid, hydrolysis is initiated by attacking of OH⁻ ion to positively charged carbon of C=N group of imidazolidine ring of IMI so that =N-NO₂ is converted to =O producing 1-[6-chloro-3-pyridinyl]methyl]-2-imidazolidone as major metabolite [75].

Photodegradation of imidacloprid under UV or natural sun light occurs rapidly in aqueous solution and on the surface of plant leaves with a half life ranged from 43 min to

1.4 day depending on light intensity, which render foliar application of imidacloprid and thiamethoxam less systemic. The major metabolites include 6-chloro-nicotinaldehyde, N-methylnicotinacidamide, 1-6-chloronicotinyll imidazolidone and 6-chloro-3-pyridyl-methylethylendiamine [76-78].

1.4.3. Mode of action

Neonicotinoid exerts toxic effect by disrupting neurotransmission through the nicotinic cholinergic synapse. Neurotransmission is mediated by acetylcholine (ACh) which is the endogenous agonist and excitatory neurotransmitter of the cholinergic nervous system. ACh released from presynaptic membrane binds the site located at the extracellular domain of the nAChR/ion channel complex, causing disruption of the equilibrium status of the membrane potential. nAChRs are the primary target molecules for neonicotinoids insecticides. In insects, nAChRs are confined to the nervous system. nAChRs are transmembrane, ligand-gated cation channels composed of combination of five subunits which are encoded by the nAChR subunit gene family. Those subunits are classified into two subfamilies on the basis of their sequence homology with muscle nAChR. The α -type subunits have a substantial homology with the α subunit of muscle nAChR whereas β subunits or simply called non- α subunits do not show sequence homology with β subunit of muscle nAChR [79]. To date, several laboratories around the world have cloned and sequenced a series of insect α and non α subunits as an attempt to elucidate pharmacological profiles of insect nAChRs.

Early electrophysiological studies on the mode of action of nitromethylenes using the nervous system of the cockroach (*Periplaneta americana*), housefly (*Musca domestica*)

and locust (*Schistocerca gregaria*) and *in vivo* symptomology revealed that nitromethylene heterocycles effectively block nerve impulse conduction at nAChR as a selective agonist for insect nAChRs [80-84]. The conclusive evidence in molecular basis for the action of nitromethylenes on insect nAChRs was shown by expressing functional homooligomer of locust (*Schistocerca gregaria*) α subunit (α L1) of nAChR in *Xenopus* oocytes. Expressed oligomeric complex was activated by nicotine and blocked by α -bungarotoxin and depolarized by nitromethylene, which support that nAChR is the primary sites of nitromethylene binding and binding site reside on α subunit of insect nAChR [85].

Radioligand binding and electrophysiological studies have revealed that imidacloprid also acts on postsynaptic nAChRs as an agonist and has selective toxicity on insects over mammals. Potency of imidacloprid for displacing [¹²⁵I] α -bungarotoxin bound to cockroach motor neuron and inhibiting [³H] imidacloprid binding to housefly head nAChR indicates that neonicotinoids primary act on insect nAChRs. In addition, imidacloprid effectively depolarizes cell body of motor neuron but shows no antagonist actions. Imidacloprid shows high affinity to head membranes of various insect species but with only low affinity to mammalian brain membrane, which reveal that favorable selective toxicity of imidacloprid to insect versus mammals is attributed to difference in their binding affinity to nAChRs [86, 87]. Electrophysiological studies using cockroach (*Periplaneta Americana*) neurons showed that imidacloprid behaved largely as an agonist on at least two distinct populations of nAChRs that are sensitive or insensitive to α -bungarotoxin, respectively [88].

Studies on binding of imidacloprid, nACh, and nicotine to nAChR revealed that those compound bind to the same subsite of insect nAChR, which led to attempts to elucidate binding mechanism on structural basis. Kagabu and Matsuno carried out X-ray crystal analysis of imidacloprid and analogous compound and showed the distance between the nitrogen atom of the 3-pyridinylmethylamine moiety and the 1-nitron (N1) of the imidazolidine ring is 5.45-6.06 Å which coincides with the distance between the ammonium nitrogen and carboxyl oxygen of ACh, between the nitrogen atoms of nicotine, and between oxygen atom of nitro group and N1 nitrogen [89]. N1 nitrogen plays an important role in binding of imidacloprid to nAChR. N1 atom with partial positive charge resulted from electron-withdrawing nitro group interacts with electron rich subsite of nAChR in which resides with aromatic ring such as cystein or lysine are involved while nitrogen on 3-pyridinyl ring or oxygen of nitro group form hydrogen bonding with H donor. The strength in binding is correlated with the degree of positive charge of N1. However, compound with stronger positive charge are less effective in tissue penetration because penetration is dependent on hydrophobicity of compound [90-92].

1.4.4. Analysis of neonicotinoids

Several GC-MS methods have been developed for the analysis of imidacloprid and its metabolites from various matrices even though the fact that imidacloprid is highly polar and thermolabile makes it difficult to analyze these compounds with GC or GC-MS. Table 1.1 summarizes the methods for the analysis of neonicotinoids. Since the injection of imidacloprid under normal GC conditions gives a chromatogram of complex fragment

peaks, for the utilization of GC or GC-MS, neonicotinoids should be derivatized into more volatile ones prior to analysis.

Valverde-Garcia *et al.* attempted to use supercritical fluid extraction (SFE) method to extract imidacloprid and other six compounds from water containing samples using water absorbent and GC for the determination of residue. Although this extraction method showed good recoveries for other compounds (>80%), the recovery for imidacloprid at various extraction conditions was zero, which did not allow them to perform GC analysis. However, Eskilsson and Mathiasson effectively extracted imidacloprid in dust waste through careful selection of modifier condition. They added 5% of methanol as a modifier into supercritical carbon dioxide and showed 97% of recovery [93, 94].

Vilchez *et al.* first developed GC-MS method for the determination of imidacloprid in water and soil samples where imidacloprid extract was transformed into a volatile compound (1-(6-chloro-3-pyridylmethyl)-imidazolidin-2-one) by hydrolysis in a basic medium. The LOD was 0.16 ppb for water and 1.0 ppb for soil samples [95].

MacDonald and Meyer extended the applicability of GC-MS method to more complex matrices, white pine foliage. They analyzed imidacloprid and two tritazole fungicides simultaneously at low LOD value. Sample preparation in this method involves the use of SPE cleanup and conversion of imidacloprid extract to the heptafluorobutyryl derivative [96].

Uroz *et al.* developed a new sensitive GC-MS-MS method to monitor 6-chloronicotinic acid which is a major metabolite of imidacloprid. This metabolite may act as an important biomarker to estimate the human exposure to imidacloprid. They

extracted imidacloprid from human urine using SPE and analyzed the derivative of 6-chloronicotinic acid [97].

That imidacloprid is metabolized into several compounds such as 5-hydroxy-, olefin-, and desnitro-forms led to the development of new GC-MS method for the determination of total residues in plant by Lodevico and Li [98]. They extracted imidacloprid and its metabolites and oxidized them into 6-chloronicotinic acid, followed by derivatization and determination. Although this method allows the determination of total residues in coffee sample, it is not possible to determine the concentration of imidacloprid or a particular metabolite present in sample.

In contrast to GC or GC-MS, HPLC could be a suitable method for the determination of imidacloprid due to strong absorbance at 270 nm. HPLC-UV or diode array detector and LC-MS have been used for the determination of neonicotinoids and metabolites. However, these methods of which sensitivity of UV or diode array detectors relatively low require a sequence of cleanup steps that are tedious and laborious and requires high solvent consumption.

Isihii *et al.* first reported HPLC-UV method for the determination of imidacloprid in various corps and rice straw and validated this method by monitoring disappearance of imidacloprid in cucumber plant [99]. Fernandez-Alba *et al.* developed another HPLC-DAD method to determine imidacloprid in vegetables [100]. Two methods consisted of extracting with appropriate solvent, partition with dichloromethane, SPE or CC clean-up, and finally HPLC analysis. Overall LOD and recoveries of two methods were 0.01 ppm and 75-109%, respectively. For the analysis of imidacloprid in water or soil which contain relative less interference than plant samples, a sequence of clean-up steps can be

simplified. Baskaran *et al.* showed the use of SPE bypassing partition for sample cleanup and HPLC-UV gave a satisfactory low LOD value (0.05 ppm) [101].

Two laboratories further extended applicability of HPLC-DAD method to the simultaneous determination of imidacloprid and its major metabolite, 6-chloronichotinic acid in grounder water and greenhouse air. However, the feasibility of these methods was validated with only spiked samples [101, 102]. Garrido *et al.* showed that the combination of advanced computational capability with the HPLC-DAD is a powerful tool to improve resolution of overlapping peaks of two analytes.

Obana *et al.* developed HPLC-DAD method for the simultaneous determination of three neonicotinoids (imidacloprid, acetamiprid and nitenpyram), which involves a sequential passage of extract through a weak anion-exchange and silica gel cartridge [103].

Use of LC-MS could be an alternative to HPLC in terms of performing multiresidue analysis with simple clean-up from food samples and monitoring the fate of imidacloprid in the environment [104, 105].

1.4.5. Neonicotinoid immunoassays

Polyclonal or monoclonal antibodies were produced against imidacloprid (Table 1.2). Li and Li reported two ELISAs, one for imidacloprid and the other for imidacloprid and its metabolites with two sets of polyclonal antibodies raised with two different haptens [106]. These compound and class specific ELISAs showed I_{50} of 35 and 73-88 ppb, respectively. Use of antibody with broad specificity is useful due to the fact that

Table 1.1. Analysis of imidacloprid with conventional methods

Analyte(s)	Matrices	Sample preparation	Analysis	LOD (ppb)	Reference
imidacloprid	water/soil	hydrolysis/LLE ^a	GC-MS (SIM)	0.16/1.0	Vilchez <i>et al.</i> , 1996
imidacloprid	white pine	derivatization/SPE ^b	GC-MS	0.01	MacDonald and Meyer. 1998
6-chloronicotinic acid	human urine	derivatization/SPE	GC-MS-MS	0.016	Uroz <i>et al.</i> , 2001
imidacloprid and metabolites	coffee	oxidization/derivatization	GC-MS		Lodevico and Li. 2002
imidacloprid	crops/rice straw/soil	LLE/CC ^c	HPLC-UV	0.005/0.01 0.02	Ishii <i>et al.</i> , 1994
imidacloprid	vegetables	LLE/SPE	HPLC-DAD	0.01	Fernandez-Alba <i>et al.</i> , 1996
imidacloprid	water/soil	SPE	HPLC-UV	0.005	Baskaran <i>et al.</i> , 1997
imidacloprid 6-chloronicotinic acid	water	LLE	HPLC-DAD		Galera <i>et al.</i> , 1998

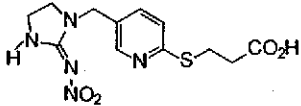
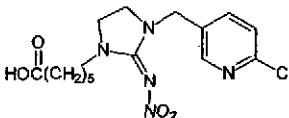
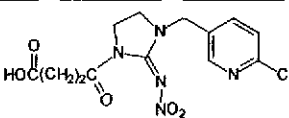
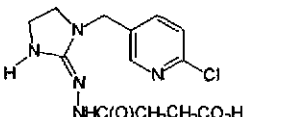
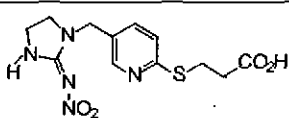
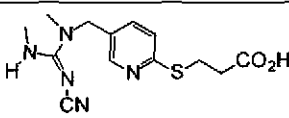
imidacloprid 6-chloronicotinic acid	air	amberlite/ soxhlet	HPLC-DAD	0.08	Frenich <i>et al.</i> , 2000
acetamiprid imidacloprid nitenpyram	vegetables	two SPEs	HPLC-DAD	0.001 0.01 0.02	Obana <i>et al.</i> , 2002
imidacloprid	fruits vegetables		LC-MS -SIM LC-MS-SCAN	0.001 0.01	Fernández-Alba <i>et al.</i> , 2000
imidacloprid	peaches /nectarines		LC-MS	0.02	Blasco <i>et al.</i> , 2002
imidacloprid	soil/ plant/ pollen	SPE	LC-APCI-MS- MS	0.0001	Bonmatin <i>et al.</i> , 2003

^aLLE=liquid-liquid extraction; ^bSPE=solid phase extraction; ^cCC=column chromatography

metabolites of imidacloprid have nearly equal toxicity to imidacloprid. Lee *et al.* made an attempt to elicit two sets of polyclonal antibodies with two different haptens [107].

Although they developed a sensitive ELISA with one hapten, they failed to produce antibodies against another hapten which the nitro group was replaced with a linker, suggesting that nitro group on imidazolidinyl ring is important to elicit compound specific antibody. Wanatabe *et al.* developed another monoclonal antibody-based dcELISA for acetamiprid. The I_{50} and cross reactivity to other neonicotinoids and structurally related compounds were 1 ppb and < 0.5, respectively [108].

Table 1.2. ELISAs for the neonicotinoid insecticides imidacloprid, acetamiprid and thiamethoxam

Target chemical	Hapten structure	Antibody type	Assay format ^b	I ₅₀	CR, ^c %	Reference
		PAb ^a	icELISA	138 nM (35 ppb)	≤ 16%	Li and Li 2000 [106]
		PAb	icELISA	287 nM (73 ppb)	≤ 86%	Li and Li, 2000 [106]
Imidacloprid		PAb	icELISA	68 nM (17 ppb)		Lee <i>et al.</i> , 2001 [107]
		PAb		NR ^d		Lee <i>et al.</i> , 2001 [107]
		PAb	dcELISA	13 nM (3 ppb)	< 0.5%	Wanatabe <i>et al.</i> , 2001 [108]
Acetamiprid		MAB ^b	dcELISA	4 nM (1 ppb)	< 0.5%	Wanatabe <i>et al.</i> , 2001 [108]

^aPAb= Polyclonal antibody; MAb = monoclonal antibody

^b icELISA = indirect competitive ELISA; dcELISA = direct competitive ELISA.

^c CR = Cross reactivity.

^d NR = no recognition.

1.5. WORKING HYPOTHESES AND OBJECTIVE

As described in the introduction, an overall objective of this research is to develop ELISAs for the neonicotinoid insecticides imidacloprid and thiamethoxam using antisera and monoclonal antibodies. For this purpose, there are the following working hypotheses:

- 1) Is it possible to improve an assay sensitivity utilizing different assay formats?
- 2) Can we explain correlation between structure of analyte and antibody binding with computer modeling?
- 3) Can we design hapten for the production of compound specific antibody based on computer modeling and develop compound specific ELISAs?
- 4) Can we determine kinetic of antibody with kinetic exclusion assay (KinExA)?

To test these hypotheses,

- Aim one is to purify antiserum, optimize assay conditions and compare assay sensitivity between icELISA and dcELISA.
- Aim two is to includes designing and synthesizing hapten according to the computer modeling result, producing antibody, and developing ELISA
- Aim three is to study the kinetic properties between imidacloprid antibody and several neonicotinoids.

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Chapter 2

Improved Enzyme-linked Immunosorbent Assay for the Insecticide Imidacloprid

ABSTRACT

Imidacloprid, 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine, is a systemic insecticide used worldwide. The sensitivity of an enzyme-linked immunosorbent assay (ELISA) for imidacloprid was considerably improved with a purified polyclonal antibody (designated as Ab-IIa) and a direct competitive assay format. The assay conditions were optimized for buffer concentrations and pH, solvents, and Tween 20 concentrations. Under the optimized conditions, the half-maximal inhibition concentration and the limit of detection were approximately 1 $\mu\text{g/L}$ and 0.06 $\mu\text{g/L}$, respectively. This means a 35-fold improvement in the assay detectability compared with the assay previously reported (Li and Li, *J. Agric. Food Chem.*, 2000, 48:3378-3382). The assay was very specific to imidacloprid and showed little cross-reactivity with other structural analogs. Computational analysis suggests that the antibody specificity primarily relate to the dihedral angles between the two rings, steric hindrance and electrostatic charges on the imidazolidinyl ring. Such information is useful for hapten design. The ELISA analysis of water samples fortified with imidacloprid showed a satisfactory correlation with the fortified levels. This assay can be a rapid and sensitive method for monitoring imidacloprid residues in the environment.

2.1. INTRODUCTION

Imidacloprid, 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine, is a systemic insecticide for effective control of sucking insects. Imidacloprid blocks the nicotinic receptor in insects, causing death (1-3). Current methods for the analysis of imidacloprid residues in environmental and biological matrices include high performance liquid chromatography (HPLC), gas chromatography (GC), HPLC-mass spectrometry (HPLC-MS) and GC-MS. Imidacloprid is thermolabile and polar, and thus derivatization is required for GC and GC-MS determinations (4-11). Immunoassays are valuable methods for environmental monitoring of pesticides (12-13). Polyclonal antibody-based indirect competitive ELISAs (cELISAs) were reported for the analysis of imidacloprid (14). One assay is specific to imidacloprid with a half-maximal inhibition concentration (I_{50}) of 35 ng/mL. Another can measure both imidacloprid and its major metabolites although the limit of detection is relatively high. Monoclonal antibody-based ELISAs were also recently developed for the chloronicotinoid insecticides imidacloprid and acetamiprid (15). In this study, several assay parameters were optimized to improve the assay sensitivity and analysis time. Computer modeling of imidacloprid and its structural analogs was used to aid understanding of the antibody specificity.

2.2. MATERIALS AND METHODS

2.2.1 Reagents

All reagents were of analytical grade unless specified otherwise. Imidacloprid (96.9% purity) and its metabolite standards which are imidacloprid olefin (99%), 5-hydroxyimidacloprid (100%) and 6-chloronicotinic acid (99%) were obtained from Bayer

Corp, Stillwell, KS. Chemicals purchased from Sigma (St. Louis, MO) were goat anti-rabbit IgG-horse radishperoxidase (IgG-HRP) (A-6154), ovalbumin (OVA) (A-5503), protein A (P-6031), HRP (P-6782), phosphate-citrate buffer capsules with sodium perborate (P-4922) and carbonate-bicarbonate buffer capsules (C-3401), and o-phenylenediamine (OPD) (P-9029). The ELISAs were carried out in 96-well polystyrene microplates (MaxiSorp F96, Nalge Nunc International, Denmark). The haptens, hapten-II-OVA and hapten-II-HRP conjugates were synthesized according to the procedure previously described (14). The antiserum (designated as Ab-IIa), previously described (14), was purified with an immunopure IgG purification kit (Pierce, Rockford, IL) according to the manufacture's instruction. Concentration of IgG in the final preparation was determined with a BSA assay (Pierce, Rockford, IL). The purified IgG in 0.02 M of phosphate-buffered saline (PBS) was stored at -20°C .

2.2.2. Synthesis of 1-[(6-chloro-3-pyridinyl)methyl]-3-carboxypentyl-N-nitro-2-imidazolidinimine (hapten I).

To a stirred solution of imidacloprid (1.03 g, 4 mmol) in dimethyl formamide (DMF, 15 mL) was added sodium hydride (0.21 g of 50% oil dispersion, 4.3 mmol) at 0°C . After 1 h, ethyl-6-bromohexanoate (2.23 g, 10 mmol) was added dropwise, and stirring was continued for 12 h at ambient temperature. The reaction mixture was poured into 80 mL of water and pH of the solution was adjusted to 6-7. The mixture was extracted with chloroform (2 x 80 mL) and the organic layer was washed with water and dried over anhydrous sodium sulfate. After the solvent was removed by a rotary evaporator under reduced pressure, the crude product was hydrolyzed in acidic aqueous solution (2 N HCl,

60-70 °C, 4 h). After the reaction mixture was adjusted to pH 6 using 1 N NaOH solution, it was extracted with ethyl acetate (2 x 50 mL). The solvent was removed with a rotary evaporator and the residue was purified with silica gel chromatography (ethyl acetate : methanol, 9:1). Hapten I was obtained as white solid (yield 35%). ¹H NMR (CDCl₃): δ 2.34 (t, J=7.6, 2H), 3.30 (t, J=7.3, 2H), 3.52 (t, J=8.8, 2H), 3.71 (t, J=7.3, 2H), 4.47 (s, 2H), 7.36 (d, J=8.0, 1H), 7.71 (dd, J=2.4, 8.3, 1H), 8.31 (d, J=2.2, 1H). LRMS (FAB) m/z: 370 (MH⁺, 100). HRMS (FAB) calcd C₁₅H₂₀N₅O₄ClH⁺ 370.1282, found 370.1281.

2.2.3. Synthesis of 1-[(6-carboxylethylthio-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine (hapten II).

To a solution of imidacloprid (1.03 g, 4 mmol) in dimethyl sulfoxide (DMSO, 15 mL) was added dropwise a DMSO solution (5 mL) containing 0.42 g of 3-mercaptopropionic acid (4 mmol) and 0.45 g of KOH (8 mmol). The reaction mixture was stirred while the temperature was gradually increased to 100 °C, and the temperature was maintained for 1 h. Water (50 mL) was added after the reaction mixture was cooled to room temperature. The solution was adjusted to pH 3 using 6 N HCl and extracted with ethyl acetate (100 mL). The organic extract was washed with water and dried over anhydrous sodium sulfate, then ethyl acetate was evaporated with a rotary evaporator under reduced pressure. The residue was purified with silica gel chromatography (ethyl acetate : methanol, 9:1). Hapten II was obtained as white solid (yield 45%). ¹H NMR (CDCl₃): δ 2.86 (t, J=7.1, 2H), 3.41 (t, J=7.0, 2H), 3.52 (t, J=8.9, 2H), 3.80 (t, J=8.0, 2H), 4.50 (s, 2H), 7.20 (d, J=8.8, 1H), 7.53 (dd, J=2.2, 8.3, 1H), 8.34 (d, J=2.2, 1H).

2.2.4. Direct competitive ELISA

A microtiter plate was coated with protein A (0.1 μg in 100 μL /well in 0.1 M carbonate-bicarbonate buffer, pH 9.6) overnight at 4 $^{\circ}\text{C}$. The plate was washed with 0.02 M PBS containing 0.05% Tween 20 (PBST) for four times and subsequently coated with purified Ab-IIa (25 ng in 100 μL /well) for 12 h at 4 $^{\circ}\text{C}$. After washing for five times, the plate was blocked with 1% BSA in PBST (200 μL /well) for 1 h at room temperature. After the plate was washed with PBST (5x) again, 50 μL of the standard or sample at various concentrations in PBST were added, followed by addition of an enzyme tracer (1/16,000 dilution in PBST, 50 μL /well). The plate was incubated for 30 min at 37 $^{\circ}\text{C}$, then washed with PBST (5x). The substrate solution (1.0 mg/mL of OPD in 0.05 M citrate-phosphate with 0.03% sodium perborate, pH 5.0) was added (100 μL /well), and the enzymatic reaction was stopped with sulfuric acid (2 M, 50 μL /well) after 15-20 min at room temperature. The absorbance was read with a Vmax microplate reader at 490 nm (Molecular Devices, Sunnyvale, CA). Inhibition curves were analyzed by mathematical fitting of experimental data to a four-parameter logistic equation with Softmax software.

A microtiter plate was coated with protein A (0.1 μg in 100 μL /well in 0.1 M

2.2.5. Determination of Cross-Reactivity

The ability of the antibody Ab-IIa to recognize imidacloprid analogs was assessed by performing direct cELISAs to determine their respective I_{50} values. Cross-reactivity (CR) was calculated as $(I_{50} \text{ by imidacloprid} / I_{50} \text{ by a test compound}) \times 100$.

2.2.6. Molecular Modeling

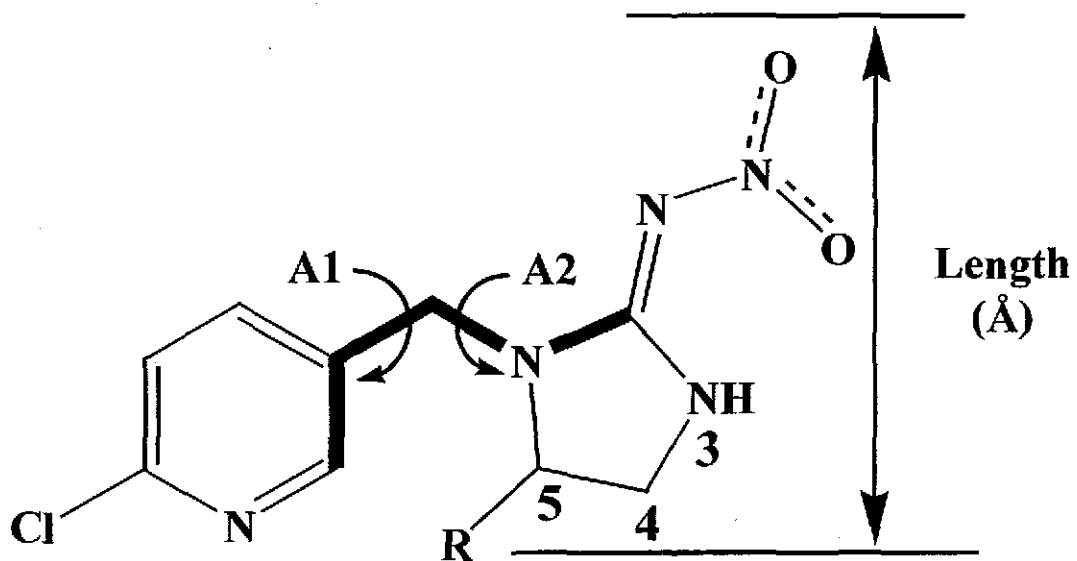


Figure 2.1. Length and dihedral angles of imidacloprid and its metabolites. Length is measured as the distance between the nitro oxygen and the far end atom of the R substituent. Dihedral angles (A-1 and A-2) are the angles between bold bonds.

A global energy minimum structure was searched with HyperChem 5.0 and HyperSpin, an add-on program (Hypercube Inc., Gainesville, FL) running on an IBM personal computer. After the initial optimization with MM+ force field, low energy conformations were searched by sequentially rotating dihedral angles through 360° in 10° increments (Figure 2.1). The lowest energy conformation was selected from several local energy minimum structures through the optimization by a semi-empirical PM3 force field which was also used to calculate partial charge of each atom (16).

Electrostatic potentials of the global energy minimum structure were calculated with the CAChe Worksystem (Fujitsu, Beaverton, OR) using the PM3 force field (Plate 1). The results were visualized by the CAChe Tabulator application. Octanol -water partition coefficients were calculated from atomic partial charges with the CNDO force field (17) using Molecular Modeling Pro (ChemSW Software Inc., N. Fairfield, CA).

2.2.7. Fortification of Imidacloprid in Water

The optimized ELISA was used to determine imidacloprid concentrations in tap water samples fortified with imidacloprid up to 40 ng/mL. Final ionic strengths, as buffer concentrations, of the samples were adjusted with four-fold concentrated PBST.

2.3. RESULTS AND DISCUSSION

Assay sensitivity and analysis time directly relate to assay formats and conditions. The objectives of this work were to improve the assay characteristics by optimizing assay conditions and using a direct cELISA, and to further confirm the high selectivity of Ab-

Ila to imidacloprid. Computer modeling was used to propose structural geometry and electrostatic potential surface of different test molecules. I_{50} values of different chemicals to Ab-Ila were correlated with their electrostatic potential and geometry, in order to understand possible contributors of hapten features to the high antibody specificity.

2.3.1. Effect of Assay Buffer Ionic Strength

Large effects of ionic strength on immunoassays were often observed for many polar and nonpolar molecules (18-22). The assays were run in PBST varying concentrations from 0.02 M to 0.4 M and in deionized water as a control. All buffers contained 0.05% Tween-20 and had a pH of 7.5. Competitive imidacloprid inhibition curves generated at various buffer concentrations are shown in Figure 2.2A. There was no significant change in I_{50} values (I_{50S}) as the buffer concentration (*i.e.*, ionic strength) increased. Ionic strength slightly altered the inhibition curves only at low levels (0.06-1 $\mu\text{g/L}$) of imidacloprid (Figure 2.2A). The imidacloprid inhibition in deionized water was higher than that in PBST. Maximum absorbance of the assay was slightly higher in 0.02 and 0.04 M PBST than that in 4-20-fold-concentrated buffers and deionized water (data not shown).

2.3.2. pH Effect

Because the antigen-antibody binding occurs through weakly intermolecular interactions (23), and pH derived alteration of an analyte may lead to the poor recognition by an antibody (24), the evaluation of pH effect on the assay is a necessary step.

Imidacloprid and the antibody were diluted in buffers of different pH values but with the same ionic strength. The buffer pH varying from 5.5 to 9.5 had negligible effect on inhibition curves (Figure 2.2B). The antibody activity was significantly reduced at pH 4.5. The assay signals (absorbance) also varied considerably as the pH was altered. Maximum signals were around pH 6.5-7.5. The results indicate that the assay is applicable in PBS with pH 5.5–9.5. However, it is noted that adjustment of buffer pH and concentrations (ionic strength) among samples is required to minimize errors due to their effect on absorbance. The buffer pH was kept at 7.0 for the subsequent work.

2.3.3. Effect of Tween 20

Tween 20 is a nonionic detergent commonly used in ELISAs to reduce non-specific interactions and often has strong effects on antibodies and assay characteristics (22, 25-26). In some circumstances, the assay detectability was considerably enhanced in detergent-free buffers (22, 25). Tween 20 (0.01-0.05%) showed little effect on this imidacloprid assay (Figure 2.2C). PBS containing 0.05% Tween-20 was used for the remainder of the study.

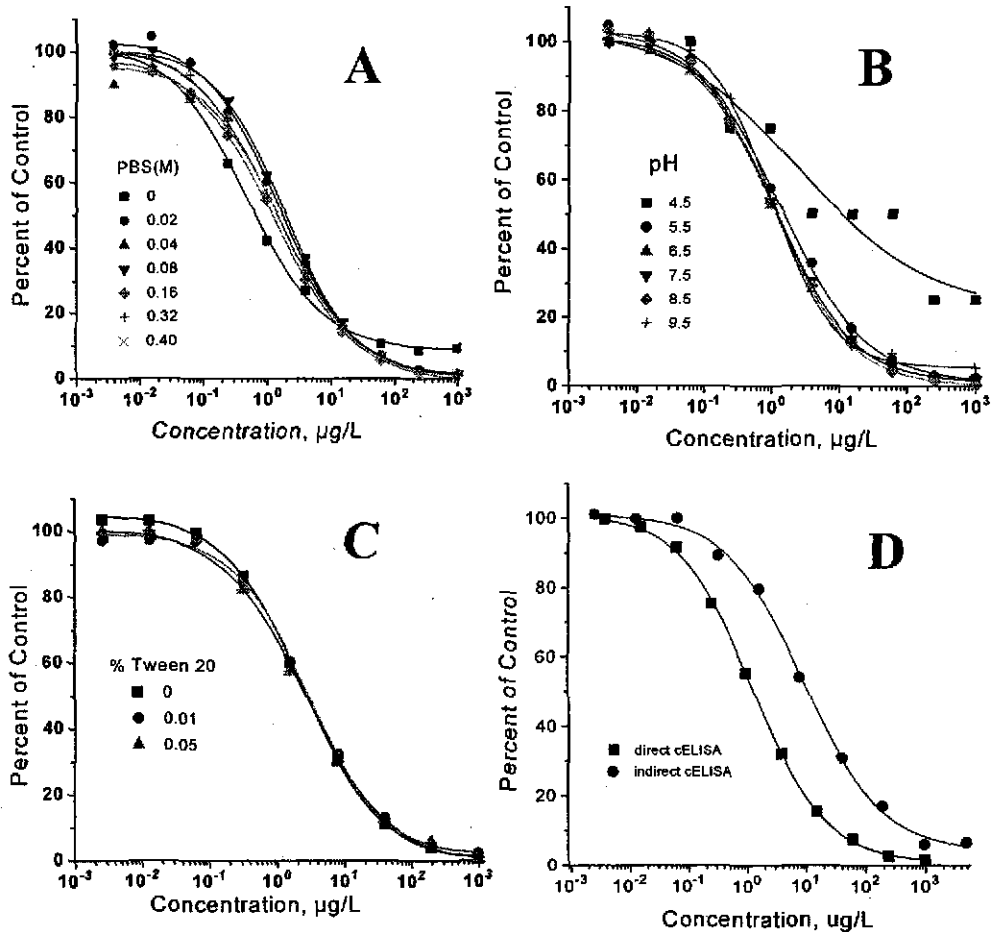


Figure 2.2. Effects of assay buffer concentration (A), pH (B) and Tween 20 (C) on the assay, and typical imidacloprid inhibition curves of direct and indirect cELISAs (D) at the optimized condition.

2.3.4. Typical Inhibition Curve

With the purified Ab-IIa, a direct cELISA format was employed to improve the assay characteristics since it was generally more sensitive than the indirect format. Figure 2.2D shows representative standard curves for imidacloprid obtained with direct and indirect cELISAs at the optimized assay conditions. The linear range of the direct and indirect cELISAs was approximately 0.15-15 $\mu\text{g/L}$ and 1-200 of imidacloprid, respectively. Concentrations giving 50% and 20% of inhibition with the direct cELISA were approximately 1.1 and 0.15 $\mu\text{g/L}$ of imidacloprid, respectively, improving the sensitivity by over 35-fold compared with that of an indirect cELISA previously reported [14]. Indirect cELISAs were tested with the purified Ab-IIa and hapten-II-OVA as a coating antigen to estimate the contribution of antibody purification to the assay improvement. The indirect cELISA gave competitive curves with an I_{50} of approximately 10 $\mu\text{g/L}$, which indicated that the antibody purification improved the assay sensitivity by 3- to 4-fold (14, and Figure 2.2D) when the same assay format and coating antigen were used. Antibody purification also reduced the assay background signal.

2.3.5. Solvent Effect

Antibody tolerance to solvents has been studied in many immunoassays (27-31). High solvent-tolerable ELISAs are very convenient and desirable for applications where sample extractions involve use of organic solvents. The effects of methanol (MeOH), acetone, acetonitrile, and dimethyl sulfoxide (DMSO) on the optimized assay were studied because they are commonly used in ELISA procedures. The solvent concentrations in the final assay varied from 0% to 40% in 0.02 M PBST. In general, all

the solvents affected the assay signals. However, MeOH and DMSO have much smaller effects on the inhibition curves (shape and position) than acetone and acetonitrile (Figure 2.3).

2.3.6. Cross-Reactivity

Antibodies intrinsically recognize compounds structurally similar to the immunizing hapten (23). Five structural analogs of imidacloprid were examined for their cross-reactivity with the antibodies (Table 2.1, Plate 1). Antiserum Ab-IIa was specific to imidacloprid and had low cross-reactivity ($\leq 9\%$) for the metabolites and hapten-I (Table 2.1). Ab-IIa highly recognized hapten-II, the immunizing hapten. It should be mentioned that Ab-IIa showed no recognition with other imidacloprid metabolites such as imidacloprid-guanidine and imidacloprid-urea, however, their purity was not confirmed (data not shown). The antibody specificity obtained with this direct cELISA agreed with that obtained with an indirect cELISA using purified Ab-IIa in this study (data not shown) and that previously determined with the indirect cELISA using the crude antiserum Ab-IIa (14).

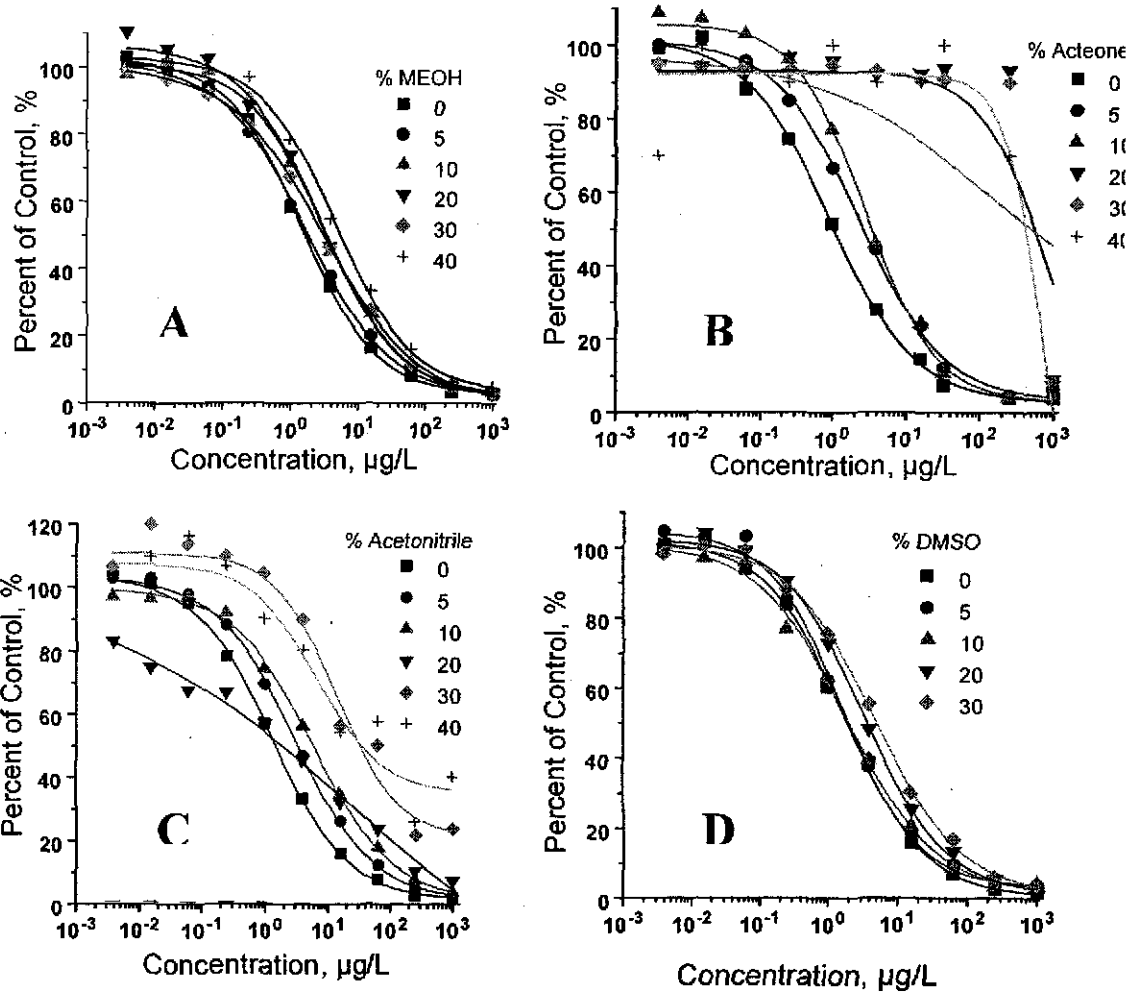


Figure 2.3. Effects of methanol (A), acetone (B), acetonitrile (C) and DMSO (D) on the assay.

Table 2.1 Comparison of Assay Cross-Reactivity and Length and Dihedral Angles of Imidacloprid and Its Structural Analogs

Chemical	I ₅₀ , ng/mL	CR, ^a %	Length, ^b Å	Dihedral angle ^b	
				A-1	A-2
Imidacloprid (A)	1	100	6.525	70.03	66.59
Hapten-I (B)	nc ^c	0	6.525	73.23	70.34
Hapten-II (C)	0.7	143	6.526	69.81	68.23
Imidacloprid olefin (D)	11	9	6.755	86.03	86.57
5-Hydroxyimidacloprid (E)	47	2	7.485	75.24	86.31
6-Chloronicotinic acid (F)	nc ^c	0	–	–	–

^a CR = cross reactivity.

^b See Figure 1 for the length and dihedral angle.

^c nc = no competition.

2.3.7. Molecular Modeling of Imidacloprid Analogs

Three dimensional geometry, hydrophobicity and electrostatic properties are calculated in order to understand the selective binding of Ab-IIa to imidacloprid (Figure 2.1, Plate 1 and Table 2.1). No recognition to 6-chloronicotinic acid suggests that the whole molecule or imidazolidinyl ring is the essential binding moiety. The large I_{50} differences between imidacloprid and the other analytes (**B**, **D-F** in Table I) indicate that 3-dimensional geometry of the whole molecule is important for the selective recognition. The molecular configuration between the imidazolidinyl and pyridinyl rings (*i.e.*, angles A1 and A2 in Figure 2.1) and the distance (designated as length) between the NO₂ oxygen and the end atom of the substituent R at the 5 position of the imidazolidinyl ring may play an important role in the antibody selectivity. The differences of the corresponding dihedral angles between imidacloprid (**A**) and hapten-II (**C**) (-0.22° and 1.64° for A1 and A2, respectively) are much smaller than those between **A** and hapten-I (**B**), imidacloprid olefin (**D**), or 5-hydroxyimidacloprid (**E**) (3.2-16° and 3.75-11.98° for A1 and A2, respectively). These computational results support the antibody selectivity, *i.e.*, low I_{50} s for **A** and **C**, high I_{50} s for **D** and **E** and no competition for **B** and **F** (Table 2.1).

Steric hindrance is also important to antibody recognition. The lengths of **A**, **B** and **C** are virtually identical, and notably shorter than those of **D** and **E**. The lack of Ab-IIa's recognition of **E** suggests the strict steric requirement for the antibody binding. The length of **E** is approximately 0.96 Å longer than that of **A**, in which the hydroxyl group may cause steric exclusion in antibody binding. When compared with **A**, **B** has a long and bulky hexanoic acid linker (6.734 Å) that presumably prevents binding (Figure 2.4).

Electrostatic properties of ligands play an important role in protein-ligand interactions [122-126]. The strong negative charges on the nitro oxygen (from -0.567 to -0.679) and large dipole moment (-3.59 D) [37] may have a strong electrostatic interaction with amino acid residues (*e.g.*, asparagine) in the antibody binding pocket [32]. Charge distribution of **D**, having a similar molecular size as **A**, largely differs from that of **A**, particularly on the imidazolidinyl ring (Figure 2.4). The partial charges of hydrogen atoms in the 4 and 5 positions of imidazolidinyl in **A** (0.10) are half of that (0.20) in **D**. The calculated charge difference between the carbon and hydrogen atoms correspondingly at the 4 and 5 positions also shows that the 4,5-double bond in **D** is more polarized than the single bond in **A**. The 4,5-double bond may also induce the hydrogen at the N3 position to be more positive. Negative potentials from the 4,5-double bond in **D** can cause it to be more hydrophilic. Octanol-water partition coefficients (k_{ow}) of **A** and **D** are 5.02 and 2.92 , respectively, which suggests that the double bond increases the hydrophilicity of **D**. Introduction of a hydroxyl group at the 5 position also increases the hydrophilicity of **E**.

The electrostatic potential surfaces on pyridinyl rings in **A** and **C** are considerably different, but almost identical on imidazolidinyl rings. The I_{50} values are almost the same for **A** and **C**. Therefore, the imidazolidinyl ring may primarily govern the high antibody specificity, and the pyridinyl ring on which the hapten spacer is attached may affect the molecular geometry for proper binding. The results of this study support the usefulness of molecular modeling aiding rational designs of haptens and immunoassay development [38-40].

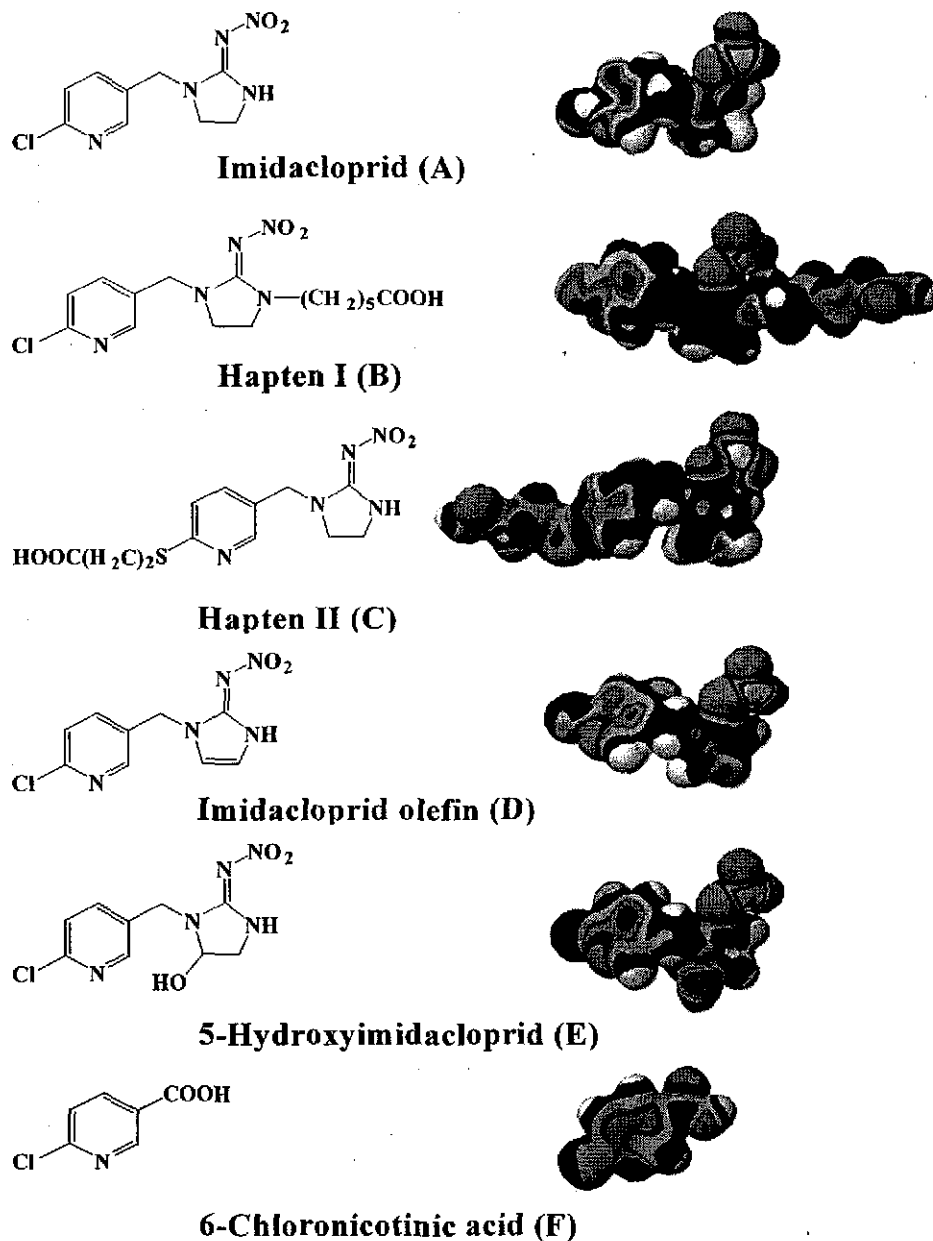


Figure 2.4. Structures and electrostatic potentials mapped on electron density isosurfaces of imidacloprid and its analogs. The energy values in atomic unit on each surface color are: white, $> +1$; red, $+0.09$ to 0.03 ; yellow, $+0.03$ to $+0.01$; pale green, $+0.01$ to 0.00 ; pale blue, 0.00 to -0.01 ; blue -0.01 to -0.03 ; violet, -0.03 to -0.06 ; and black, < -0.06 .

2.3.8. Analysis of Imidacloprid in Water

Tap water was fortified with imidacloprid at different levels up to 40 ng/mL and assayed with the optimized direct cELISA. The concentrations of imidacloprid determined by ELISA correlated very well with the fortification values with a slope of 1.0 and a correlation coefficient of 1.00 (Figure 2.5). The results showed that the ELISA could accurately measure the concentration of imidacloprid in water. The direct cELISA took a much shorter time than the indirect cELISA previously reported [14].

2.4. CONCLUSION

The detectability and analysis speed of an imidacloprid ELISA were significantly improved by purification of the polyclonal antibody, use of a direct cELISA format, and optimization of assay conditions. The assay was specific to imidacloprid with an I_{50} of approximately 1 ng/mL and had minimal cross reactivity with major imidacloprid metabolites. The antiserum (Ab-IIa) was raised from rabbits immunized with an immunogen of which the hapten had a spacer on the pyridinyl ring of imidacloprid. Computational analysis suggests that the antibody specificity primarily relate to the dihedral angles between the two rings, steric hindrance and electrostatic charges on the imidazolidinyl ring. The distance between the two extremities of the imidazolidinyl ring may be critical for proper binding. The strong negative charges of the nitro group and large dipole moment may also be important for imidacloprid binding. Low antibody cross-reactivity to the analytes having a double bond or a hydroxyl group on the imidazolidinyl ring further suggests that the imidazolidinyl moiety is critical for proper antibody binding.

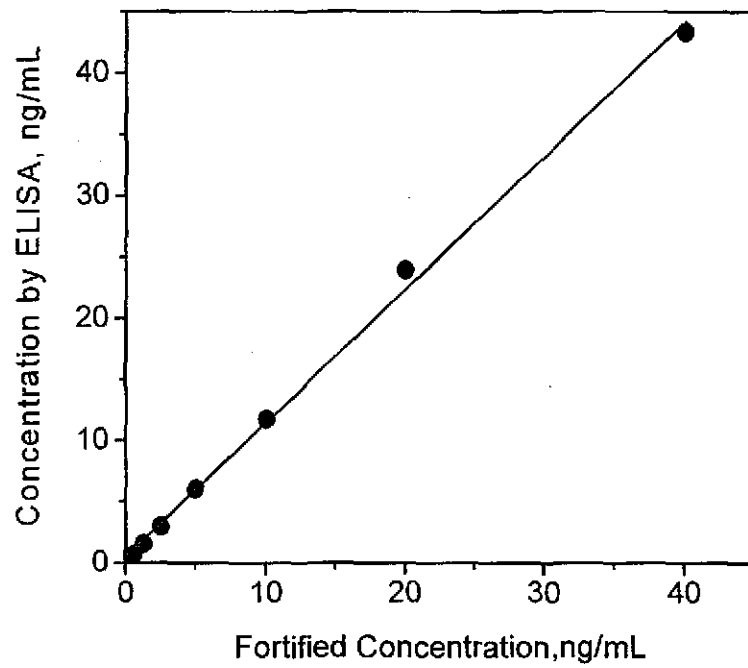


Figure 2.5. Correlation between concentrations of imidacloprid determined by ELISA and those fortified in tap water.

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Chapter 3

Development of an Enzyme-Linked Immunosorbent Assay for the Insecticide Thiamethoxam

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed for the neonicotinoid insecticide thiamethoxam, 3-(2-chlorothiazol-5-ylmethyl)-5-methyl-4-nitroimino-1,3,5-oxadiazinane. Three antisera were raised from rabbits immunized with the hapten-KLH conjugate. Based on the computational analysis of hapten candidates, the hapten with a spacer arm on the thiazolyl ring of thiamethoxam was synthesized to elicit thiamethoxam-specific antisera. The hapten was 3-(2-(2-carboxyethylthio)-5-ylmethyl)-5-methyl-4-nitroimino-1,3,5-oxadiazinane. Antisera were characterized with indirect competitive ELISA. Cross-reactivity and effects of organic solvents, pH, and ionic strengths were evaluated. The antiserum was specific for thiamethoxam and tolerant of up to 5% acetonitrile and 5% acetone. Varying ionic strength and pH in the tested ranges had negligible effect on the assay performance. Under the optimized conditions, the half-maximal inhibition concentration (IC_{50}) and the limit of detection were approximately 9.0 and 0.1 $\mu\text{g/L}$ of thiamethoxam, respectively. The ELISA analysis of stream and tap water samples showed an excellent correlation with the fortification levels.

Keyword: ELISA; immunoassay; thiamethoxam; neonicotinoid insecticide; hapten design

3.1. INTRODUCTION

Thiamethoxam 3-(2-chlorothiazol-5-ylmethyl)-5-methyl-4-nitroimino-1,3,5-oxadiazinane, belongs to a relatively new class of insecticides known as neonicotinoids. Thiamethoxam, imidacloprid, and related neonicotinoids have several advantages over pyrethroid, organophosphate and carbamate insecticides, and are rapidly replacing them worldwide.

Pyrethroids interact mainly with pre-synaptic sodium channels in vertebrates as well as insects [1-3]. Organophosphate and carbamates act by inhibiting acetylcholine esterases and thus are toxic to vertebrates, insects, and other invertebrates [4]. By contrast, neonicotinoids act similarly to nicotinic acid as agonists of the post-synaptic nicotinic acetylcholine receptors (nAChR). Neonicotinoids are 100-fold or more selective for insect nAChRs than for vertebrate nAChRs [5,6]. Several research groups worldwide have published evidence related to the sub-molecular basis for this selectivity, from the standpoint of the receptor subunit composition and properties, as well as the steric and charge distribution characteristics of the neonicotinoids [6-18].

The U.S. EPA promulgated rules for thiamethoxam use beginning in 2000 [19-22]. During 2001, thiamethoxam was approved for various uses by the Massachusetts Department of Food and Agriculture, the Canadian Pest Management Regulatory Agency, and Australian National Registration Authority for Agricultural and Veterinary Chemicals. Abundant details of the chemistry, metabolism, bioavailability, and human- and ecotoxicology, as well as approved uses, analytical procedures, and potential risks are presented in refs 23-25. The major concerns are wind drift and leaching of

thiamethoxam into surface water and groundwater [23], toxicities to a few vulnerable aquatic species such as mysid shrimp and rainbow trout embryos [25], toxicity to honey bees, parasitic wasps, and other beneficial insects [25], and the development of resistance by target insects [24]. In addition, thiamethoxam in the formulation known as Actara25-WG may lose activity or become phytotoxic when mixed with certain fungicides such as Mancozeb, Metalaxyl, and Folpet [26]. Despite these drawbacks, thiamethoxam, imidacloprid, and other neonicotinoids are expected to replace organophosphates as the most widely used insecticides worldwide [23].

Thiamethoxam and related neonicotinoids also have low acute dermal and inhalation toxicity, and in normal use do not cause allergic reactions, or skin or eye irritation in humans and animals. Because of their high affinity for insect nAChRs [9], neonicotinoids are very potent, and application of 5-8 ounces per acre is effective for some soil and foliar applications.

Currently two generations of neonicotinoid insecticides are in use. These are the chlorothiazolyl derivatives such as imidacloprid, and the thianicotinyl derivatives, exemplified by thiamethoxam. High performance liquid chromatography (HPLC) and HPLC-mass spectrometry (HPLC-MS) are currently preferred for determination of neonicotinoids in environmental samples [23]. The thermolability and high polarity of neonicotinoids make them difficult to analyze with gas chromatography (GC) or GC-MS [27-29]. However, the water solubility, asymmetric structure, and potential for leaching into surface water and groundwater make immunoassay (ELISA) potentially a good analytical method. To date, our laboratory and one other developed haptens and ELISAs based on rabbit sera, and Watanabe *et al.* derived another hapten and monoclonal mouse

antibodies for ELISA of the most heavily used chlorothiazolyl neonicotinoid insecticides, imidacloprid and acetamiprid [30-32]. In this paper we describe the design of a hapten and development of serum-based direct and indirect competition ELISAs for thiamethoxam, the most used thianicotinyl insecticide in this class. Hapten design was facilitated by computational modeling of the energy-minimized structures and their charge distributions.

3.2. MATERIALS AND METHODS

3.2.1. Reagents

All reagents were of analytical grade unless specified otherwise. Reference standards of clothianidin (99.9%), acetamiprid (99.5%), and dinotefuran (99.7%) were kindly provided by the National Institute of Agricultural Science and Technology, South Korea. Primary stock solutions were prepared by dissolving each reference standard in methanol at a concentration of 0.5 mg/mL. Chemicals purchased from Sigma (St. Louis, MO) were goat anti-rabbit IgG-horseradish peroxidase (IgG-HRP), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), HRP, phosphate-citrate buffer capsules with sodium perborate, carbonate-bicarbonate buffer capsules, and o-phenylenediamine (OPD). TiterMax Gold adjuvant was obtained from CytRx Corp. (Norcross, GA). The ELISAs were carried out in 96-well polystyrene microplates (MaxiSorp F96, Nalge Nunc International, Denmark). Thiamethoxam, the thiamethoxam hapten, and the hapten conjugates were synthesized in this laboratory. The antiserum was purified with a

HiTrap Protein G HP column (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Concentration of antibody in the final preparation was determined with the Bio-Rad Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). The purified IgG in phosphate-buffered saline (PBS, 12 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH 7.5) was stored at $-20\text{ }^{\circ}\text{C}$.

3.2.2. Spectroscopy and Chromatography

^1H NMR spectra were obtained on a Nicolet NT-300 MHz for solutions in CDCl_3 , and are described as multiplicity, coupling constant (J) in hertz (Hz), number of protons, and assignment. Chemical shifts (δ , ppm) are relative to internal tetramethylsilane (TMS). Low-resolution mass spectra were recorded with a Hewlett-Packard 5958 GC/MS system with an electron impact (EI, 70 eV) ionization and are given as $[\text{M}]^+$.

3.2.3. Molecular Modeling

Global energy minimum structures were searched by preliminary conformational analysis with all the rotatable bonds, and refined energy minimization was done by PM5 force field. To evaluate the structural similarity, root mean square (RMS) deviations were calculated by superimposing other chemicals on thiamethoxam. Atomic properties, including superdelocalizability, partial charge and electron density, were calculated with the same force field. Electron density isosurfaces colored by electrostatic potential were also calculated to evaluate the electron distribution over the molecules. All the calculations were done by CAChe Worksystem Pro (Version 5.0, Fujitsu, Japan). Partial dipole moment, molecular dimension and hydrogen bonding properties were calculated

with Molecular Modeling Pro (Version 5.0.8, ChemSW Inc., USA). Atomic polarizability and partial π and σ charges were calculated with PETRA [33].

3.2.4. Safety Precaution

Hapten syntheses were done in a chemical fume hood with charcoal filters. All synthetic byproducts and wastes and solutions of thiamethoxam analytes were disposed of as hazardous materials. Goggles, spill-resistant gowns, and chemical-impermeable nitrile gloves were worn.

3.2.5. Synthesis of Hapten (Figure 3.1)

In general, the final hapten, thiamethoxam and intermediates were synthesized according to established methods [30, 34-37].

3.2.5.1. Preparation of 3-methyl-4-nitroimino-1,3,5-oxadiazinane (I)

A solution of N-methyl-N'-nitroguanidinene (6.0 g) and 37% aqueous formaldehyde solution (37.5 mL) was added to 37.5 mL of 90% formic acid, and heated to 80 °C for 16 h. After cooling to 0 °C, aqueous sodium hydroxide solution (300 g/L, *ca* 70 mL) was added to adjust the solution to pH 8.0. The resulting mixture was extracted with CH₂Cl₂. The organic phase was washed with saturated sodium chloride, dried by anhydrous sodium sulfate and evaporated to dryness. The solid residue was recrystallized from ethyl acetate and ethyl ether to get white solid 5.5 g, yield 67%; m.p. 141-143 °C; GC-MS: C₄H₈N₄O₃, 160 (M⁺).

3.2.5.2. Preparation of 2-chloro-5-methylthiazole (II)

A solution of 2-amino-5-methylthiazole (10 g) in 40 mL of concentrated HCl and 20 mL of water was cooled to 0 °C. Aqueous sodium nitrite (7 g in 15 mL of water) was added drop-wise to the reaction mixture. After the mixture was maintained at 0 °C for 3 h, the solution was heated to 80 °C for 3 h, then cooled to room temperature. The reaction mixture was extracted with chloroform (400 mL x 3), and the extracts were combined and dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure, and the residue was purified with a silica gel column and eluted with CH₂Cl₂. GC-MS: C₄H₄CINSH⁺, 134 (MH⁺).

3.2.5.3. Preparation of 2-chloro-5-bromomethylthiazole (III)

In a 50-mL round-bottomed flask, a solution of II (8.0 g), N-bromosuccinimide (NBS) (12.8 g), benzoyl peroxide (AIBN) (28.8 mg) in CCl₄ (200 mL) was heated to 80 °C for 36 h, cooled to room temperature, and filtered. The filtrate was washed to neutral with water, 5% sodium bicarbonate and water, dried by anhydrous sodium sulfate. After solvent removal, the residue was purified by flash chromatography (ethyl acetate: hexane = 1:9). Yield 85%; GC-MS: C₄H₃BrCINSH⁺, 213 (MH⁺).

3.2.5.4. Preparation of thiamethoxam (IV)

A solution of I (3.0 g), III (5.0 g) and potassium carbonate (6.25 g) in DMF (20 mL) was heated to 30 °C for overnight, then to 50 °C for 16 h. After the reaction mixture was filtered through celite, DMF was removed at reduced pressure. The residue was purified by flash chromatography (CH₂Cl₂ : CH₃OH = 95 : 5). The purified product has m.p. 140-

142 °C; LC-MS: $C_8H_{10}ClO_3SH^+$, 292 (MH^+); 1H NMR (DMSO- d_6): δ 2.832 (3H, s, CH_3), 4.746 (2H, s, CH_2), 4.968 (2H, s, CH_2), 5.039 (2H, s, CH_2), and 7.639 (1H, s, $CH=$).

3.2.5.5. Preparation of 3-(2-(2-carboxyethylthio)-5-ylmethyl)-5-methyl-4-nitroimino-1,3,5-oxadiazinane (V)

In a 50-mL round-bottomed flask, a solution of thiamethoxam (0.5 g), 3-mercaptopropionic acid (0.2 g), and 85% potassium hydroxide (powder, 0.2 g) in DMSO (15 mL) was heated to 75 °C for 3 days. The solution was poured into water (100 mL), adjusted to pH 3 with 1 N HCl, and then extracted with ethyl acetate (30 mL x 3). The organic phase was combined, washed with brine solution, and dried with anhydrous Na_2SO_4 . After the solvent was removed at reduced pressure, the residue was dissolved in methanol. The product was separated by preparative thin layer chromatography (CH_2Cl_2 : CH_3OH = 9 : 1). The yield was 55.6%; LC-MS: $C_{11}H_{15}N_5O_5S_2H^+$, 362 (MH^+); 1H NMR (DMSO- d_6): δ 2.756 (2H, t, CH_2 , J = 7.0 Hz), 2.980 (3H, s, CH_3), 3.406 (2H, t, CH_2 , J = 7.0 Hz), 4.806 (2H, s, CH_2), 4.976 (2H, s, CH_2), 5.032 (2H, s, CH_2), and 7.616 (1H, s, $CH=$).

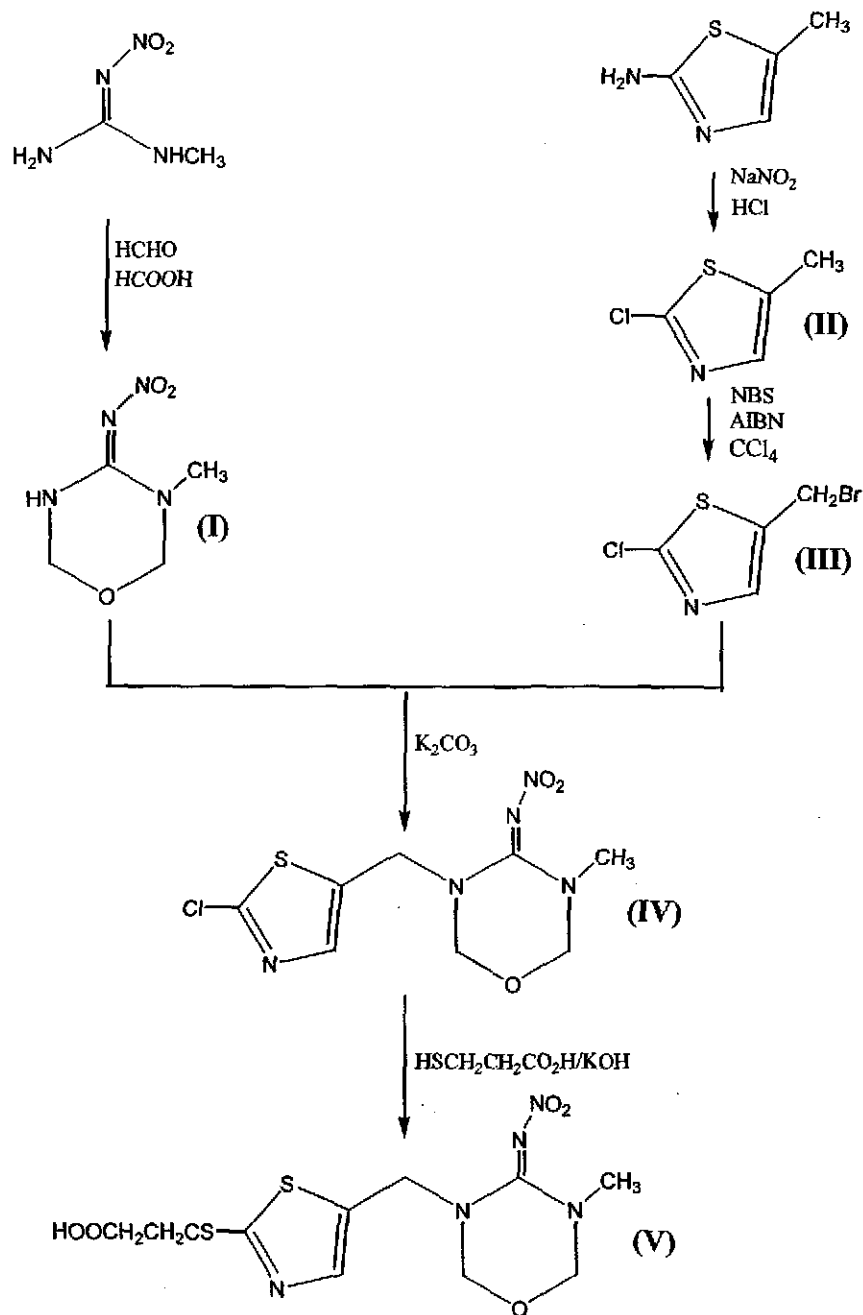


Figure 3.1. Synthetic scheme of thiamethoxam hapten.

3.2.6. Preparation of Protein-Hapten Conjugates

3.2.6.1 Hapten-KLH and BSA conjugation

The hapten (V) was conjugated to KLH and BSA as previously described [30] with slight modification. To 0.05 mmol of the hapten was added 500 μ L of DMF solution containing 0.1 mmol each of N-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (DEC). The mixture was stirred at room temperature for 4 h and then centrifuged to remove precipitated urea. The clear supernatant was slowly added, a few μ L per 20 min intervals, to KLH solution (30 mg KLH in 10 mL of 0.1 M borate buffer, pH 9.0) or BSA solution (150 mg BSA in 10 mL of 0.1 M borate buffer, pH 9.0). The reaction mixture was stirred overnight at 4 °C, then dialyzed against PBS (1.5 L) for 3 days at 4 °C with two buffer changes per day.

3.2.6.2. Preparation of Enzyme Tracer

The hapten-HRP conjugate was prepared as above with the following exception. Hapten (6 μ mol) was activated with NHS (30 μ mol) and DEC (60 μ mol) in 260 μ L of DMF, and the reaction mixture was added to HRP (2 mg) in 3 mL of 0.13 M NaHCO₃ buffer. After the dialysis against PBS, the crude hapten-HRP conjugate was diluted with an equal volume of glycerol and stored at -20 °C.

3.2.7. Immunization

The hapten-KLH conjugate was used to immunize rabbits. A biochemically defined adjuvant, TiterMax Gold was selected due to its ability to produce high titers of antisera

[38]. Three New Zealand white rabbits (2-4 kg) were immunized with hapten-KLH emulsified in TiterMax Gold as previously described [30]. One received 60 µg, and the others received 100 µg of emulsion in 0.4 mL. Two booster injections were given at 4-week intervals. Injections were made intradermally and subcutaneously at multiple sites on the animals' backs. The titers of the antisera were monitored by ELISA using checkerboard titration. Seven days after the last injection, the rabbits were bled, and the antisera were collected and stored at -80 °C. Inhibition of antibody binding by free thiamethoxam was monitored with an indirect competitive ELISA (icELISA) [30].

3.2.8. Direct Competitive ELISA (dcELISA)

A microtiter plate was coated with rabbit anti-thiamethoxam-KLH antibody (100 µL of 2.0 µg antibody/well in 0.1 M carbonate-bicarbonate buffer, pH 9.6) overnight at 4 °C. The following day, the plate was washed four times with PBS containing 0.05% Tween 20 (PBST) and then blocked with 1% BSA in PBS (200 µL/well) for 1 h at room temperature. After the plate was washed (5x) with PBST, a solution of 50 µL of the analytes or standards diluted in PBST and 50 µL of thiamethoxam-HRP conjugate (0.16 ng/µL in PBST) was added. After 40 min incubation at 37 °C and another five washings, the substrate solution (100 µL per well, containing 1.0 mg/mL of OPD in 0.05 M citrate-phosphate and 0.03% sodium perborate, pH 5.0) was added. The reaction was stopped with sulfuric acid (4 N, 50 µL/well) after 15 min at room temperature, and absorbance at 490 nm was read with a Vmax microplate reader (Molecular Devices, Sunnyvale, CA). Inhibition curves were fit to a four-parameter logistic equation with Softmax software V 2.35 (Molecular Devices).

3.2.9. Fortification of Thiamethoxam in Water

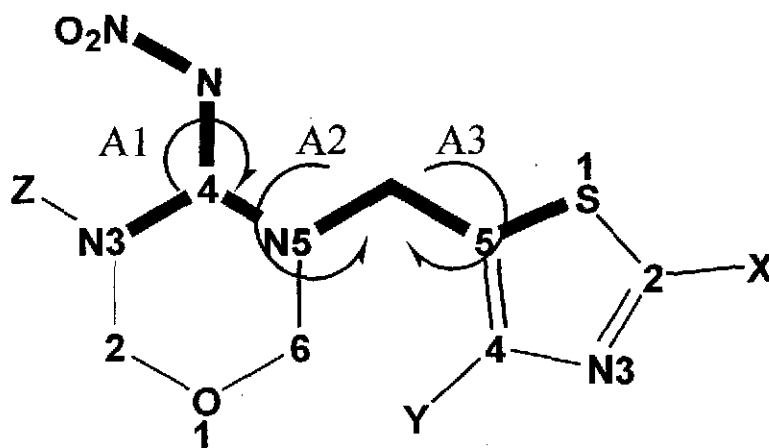
Water samples were from a tap in the laboratory and were collected from Manoa stream in Honolulu, Hawaii. Tap and stream water samples were fortified with thiamethoxam up to 100 ng/mL. The optimized dcELISA was used to determine thiamethoxam concentrations in the fortified water samples. The samples were buffered with four-fold concentrated PBST.

3.3. RESULTS AND DISCUSSION

3.3.1. Hapten Design and Synthesis

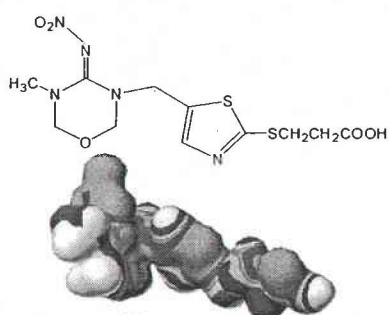
There are several possible linker positions (X, Y and Z) in thiamethoxam for hapten preparation (Figure 3.2). To obtain compound-specific antibodies, a good option is to place a linker on a remote part of the molecule and to maximally reserve the parent molecular property [39, 40]. The hapten design was aided with computational analysis of hapten candidates. Various chemical and physical properties of thiamethoxam and its analogues were predicted with computational modeling to identify a suitable linker position (Figures 3.2 and 3.3, Table 3.1). The computational analysis suggested that a thioalkyl spacer on the 2-position of the thiazolyl ring alters the molecular properties much less than one on the 4-position of the thiazolyl ring or the 5-position of the oxadiazinane ring (Figures 3.2 and 3.3, Table 3.1). In addition, replacing the N-methyl group with a spacer (proposed hapten C) may not produce a suitable hapten because clothianidin and thiamethoxam share the same thiazole structure, located at the end of the molecule remote from the spacer. The proposed hapten B may not be an adequate hapten

either because a large steric hindrance of the linker at the 4-position of thiazole significantly distorts the geometry from that of thiamethoxam (Figure 3.3, Table 3.1). These considerations suggest that the proposed haptens B and C would not elicit thiamethoxam-specific antibodies. The computational modeling indicated that the hapten A has almost the same geometric overlap and electrostatic properties as thiamethoxam (Figure 3.3, Table 3.1) and is a suitable hapten candidate. Therefore, it was synthesized and used to induce antibodies. Thiamethoxam (IV) and three intermediates (I, II and III, Figure 3.1) were synthesized according to established methods [34-37]. Thiamethoxam was prepared by reaction of compounds I and III as shown in Figure 3.1. Introduction of the spacer to the selected site of thiamethoxam was carried out under a mild alkaline condition because conversion of =N-NO₂ to =O at a strong alkaline condition was observed in the previous work [30]. The molecular ratio of hapten to protein was estimated by UV absorbance and was 10 and 43 for hapten-BSA and hapten-KLH, respectively.

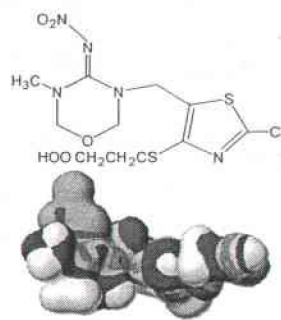


Chemical Name	X	Y	Z
Thiamethoxam	Cl	H	CH ₃
Hapten A (synthesized)	SCH ₂ CH ₂ COOH	H	CH ₃
Hapten B (proposed)	Cl	SCH ₂ CH ₂ COOH	CH ₃
Hapten C (proposed)	Cl	H	CH ₂ CH ₂ CH ₂ COOH

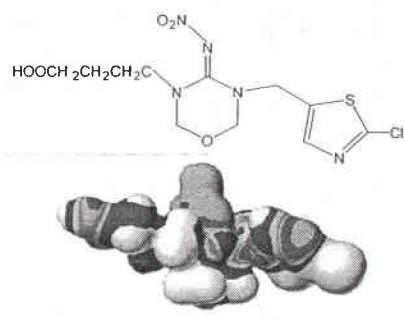
Figure 3.2. Structures and dihedral angles of thiamethoxam and hapten candidates.



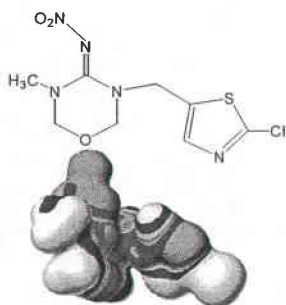
Hapten A
 $I_{50} = 14.8 \text{ nM}$ (10 ppb)



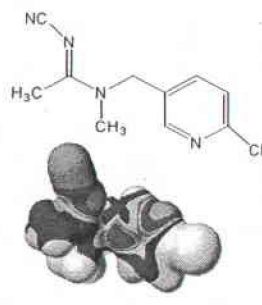
Proposed hapten B



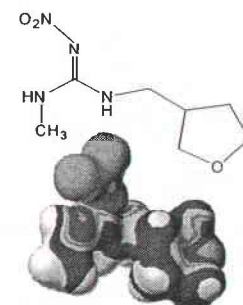
Proposed hapten C



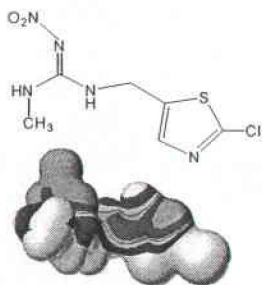
Thiamethoxam
 $I_{50} = 29.3 \text{ nM}$ (9 ppb)



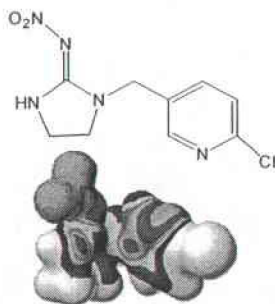
Acetamiprid
 $I_{50} > 6,740 \text{ nM}$ (> 1,500 ppb)



Dinotefuran
 $I_{50} > 11,510 \text{ nM}$ (> 2,500 ppb)



Clothianidin
 $I_{50} = 1,587 \text{ nM}$ (420 ppb)



Imidacloprid
 $I_{50} = 3,462 \text{ nM}$ (885 ppb)

Figure 3.3. Electron density isosurfaces colored by electrostatic potential; color legends; white > 0.09; 0.03 < red < 0.09; 0.01 < yellow < 0.03; 0.00 < pale green < 0.01; -0.01 < pale blue < 0.00; -0.03 < blue < -0.01; -0.06 < pink < -0.03; gray < -0.06. The structures of hapten B and C were proposed, but not synthesized.

Table 3.1. RMS errors of the superimposition on thiamethoxam and molecular dimensions of global energy minimum structures

Name	RMS deviation	Dihedral angle (Degree)			Molecular dimension (Å)		
		A1	A2	A3	Length	Width	Depth
Acetamiprid	0.1092	-1.2	70.1	-111.1	11.35	8.77	7.62
Clothianidin	0.0982	-61.8	74.3	-125.8	10.80	8.78	7.67
Dinotefuran R	0.2813	-3.2	-169.9	173.2	11.20	8.82	6.16
Dinotefuran S	0.2796	-3.4	115.2	-71.7	11.10	8.82	6.35
Imidacloprid	0.1127	-0.2	69.0	-90.0	10.68	9.18	7.98
Thiamethoxam	0.0000	-119.3	82.9	-72.9	12.17	9.01	7.91
Hapten A (synthesized)	0.0242	-119.7	82.3	-73.9	12.16	9.03	7.92
Hapten B (proposed)	0.2868	-89.1	155.2	-79.8	12.35	9.12	7.82
Hapten C (proposed)	0.0562	-105.2	84.4	-73.8	12.15	9.11	7.53

3.3.2. Immunization and Characterization of Antisera

Table 3.2 shows the titers and IC₅₀ values of the three antisera for free thiamethoxam. The titer of antiserum-II (Ab-II) from rabbit II was five- and ten-fold higher than that from rabbit I (Ab-I) and III (Ab-III), respectively. However, the antiserum-I (Ab-I) from rabbit I had the lowest IC₅₀ (278 ng/mL) in an icELISA (Table 3.2). Protein G affinity-purified Ab-I was stored at -20 °C, and used throughout this work. When compared with the titers of imidacloprid antisera obtained with the same procedure [30], the titers of these thiamethoxam antisera were low, which may be due to the lower ratio of thiamethoxam hapten to KLH, and differences in the hapten structures and individual animals.

Table 3.2. Titer and apparent binding affinity of antisera

Antiserum	Titer ^a	IC ₅₀ (ng/mL) ^b
I	8,000	280
II	40,000	> 1,500
III	4,000	> 1,200

- The titer of antiserum is defined as the antiserum dilution that produced an absorbance of 1 at 490 nm, 20 min after addition of the substrate.
- IC₅₀ values represented the concentration of thiamethoxam in ng/mL that produced 50% inhibition of antiserum binding to the hapten conjugate using icELISA. The wells were coated with 100 µL of conjugate of 25 ng/mL.

3.3.3. Competitive Inhibition

Figure 3.4 shows representative standard curves for thiamethoxam obtained with dcELISA and icELISA. The calibration ranges of the dcELISA and icELISA curves were approximately 0.1-200 and 10-5,000 $\mu\text{g/L}$, respectively. The IC_{50} values of dcELISA and icELISA were approximately 8 and 160 $\mu\text{g/L}$, respectively, indicating that use of dcELISA and purification of antibody improved the sensitivity by 20-fold compared with that of an icELISA. A similar improvement with the dcELISA format was observed with the imidacloprid polyclonal antibody. A dcELISA format using the purified Ab-I was employed throughout this study since it significantly improved the assay sensitivity.

3.3.4. Cross-Reactivity

The specificity of Ab-I was estimated by performing dcELISA with four neonicotinoid insecticides as competitors, which were imidacloprid, acetamiprid, dinotefuran, and clothianidin. Ab-I was specific for thiamethoxam, and showed < 2% cross-reactivity for imidacloprid, chlothianidin and acetamiprid (Table 3.3). No response in the assay was observed up to 12 μM of dinotefuran. These results support the hapten design suggested by the computational analysis.

The relationship between the antibody specificity and competitor structures was examined by computational modeling. The largest structural difference was found in dihedral angles (Figure 3.2 and Table 3.1). The nitro group in thiamethoxam appears to be distorted into a nearly perpendicular position (dihedral angle A1) to the thiadiazinane

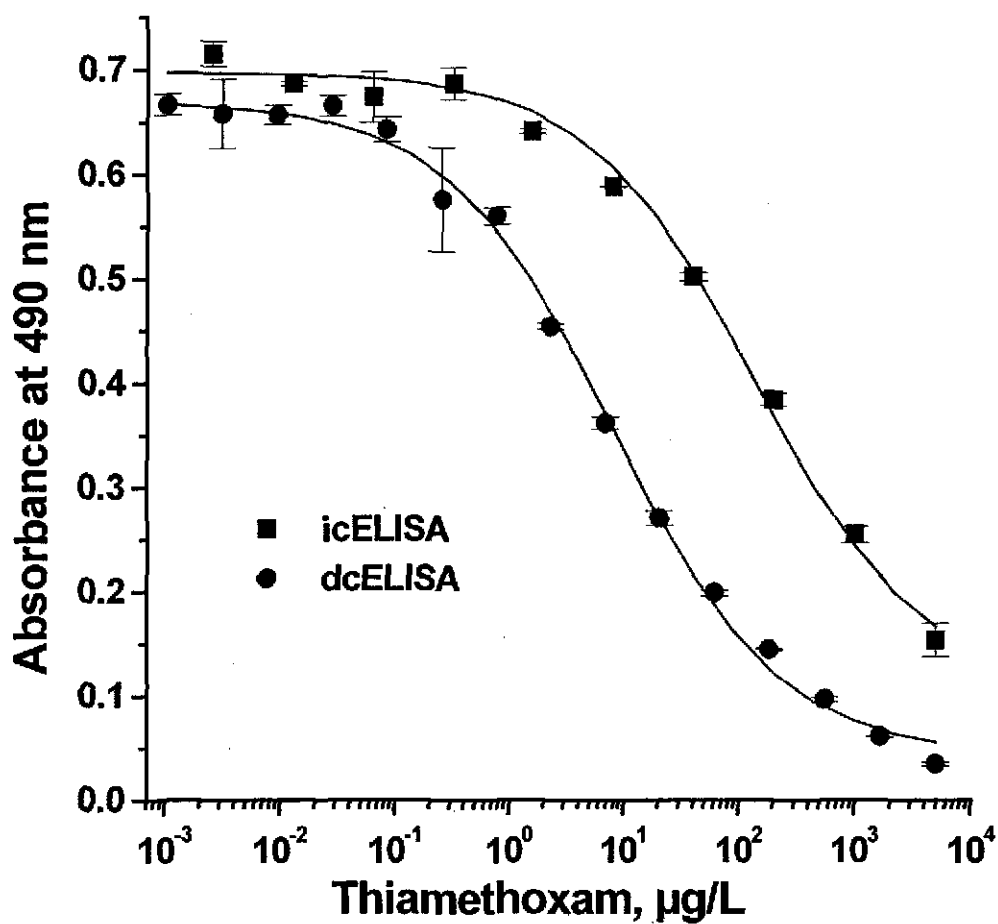


Figure 3.4. Standard inhibition curves of thiamethoxam in dcELISA and icELISA formats. Plates were coated with 100 µL of 2.0 µg of purified Ab-I for dc ELISA or 2.5 ng of hapten-BSA per well for icELISA. Each value represents the mean of four replicates.

Table 3.3. Cross-reactivity of Ab-I to thiamethoxam and its structural analogues^a

Compound	IC ₅₀ , nM (ng/mL)	Cross-reactivity, %
Thiamethoxam	29.3 (9.0)	100
Thiamethoxam hapten	14.8 (10)	198
Clothianidin	1,587 (420)	1.8
Imidacloprid	3,462 (885)	0.8
Dinotefuran	> 11,510 (>2,500)	< 0.25
Acetamiprid	> 6,740 (>1,500)	< 0.4

a. The plate was coated with 2.0 µg of affinity-purified antibody (100 µL/well).

b. Numbers in parenthesis are IC₅₀ values in ng/mL.

ring, which coincides well with crystallographic data of structurally related N-methyl imidacloprid [41]. Computational modeling suggested that the steric volume of the pyridine of acetamiprid and imidacloprid ($55.29 \text{ cm}^2/\text{mol}$) is similar to that of the thiazole of thiamethoxam and clothianidin (Figure 3.2).

In addition, there appeared to be similar electrostatic properties between the pyridine and thiazole rings. Large IC_{50} differences for thiamethoxam, clothianidin and imidacloprid indicate that the conservation of the nitroiminothiadiazinane moiety in the thiamethoxam hapten distal to the linker was important for the production of thiamethoxam-specific antibodies. Clothianidin and thiamethoxam have a chlorothiazole moiety; however, the large structural differences in their nitroimino groups probably results in low binding affinity of clothianidin to the antibody. The electrostatic properties of imidacloprid are more similar to thiamethoxam than clothianidin, but large differences exist in dihedral angle A1 among imidacloprid, clothianidin and thiamethoxam (Table 3.1). The large IC_{50} value for imidacloprid strongly suggests that the geometry of the nitroimino group is an important factor for the selectivity between chemicals (Figure 3.3, Table 3.1). The R- and S-enantiomers of dinotefuran share the same nitroimino structure with clothianidin, but dihedral angle A1 and the molecular shapes are completely different. Acetamiprid shows good geometric overlap with thiamethoxam, but the large electrostatic difference permits no cross-reactivity.

3.3.5. Chemical Effects on Assay Performance

Assay performance is often affected by ionic strength, pH, and solvent concentration of samples. The pH of aqueous samples may interfere with ELISAs due to the

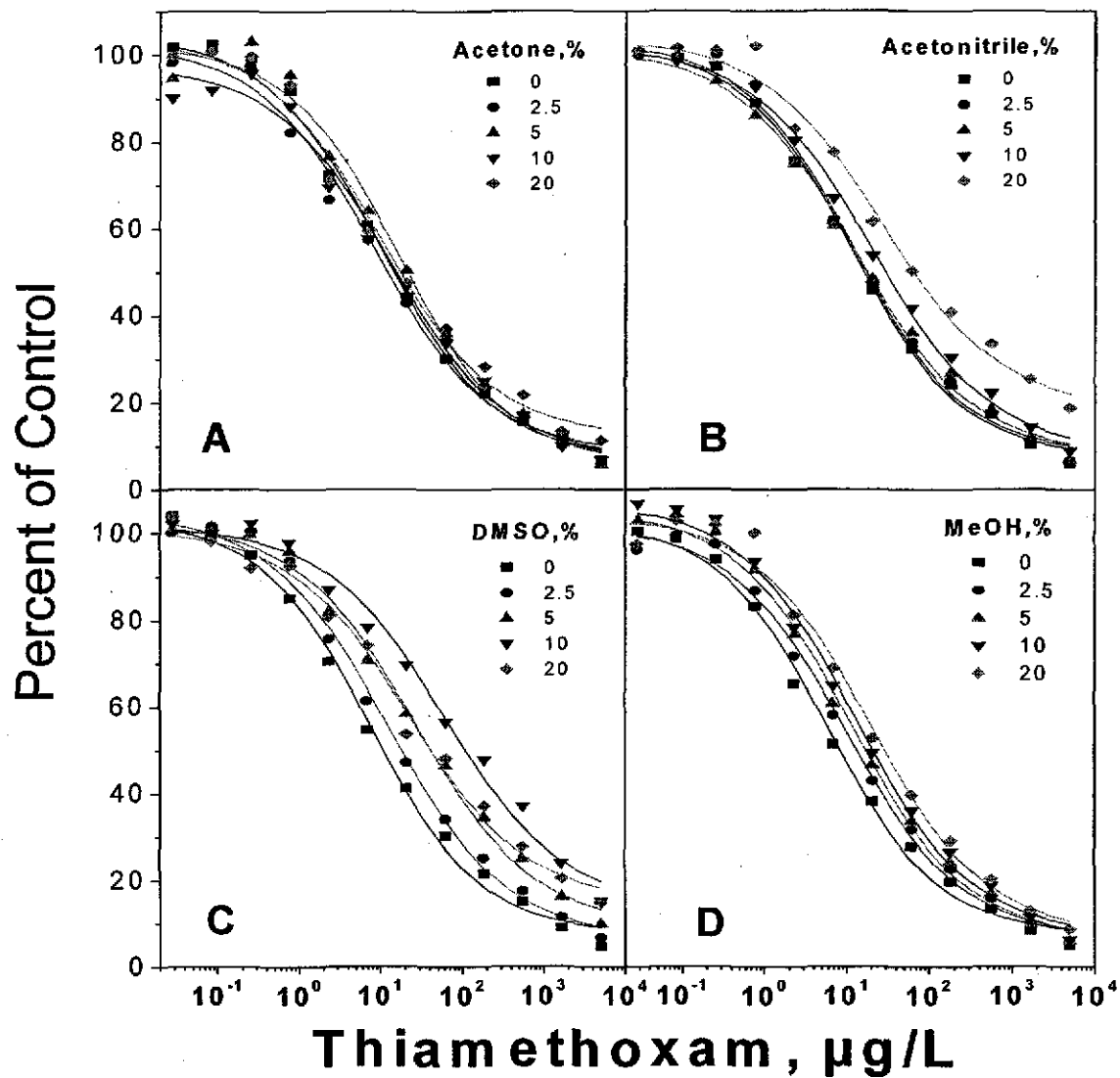


Figure 3.5. Effects of acetone (A), acetonitril (B), DMSO (C),and methanol (MeOH) on the assay. Values refer to solvent v/v concentration in the competitive assay solution. Each value represents the mean of four replicates.

disturbance of weak molecular interactions in antibody-antigen binding caused by pH derived alteration (e.g., hydrolysis, conformational change, ionization) of either an analyte or molecules (antibody or enzyme tracer) participating in the interaction [42-44]. Salt concentrations in real world samples can often affect assays [40, 44]. The effects of these parameters on the assay were evaluated.

3.3.6. Solvent Effects

The effects of DMSO, MeOH, acetone and acetonitrile were studied because they are water-miscible and are commonly used in sample extractions. Serial dilutions of thiamethoxam standard in PBST containing 0, 5, 10, 20, or 40% of organic solvent were mixed with an equal volume of antibody solution diluted in PBST for dcELISA. In general, maximum absorbance (A_{\max}) gradually decreased as the concentration of solvent increased. Acetonitrile and acetone showed less effect on the inhibition curves (shape and position) than MeOH and DMSO (Figure 3.5). Little change in IC_{50} values was observed in up to 5% of acetonitrile and 5% of acetone. However, IC_{50} values increased gradually as concentrations of MeOH and DMSO increased.

3.3.7. Effect of pH and Ionic Strength

To evaluate the influence of pH and ionic strength on assay performance, thiamethoxam was diluted in PBST of different pH values (4.0-9.0) with the same ionic strength or different ionic strength (0.15-1.5 M) at pH 7.5 and enzyme tracer in PBST, pH 7.5. A_{\max} values decreased at pH <6; however, there were negligible shifts of the inhibition curves in the pH range of 4-9 (Figure 3.6). All subsequent assays were

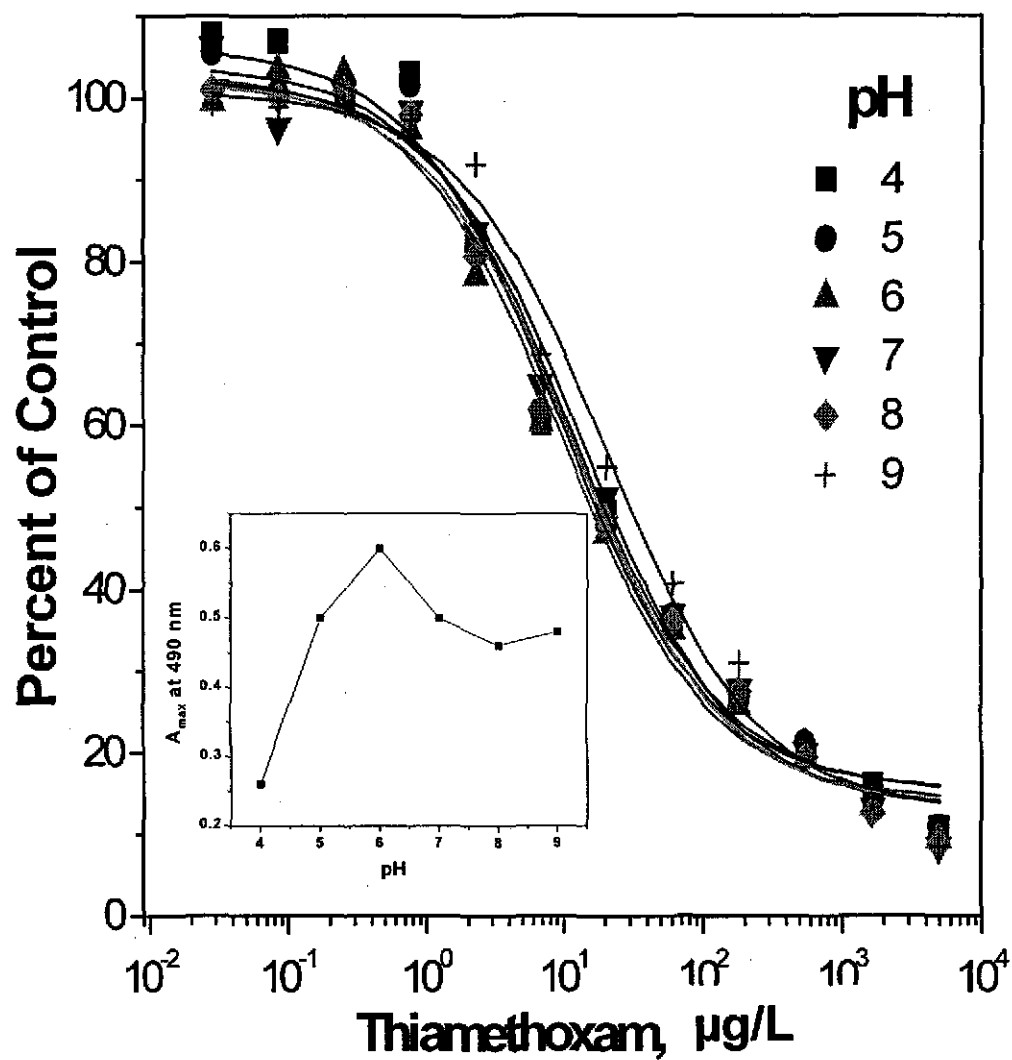


Figure 3.6. pH effect on assay sensitivity. Each value represents the mean of four replicate wells.

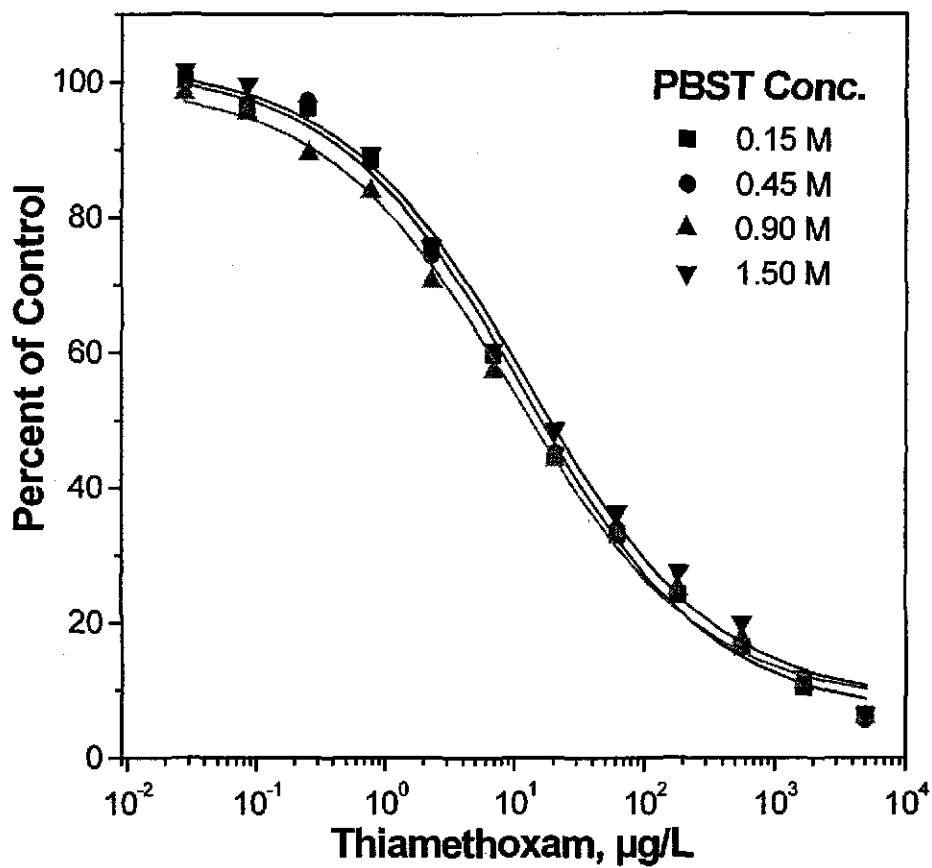


Figure 3.7. Effect of ionic strength on assay sensitivity. Each value represent the mean of four replicates.

performed at pH 7.0. There were no significant changes in IC_{50S} and A_{max} in the tested range of ionic strength (Figure 3.7).

3.3.8. Thiamethoxam-Fortified Water Samples

Thiamethoxam in water samples was analyzed with the dcELISA. The concentrations of thiamethoxam by ELISA correlated very well with the fortification values with a slope of 0.94 and correlation coefficient of 0.99 for the tap water samples and a slope of 0.94 and correlation coefficient of 0.99 for the stream water (Figure 3.8). The results showed that the ELISA can accurately measure the concentration of thiamethoxam in water.

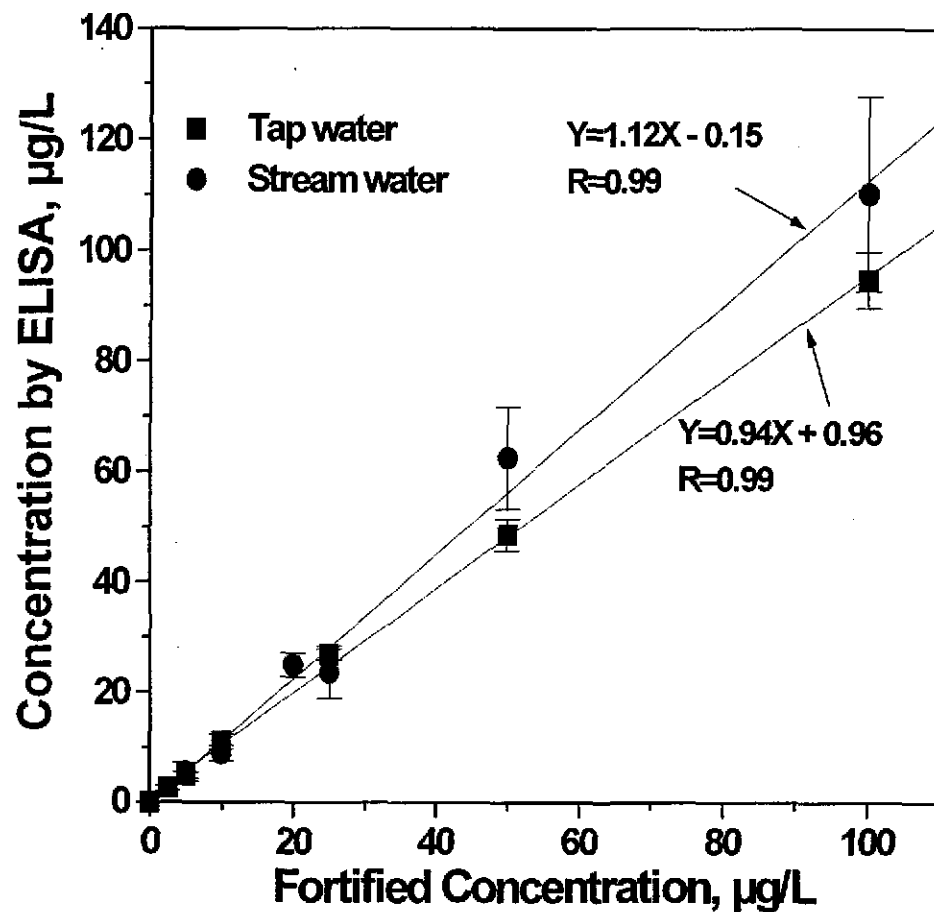


Figure 3.8. Correlation between thiamethoxam concentrations determined by ELISA and those fortified in tap water and stream water samples. The plate was coated with 2.0 µg of Ab-I in 100 µL/well. The concentration of enzyme tracer was 0.16 ng/µL in PBST, pH 7.0. Each value represents the mean of four replicates.

3.4. SUMMARY AND CONCLUSIONS

Thiamethoxam-specific polyclonal antibodies were obtained from rabbits immunized with a hapten KLH conjugate of hapten having a thioalkyl spacer at the thiazolyl ring of thiamethoxam. The hapten design was aided by computational analysis of RMS deviations, dihedral angles and molecular dimensions of candidate haptens. The experimental results were consistent with our prediction that the substitution of the chlorine atom with a thioalkyl group is a suitable position to derivatize in as well to obtain thiamethoxam-specific antibodies. All the molecules that have a flat dihedral angle (A1) showed little cross-reactivity. In addition, the nitro group configuration and presence of the oxadiazinane group may play a key role in the antigen-antibody recognition.

An ELISA was developed for the detection of thiamethoxam. The use of the protein G-purified IgG and the dcELISA format significantly improved sensitivity and analysis time for the thiamethoxam ELISA. The assay was specific for thiamethoxam with an IC_{50} of 29 nM and had < 2% cross-reactivity with structural analogues of thiamethoxam. The good recovery of thiamethoxam in water samples showed that the assay can be used for the determination of thiamethoxam residues in water, but further work is needed to validate this assay with other matrices.

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Chapter 4

A Monoclonal Antibody-Based ELISA for the Insecticide Imidacloprid

4.1. INTRODUCTION

Imidacloprid, 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine, belongs to a relatively new class of insecticides known as neonicotinoids [1-4]. Imidacloprid and other neonicotinoids act as agonists on the postsynaptic nicotinic acetylcholine receptors (nAChR), causing the modification of insect behavior and finally death [5-6]. Research on the molecular basis for the selectivity of neonicotinoids has elucidated the mechanism of nAChR-insecticide interaction and suggested that the low mammalian toxicity can be attributed to the higher selectivity of neonicotinoids for insect nAChR than for vertebrate nAChR [4, 7-16]. Because neonicotinoids have greater systemic activity, lower acute mammalian toxicity, and no cumulative long-term toxicity, they may eventually replace pyrethroid, organophosphate and carbamate insecticides. However, several properties of neonicotinoids, such as wind drift, leaching into surface water and ground water, and toxicity to honey bees and other beneficial organisms, remain to be elucidated [17-22].

Two chemical classes of neonicotinoid insecticides are currently in use. These are the chloropyridinyl derivatives such as imidacloprid, and the chlorothiazolyl derivatives, exemplified by thiamethoxam. High performance liquid chromatography (HPLC) with UV detection or HPLC-mass spectrometry (HPLC-MS) are currently preferred for the determination of imidacloprid in environmental samples [23-29]. The thermolability and high polarity of neonicotinoids make them difficult to analyze by gas chromatography (GC) or GC-MS, which requires derivatization of the analytes prior to analysis [30-32].

Immunoassay, however, has proven to be a good analytical method for rapid monitoring of agrochemicals [33, 34]. To date, several ELISAs have been developed for neonicotinoids. We have reported two ELISAs for imidacloprid and thiamethoxam that use rabbit antisera [35-37]. Lee et al. developed another ELISA for imidacloprid based on rabbit sera [38], and Watanabe et al. derived two monoclonal mouse antibodies for ELISA of imidacloprid and acetamiprid [39]. In this paper, we describe the development of an ELISA based on a new mouse MAb and ELISA for imidacloprid, developed from Hapten II that we described previously [35]. We also present solution-phase kinetics of the MAb binding to imidacloprid, the imidacloprid haptens, and other neonicotinoids, determined by kinetic exclusion assay (KinExA).

4.2. EXPERIMENTAL

4.2.1 Materials and reagents

All reagents were of analytical grade unless specified otherwise. Reference standards of clothianidin (99.9%), acetamiprid (99.5%), and dinotefuran (99.7%) were kindly provided by the National Institute of Agricultural Science and Technology, South Korea. Imidacloprid and the imidacloprid haptens were synthesized in this laboratory as previously described [35]. Chemicals purchased from Sigma (St. Louis, USA) were goat anti-mouse IgG-horseradish peroxidase (IgG-HRP), bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH), phosphate-citrate buffer capsules with sodium perborate, carbonate-bicarbonate buffer capsules, o-phenylenediamine (OPD), complete and incomplete Freund's adjuvant, DMSO, polyethylene glycol 1500

(PEG), and goat anti-mouse IgG, N-hydroxysuccinimide (NHS), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC).

3, 3', 5, 5'-Tetramethylbenzidine (TMB) was obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Maryland). The monoclonal antibody isotyping kit was purchased from Pierce (Rockford, IL). HT, HAT and RPMI 1640 supplements were bought from Life Technologies (Grand Island, NY). Fetal bovine serum from Hyclone (Logan, UT) was heat inactivated at 56 °C for 30 min prior to use. Hybridoma cloning factor (ORIGEN) was obtained from Fisher Scientific (Pittsburgh, PA). Murine myeloma cell line Sp2/0Ag14 was purchased from the American Type Culture Collection (Manassas, VA; ATCC cat. no. CRL-1581). The ELISAs were carried out in 96-well polystyrene microplates (MaxiSorp F96; Nalge Nunc International, Copenhagen, Denmark). The MAb was purified with a T-gel purification kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Concentrations of antibody in the final preparation were determined with the Bio-Rad Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). The purified IgG in phosphate-buffered saline (PBS, 5mM Na₂HPO₄, 1.8 mM KH₂PO₄, 136 mM NaCl, and 2.7 mM KCl, pH 7.5) was stored at -20 °C until use.

4.2.2. Preparation of protein-hapten conjugates

Imidacloprid haptens (Figure 4.1) were conjugated to KLH, OVA, and BSA according to the procedures previously described [35], but scaled down by 50% (hapten 0.05 mmol, NHS 0.1 mmol and EDC 0.1 mmol) in 0.5 mL DMF. All the conjugates were stored at -80 °C. Aliquots of conjugates were stored at 4 °C for daily use.

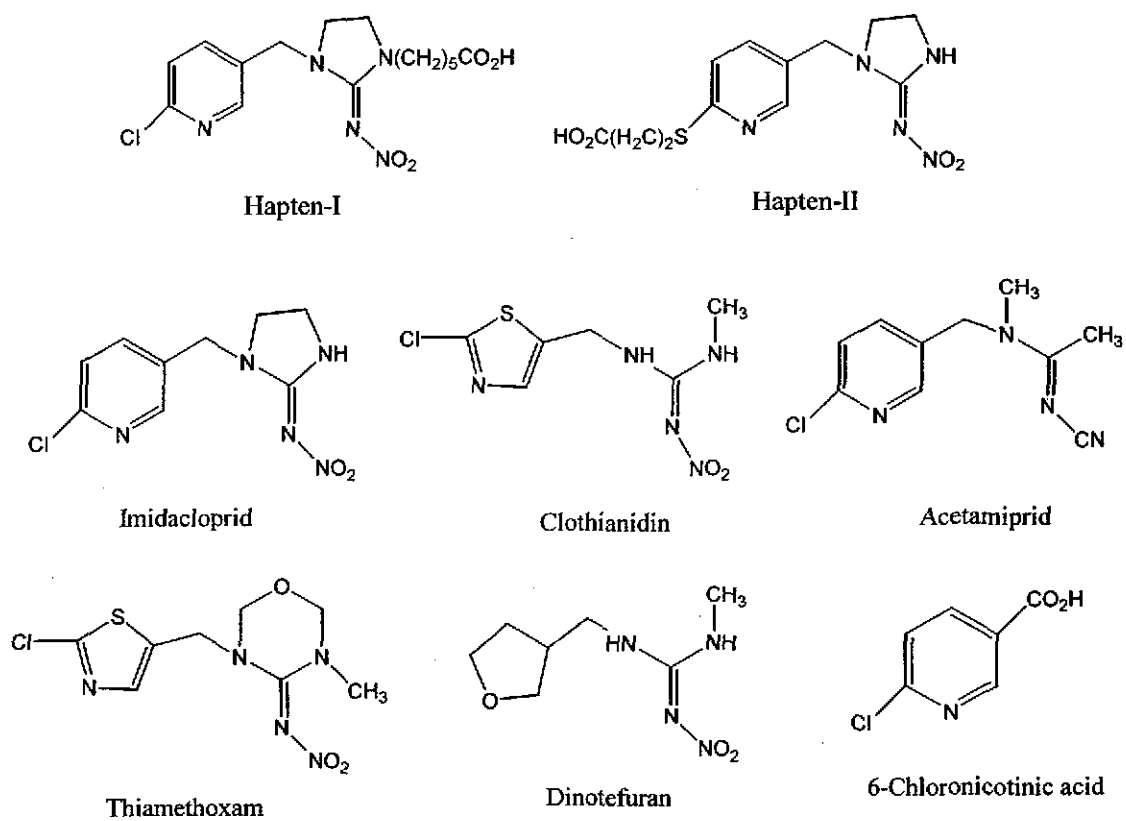


Figure 4.1. Structures of imidacloprid haptens, imidacloprid, its major metabolite 6-chloronicotinic acid and other neonicotinoids

4.2.3. Monoclonal antibody generation and characterization

The procedures for generating the immune response in mice and producing MABs were similar to those described by Shelver *et al.* [40]. Five female Balb/c mice, 6 weeks old (Jackson Labs, Bar Harbor ME), were initially immunized by intraperitoneal (i.p.) injection with 50 µg of imidacloprid hapten I-KLH or hapten II-OVA. After four monthly i.p. booster immunizations with 50 µg of hapten conjugate emulsified with incomplete Freund's adjuvant, serum samples were taken. One mouse, immunized with hapten II-OVA, developed a serum antibody titer >1:30,000 against hapten II-BSA, and the strongest competitive binding of soluble imidacloprid in an icELISA. This mouse was selected for hybridoma production. Four days prior to splenocyte harvest, this mouse was injected with the hapten II-OVA in PBS, 50 µg through the tail vein and 50 µg i.p.

Murine Sp2/0Ag14 myeloma cells were grown in RPMI 1640 supplemented with 15% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (designated as complete medium). The culture medium supernatants were saved from each Sp2/0Ag14 cell splitting and used as the conditioned medium replacing feeder cells in the fusion and cloning experiments. Splenocytes were harvested from the best-responding mouse described above, hybridomas were prepared by fusion with the Sp2/0Ag14 myelomas, and selected in complete medium containing 10 µM sodium hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine.(HAT) as previously described [40]. After plating in 96-well culture plates, the HAT selection was performed from 2 to 14 days post fusion. Two weeks after fusion the cells were screened for their ability to produce antibodies to imidacloprid utilizing concurrent indirect non-

competitive and competitive ELISAs with TMB as a substrate. One hybridoma colony designated 6C9 was selected for further study, based on its competitive binding and ability to withstand expansion and preservation in liquid nitrogen. The 6C9 cells were cloned twice by limiting dilution in complete medium with 10% ORIGEN cloning supplement. Four clones, designated E6A6, E6F3, H7F7, and H7A7, were expanded and archived for further study. Isotyping, performed with a commercial kit (Pierce, Rockford IL), showed that all four clones were IgG₁κ. Unless otherwise specified, all experiments were done with MAb E6F3 culture supernatant.

4.2.4. Assay optimization

Effects of assay buffer ionic strength, pH, Tween-20, and solvents were done according to the procedures previously described [36]. In addition, plates were incubated after mixing equal volume of MAb and imidacloprid standard diluted in PBST at various time periods (30, 60, 90, or 180 min) to estimate the effect of incubation time. The remainder of the ELISA procedures was done under optimized.

4.2.5. icELISA

Microplate wells were coated with hapten I-BSA or hapten II-BSA (4 ng in 100 μL/well in 0.05 M carbonate-bicarbonate buffer, pH 9.6) overnight at 4 °C. The following day, the plates was washed 4 times with PBS containing 0.05 % Tween 20 (PBST) and then blocked with 1% BSA in PBS (200 μL/well) by incubation for 1 h at room temperature. The plates were washed again five times, a solution of 50 μL/well of analytes or standard diluted in PBST and 50 μL/well (0.1 μg antibody/well) of

imidacloprid MAb was added and incubated at 37 °C for 40 min. Peroxidase-labeled goat anti-mouse IgG (1:10,000 in PBST; 100 µL/well) was then added, and the plates were incubated 40 min at 37 °C. and the plates were again washed five times as above, and then substrate solution (100 µL per well of 0.05 M citrate-phosphate buffer, pH 5.0, containing 0.03% sodium perborate, and 1.0 mg/mL of OPD) was added. After 20 min at room temperature, the reaction was stopped with sulfuric acid (4 N, 50 µµL/well), and absorbance at 490 nm was read with a Vmax microplate reader (Molecular Devices, Sunnyvale, CA). Samples and standards were generally analyzed in four replicate wells. Inhibition curves were fitted with the four-parameter logistic equation using Softmax version 2.35 software (Molecular Devices)

4.2.6. dcELISA

The dcELISA was done as previously described [37]. Hapten II-HRP conjugate was used as an enzyme tracer.

4.2.7. Measurements of binding kinetics

Binding kinetics were determined using kinetic exclusion fluoroimmunoassay (KinExA). The principles, and details of the KinExA 3000 instrument (Sapidyne Instruments Inc., Boise, ID) and assay procedures have been described elsewhere [41-45]. In the experiments reported here, polystyrene beads of 98-µm diameter were obtained from Sapidyne Instrument, Inc. Aliquots of dry beads (200 mg) in 1.5-mL Eppendorf tubes were suspended and settled twice in 1mL of 6 N NaOH, and then washed successively in glass distilled water, PBS, and coating buffer. The beads were

suspended in 1 mL of coating buffer containing 0.2 mg of hapten II-BSA and the tube gently rolled at room temperature for 3 h. After the conjugating solution was discarded, the beads were washed three times with PBS, resuspended in blocking buffer (1% BSA in PBS), and mixed by rolling at room temperature for 2 h. Beads were washed with PBS three times again and stored at 4 °C until they were used for assay. On the day of use, 200-mg aliquots of coated beads were uniformly suspended in 27 mL of PBS. For each sample analyzed, a new bed of hapten conjugate-coated beads exactly 4 mm high (to match the width of the excitation beam) was deposited over the mesh trap in the capillary flow cell. To determine K_d , various concentrations of analyte and a fixed amount of the MAb solution were mixed and allowed to equilibrate for 1 h at room temperature. The solutions were passed through the KinExA's beads to capture MAb with unoccupied binding sites on the immobilized hapten. After a brief wash, a solution of Cy5-labeled goat anti-mouse IgG was passed through the beads, and after a brief wash, the fluorescence of the bound secondary antibody conjugate was used to quantify the primary antibody. To measure k_{on} a constant amount of antibody and various concentrations of an analyte were mixed by simultaneous injection, and the mixture was passed through a tube of known length, and then through the capillary containing the haptened beads, at a predetermined rate. After washing with PBS, the amount of bound primary antibody on the beads was quantified with Cy5-labeled goat anti-mouse IgG, as in the K_d determination. The off rate $k_{off} = k_{on} K_d$. Detailed calculations for determination of the amount of functional MAb, and all other parameters, including standard errors, may be found in reference [45].

4.2.8. Fortification of imidacloprid in water and cucumber

Water samples were collected from a tap in the laboratory and from the Manoa stream in Honolulu, Hawaii. Aliquots were fortified with imidacloprid to 0, 2, 10, 50, and 200 ng/mL, mixed with an equal volume of 2-fold concentrated PBST (24 mM phosphate, 274 mM NaCl, 5.4 mM KCl, 0.1% Tween 20, pH 7.5) containing a pre-determined limiting amount of MAb. Cucumbers were purchased from a local grocery store and homogenized to a free-flowing puree in a blender (Waring, USA). Aliquots (10 g) of the homogenate were fortified with imidacloprid standard in DMSO to 0, 10, 20, 50, 200, and 500 ng/mL. The fortified cucumber samples were extracted with 20 mL of MeOH for 30 min, and then centrifuged for 20 min at 10,000 rpm to remove the solids. The supernatants were filtered through a 0.45 μ m membrane. The sample extracts were analyzed with icELISA.

4.3. RESULTS AND DISCUSSION

4.3.1. Characterization of MAbs

Groups of mice were immunized with one of two different haptens (Figure 1) with the linkers attached to different ends of the molecule. Hapten-I has a linker on the nitro imidazolidinyl ring exposing the 6-chloro pyridinyl moiety. In hapten-II, the linker is on the pyridinyl ring, which presents the nitro imidazolidinyl moiety for antibody recognition. Two ELISAs for imidacloprid and two major metabolites (imidacloprid olefin and 5-OH-imidacloprid) were previously developed with antisera raised with these two haptens [35]. This study was expected to produce new MAbs for imidacloprid and possibly some of its metabolites. However, none of the mice immunized with hapten I-

KLH conjugate produced MAb able to compete with imidacloprid. Four MAbs derived from the mouse immunized with hapten II-OVA were tested for their competitive binding of imidacloprid in icELISA with each of the haptens. As shown in Table 1, none of the MAbs showed competitive inhibition with hapten I-BSA. Although all four MAbs were originally selected for binding to hapten II-BSA, three competitively recognized imidacloprid, and their I_{50} values were identical within experimental error. Thus, the four clones represented a minimum of two cell lines. MAb H7A7 cannot be used for competition ELISAs, but may prove useful for applications such as immunoaffinity cleanup of imidacloprid. Hybridoma lines E6A6, E6F3, and H7F7 produced MAbs with the same IgG1 κ isotype and I_{50} values. These cell lines may be identical clones, although this was not tested in other ways. Only supernatant from hybridoma E6F3 was used for the remainder of this study.

Table 4.1. Competitive binding of MAbs to imidacloprid haptens.

Coating antigen ^a	E6A6	E6F3	H7F7	H7A7
	I_{50} (ng/mL)			
hapten I-BSA	nc ^b	nc	nc	nc
hapten II-BSA	14.3 ± 0.4 ^c	14.6 ± 0.8	15.2 ± 0.6	nc

a. The coating antigen was at 5.0 ng in 100 μ L/well. b. No competitions were observed up to 5000 ng/mL of imidacloprid. c. I_{50} values obtained in icELISA. with hybridoma culture fluid diluted 1:100 in PBST. Each value represents the mean of four replicates, \pm the standard error

4.3.2. Competitive inhibition

Figure 4.2 shows representative standard curves for imidacloprid generated by the icELISA and dcELISA. The working ranges of the icELISA and dcELISA were approximately 0.1-4.0, and 0.03-4.0 $\mu\text{g/L}$, respectively. The I_{50} values of icELISA and dcELISA were 0.8 and 0.3 $\mu\text{g/L}$, respectively. Although use of dcELISA format gave a nearly 3-fold lower I_{50} value than that of icELISA, this improvement is small compared with our previous observations where dcELISA improved assay sensitivity 20-fold [36, 37]. Wanatabe *et al.* [39] produced three different MAbs using the same hapten as the one used in this experiment. Their ELISAs showed I_{50} values from 0.8 to 6.4 $\mu\text{g/L}$ and low cross reactivity to two neonicotinoids and some metabolites. Lee *et al.* [38] synthesized two different haptens to raise imidacloprid antisera. One had a linker attached via the nitro group and the other had the same structure as hapten-I used in this experiment, except for differences in the length and functional group of the linker. The former hapten failed to produce imidacloprid specific antisera in rabbits, indicating that the nitro guanidine ($=\text{N}-\text{NO}_2$) moiety may act as a key antigenic determinant. The latter hapten elicited antisera that competitively bound imidacloprid with an I_{50} of 17.3 $\mu\text{g/L}$. The I_{50} of our dcELISA was approximately 3 and 20-fold lower than that of Wanatabe's and Lee's, respectively.

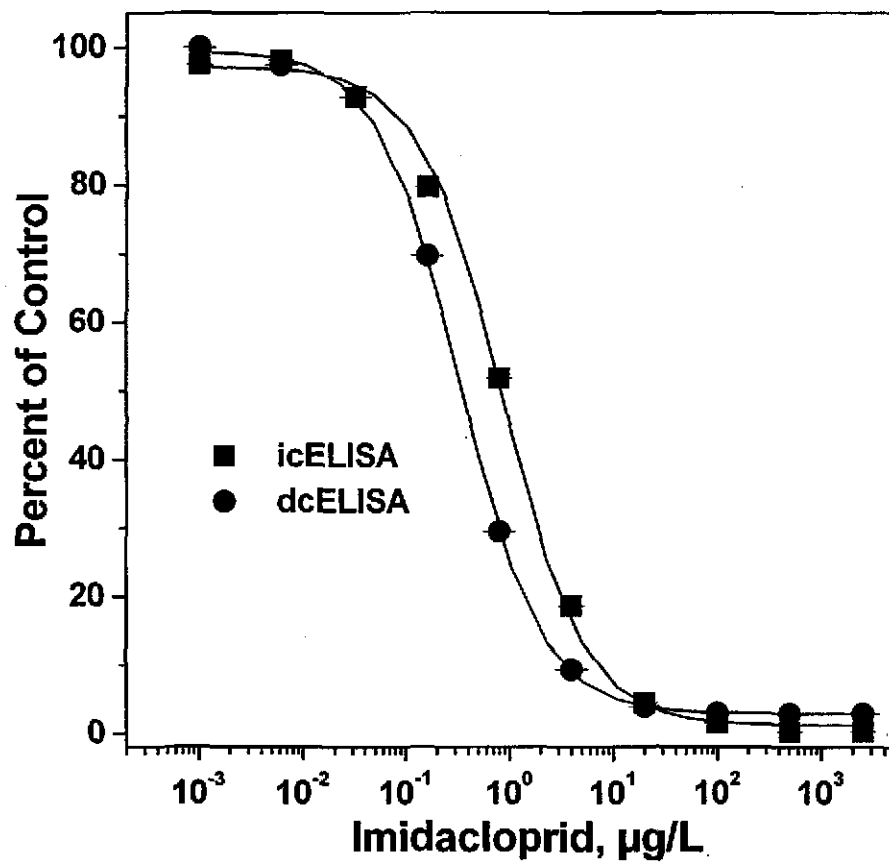


Figure 4.2. Standard inhibition curves of imidacloprid in dcELISA and icELISA formats. Plates were coated with 0.5 µg of purified MAb for dcELISA or 4.0 ng of coating antigen per well for icELISA. Each value represents the mean of four replicates

4.3.3. Physicochemical effects on assay performance

Immunoassay performance is often affected by chemical parameters such as ionic strength, pH, surfactant, organic solvent concentration, and substances in the sample matrix, as well as physical factors such as incubation time and temperature at different assay steps. The effects of these parameters were estimated by comparing I_{50} values obtained under various conditions with that of a control. The maximum absorbance (A_{max}), reflecting maximal binding to the competing hapten and the lowest I_{50} were observed at pH 7 (Figure 4.3). Although A_{max} values were lower at pH less than 6 and greater than 8, there were no significant changes in I_{50} values for assays run between pH 6 and 8. All subsequent assays were performed at pH 7.0. No significant changes in I_{50} values and A_{max} were observed over 1-10-fold concentrated PBS containing 0.05% Tween 20 (Figure 4.4). In practice, ionic strength of environmental samples can be adjusted by simple dilution with water or concentrated buffer. Although PBS with or without 0.05% Tween 20 showed no effect on assay sensitivity and A_{max} , addition of Tween 20 slightly reduced variations in absorbance among replicate wells so that Tween 20 at 0.05% was present in all assays. Incubation times of 30-180 min for the competition step did not change assay performance (Figure 4.5) so incubation at 37 °C for 40 min was adopted. In addition, the effects of DMSO, MeOH, acetone and acetonitrile were studied because these solvents are water-miscible and are commonly used in sample extractions. Figure 4.6 shows normalized dose-response curves at various solvent concentrations. In general, little effects of four solvents on A_{max} were observed when the assay was run in concentrations of solvents up to 20%. However, I_{50} values increased gradually as concentrations of acetonitrile and DMSO increased. MeOH and

acetone showed less effect on assay sensitivity (I_{50}) than acetonitrile and DMSO. I_{50} was negligibly affected by MeOH up to 20%, but it was approximately doubled in 20% acetonitrile. The presence of 20% DMSO resulted in a 38% drop in the A_{max} . The I_{50} , however, did not change appreciably. Although we observed some effect of acetonitrile and DMSO on assay sensitivity, we still obtained reproducible inhibition curves and usable I_{50} values. Thus, this assay accurately determines the concentration of imidacloprid in solvent extracts with less need for dilution. By comparison, the imidacloprid polyclonal antibody (PAb) was significantly affected by acetone and acetonitrile, but much less by MeOH and DMSO [36].

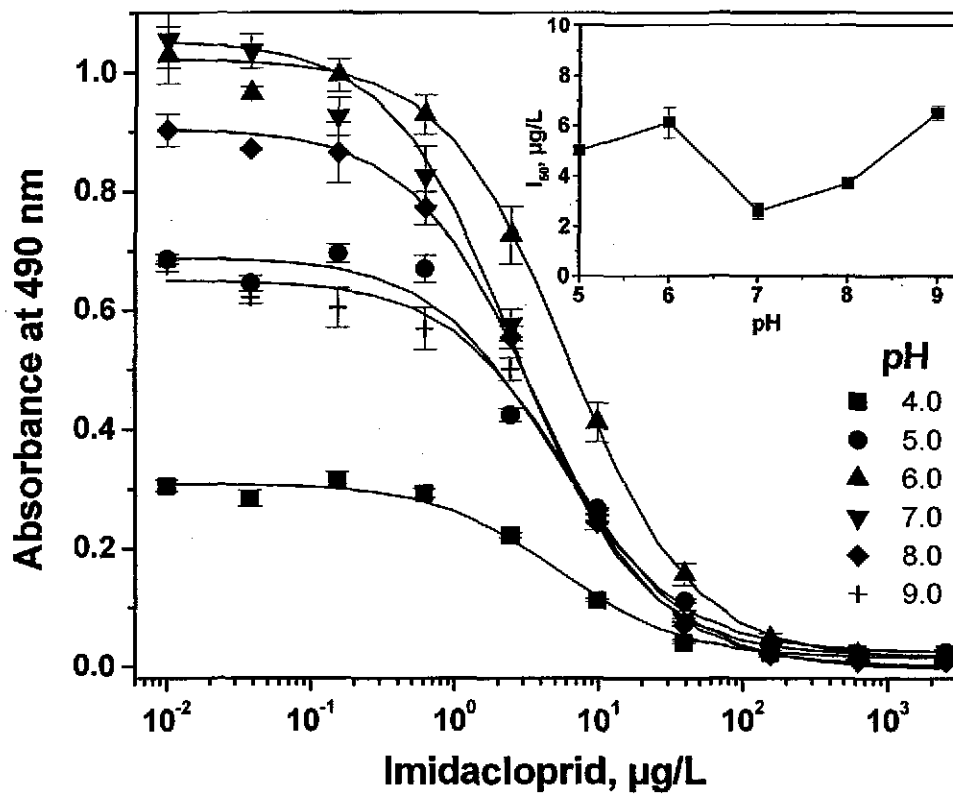


Figure 4.3. Effects of assay buffer pH. Each solid symbol represents the mean of four replicates.

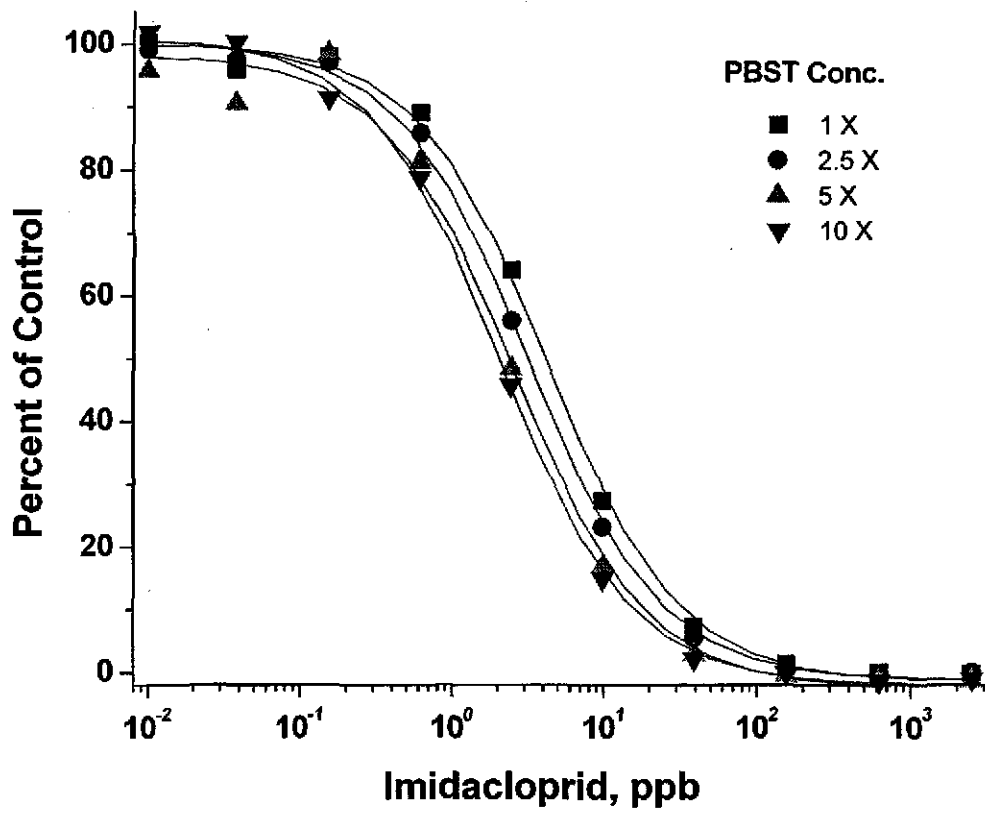


Figure 4.4. Effect of ionic strength on assay performance. Each value represent the mean of four replicates.

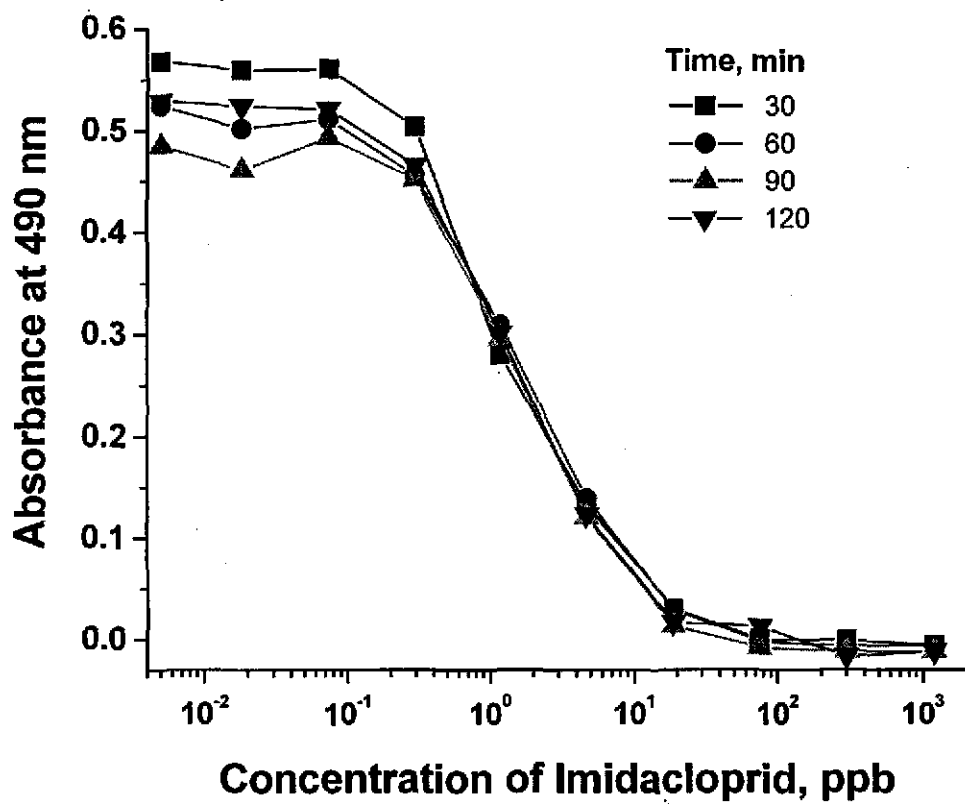


Figure 4.5. Effect of incubation time on assay sensitivity. Each value represents the mean of four replicates.

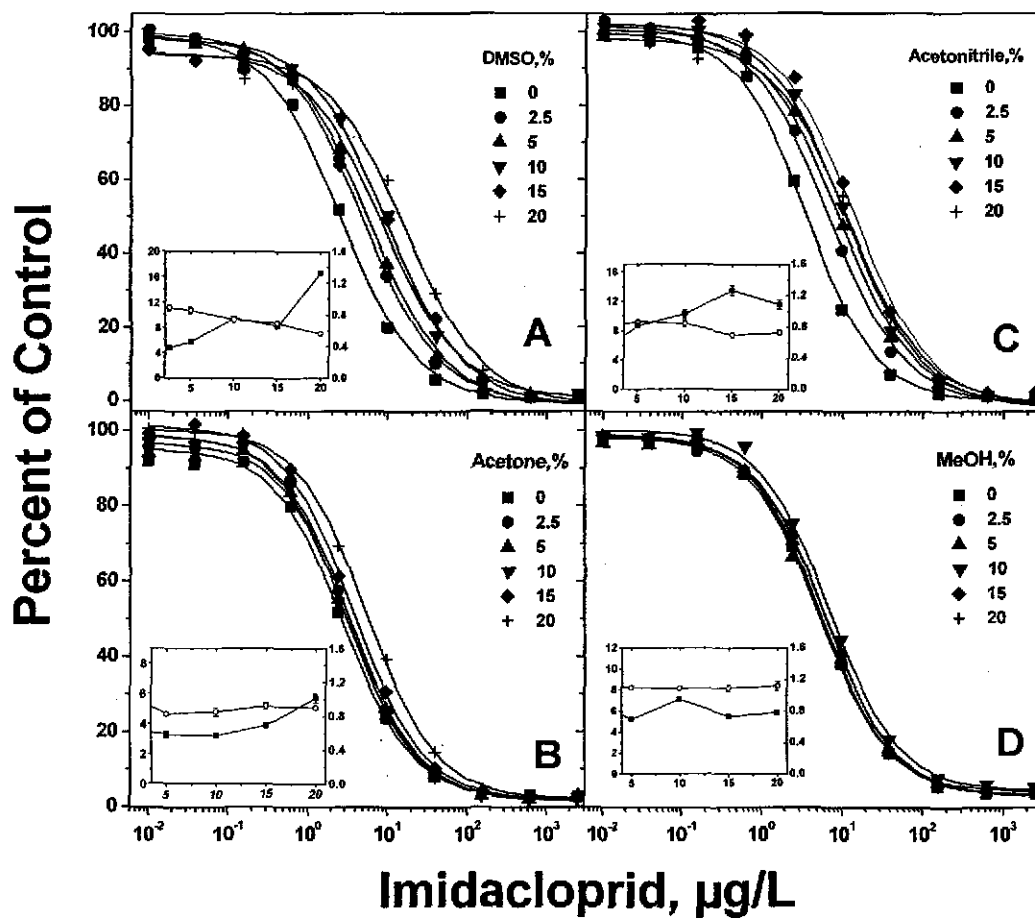


Figure 4.6. Effects of DMSO (A), acetone (B), acetonitrile (C), and MeOH (D) on the assay. Values refer to the final concentrations of solvents (v/v) in the competitive assay solution. Insets indicate the fluctuations of A_{max} (Right Y-axis) and I_{50} (Left Y-axis) (O: A_{max} , ■: I_{50}) as a function of solvent concentration (X-axis). Each value represents the mean of four replicates

4.3.4. Kinetics of MAb binding and cross reactivity

Table 4.2 shows the K_d , k_{on} , and k_{off} values determined by KinExA for the MAb E6F3. Also presented for comparison are I_{50} data obtained with icELISA. MAb E6F3 was very specific for imidacloprid with a cross reactivity of < 4.0% with other neonicotinoid insecticides (Table 4.2). The K_d values determined by KinExA for clothianidin and acetamiprid were very similar, despite a large difference in I_{50} values for these compounds in icELISA. Dinotefuran, thiamethoxam and 6-chloronicotinic acid were not detectable by MAb E6F3 in KinExA or ELISA. The on-rate (k_{on}) of imidacloprid, which was directly measured by KinExA, was approximately 10 times faster than that of clothianidin or acetamiprid. The k_{off} value of imidacloprid (calculated from K_d and k_{on}) was approximately five times slower than that of clothianidin and acetamiprid. This was somewhat unusual, because antibodies generally bind small molecules with similar, fast on-rates and different off-rates [46].

The KinExA and ELISA results may be explained in part by the computational models of the neonicotinoids and haptens that we described previously [37]. The weak affinity of MAb E6F3 for clothianidin and acetamiprid is likely due to lack of the imidazolidine ring, which probably accounts for much of imidacloprid's binding. The 2-chlorothiazole ring of clothianidin is electronically and sterically very similar to the chloropyridine ring of imidacloprid or acetamiprid and could be reasonably expected to occupy the same binding site as imidacloprid. The computational modeling suggested that steric constraints of the thiadiazinane ring prevent the nitro group of thiamethoxam from lying in the plane of the thiadiazinane ring as it is able to in imidacloprid and acetamiprid. The nitro group of thiomethoxam would be oriented perpendicularly to the

thiadiazinane ring, so that the dihedral angle of the nitro group in thiamethoxam would be quite different from that in imidacloprid [37]. Clothianidin was bound weakly by MAb E6F3, possibly due to the similar alignment of its nitro group in its structure as that of thiamethoxam. Dinotefuran has a tetrahydrofuran group that does not resemble the chlorpyridine groups in imidacloprid electronically or sterically. This may explain why MAb E6F3 does not bind dinotefuran.

4.3.5. Imidacloprid-fortified water and cucumber samples

Tap and natural stream water and cucumber samples spiked with imidacloprid were analyzed by icELISA. Ionic strength of the samples was adjusted by addition of an equal volume of 2-fold concentrated PBST containing a fixed amount of MAb. The recovered concentrations of imidacloprid by ELISA correlated well with the spike concentration, with a correlation coefficient of 0.99 for both water and cucumber samples and with overall mean recovery ranged from 70 to 120% (Table 4.3). The results showed that the ELISA can accurately measure the concentration of imidacloprid in environmental matrices.

Table 4.2. Binding characteristics of MAb E6F3 to neonicotinoid insecticides and 6-chloronicotinic acid.

Compound	KinExA			ELISA		
	K _d (nM)	CR ^a (%)	k _{on} (M ⁻¹ s ⁻¹) x 10 ⁶	k _{off} (s ⁻¹)	I ₅₀ (nM)	CR (%)
Imidacloprid	0.7 ± 0.17	100	14.40 ± 3.96	0.010	6.2	100
Clothianidin	35.7 ± 3.4	2.0	1.63 ± 0.085	0.058	175.1	3.6
Acetamiprid	44.6 ± 9.5	1.6	1.16 ± 0.113	0.052	1078	0.6
Thiamethoxam	nc ^b		nc		nc	
Dinotefuran	nc		nc		nc	
6-Chloronicotinic acid	nc		nc		nc	

^aCR = Cross reactivity

^bNo competition up to 5.0 µg/mL of each compound.

Table 3. Percentage recovery of imidacloprid fortified to water and cucumber samples by the icELISA

Sample	Imidacloprid		S.D. ^a	Mean recovery (% n = 4)
	fortified (ng/mL)	detected (ng/mL)		
Tap water	0	nd ^b		
	2	1.8	0.3	92.0
	10	8.6	0.7	85.5
	50	49.4	4.5	98.8
	200	208.0	16.8	104
Stream water	0	nd		nd
	2	2.5	0.4	113.2
	10	10.1	0.9	100.7
	50	52.7	4.5	105.5
	200	239.9	42.6	119.9
Cucumber	0	< 0.04		
	10	7.03	0.1	70
	50	39.5	0.2	79
	200	193.8	22.8	97
	500	535.7	19.1	107

^aS.D., standard deviation; ^bnd, not detected.

4.4. CONCLUSIONS

A sensitive imidacloprid-specific ELISA based on a monoclonal antibody was developed. Separate groups of mice were immunized with the different hapten conjugates, in an attempt to produce MAbs with different specificities. Hapten II-OVA conjugate elicited imidacloprid specific MAbs. However, hapten I-KLH conjugate was not effective in producing a MAb to recognize imidacloprid or its metabolites. KinExA and ELISA revealed different aspects of binding by MAb E6F3. Acetamiprid and clothianidin showed weak binding in icELISA and KinExA. Dinotefuran and thiamethoxam were not bound by E6F3 in either assay, a result consistent with their structural and electrostatic properties. The satisfactory recoveries and correlation between measured and fortified concentrations in two water samples and cucumber homogenate suggest that the assay can be used to quantify imidacloprid residues in these matrices. However, further work will be needed to validate this assay for other application.

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Chapter 5

Production and characterization of thiamethoxam specific MAbs

5.1. INTRODUCTION

In chapter 3, development of an ELISA for thiamethoxam using an antiserum was described. Three antisera were raised with the thiamethoxam hapten which was designed with computational analysis with three hapten candidates. An ELISA showed a good affinity and specificity to thiamethoxam with I_{50} of approximately $1\mu\text{g/L}$ and low cross reactivity to structurally related compounds.

To date, no MAb based ELISA for thiamethoxam has been developed so that we attempted to produce thiamethoxam specific MAbs. A MAb based immunoassay is advantageous over an antiserum based one even though use of antiserum is prevalent in environmental immunoassay. Hybridoma technology enables one to obtain a single type of pure antibody by cloning a single cell line. Growing an individual antibody-producing cells provides an unlimited source of a single MAb with a distinctive affinity and specificity. This also overcomes the limitations of polyclonal antisera, which contain a population of antibodies with various percentage of desirable and undesirable antibodies. In addition, it is impossible to obtain antisera with particular properties when immunized animals die.

In this chapter, production and characterization of thiamethoxam MAbs are described. Hapten-KLH conjugate that was used for the production of thiamethoxam antisera was again used as an immunogen for MAbs production with the expectation that this immunogen may elicit MAbs specific to thiamethoxam.

5.2. EXPERIMENTAL

The reagents that were used in this experiment were the same as those chapter 3 and 4. MAbs were generated according to the procedures described in chapter 4. icELISA and dcELISA were carried out according to the procedures described in two chapters with slight modification for the dcELISA, which the plates were coated with an excessive amount of goat anti-mouse antibody first to capture the primary antibody. Mice were immunized with the same immunogen used for the production thiamethoxam antisera in chapter 3.

5.3. RESULTS AND DISCUSSION

Through the extensive screening of hybridoma cells, six positive clones were obtained and supernatants were harvested. The checkerboard titration was carried out with each of MAb to estimate their titers and determine the optimal dilution of MAbs and coating antigen (thiamethoxam-BSA) for the competitive ELISA. Figure 5.1 shows absorbance of each MAb obtained with noncompetitive indirect ELISA at 5 ng/well coating of thiamethoxam hapten-BSA and various dilutions of MAbs. In general, absorbance was gradually decreased as dilution of MAb or coating antigen was increased. MAb C10 XI shows no binding affinity to thiamethoxam with background signal at all combination of coating antigen and C10 XI.

An icELISA was performed to test five MAbs for their binding to free thiamethoxam. On the basis of checkerboard titration results, plates were coated with 5 ng of coating antigen/well and 1:20 dilution of each MAb was mixed with various concentrations of thiamethoxam standard diluted in PBST for the competition. Table 5.2 shows the results

of icELISA. Each value was obtained from standard inhibition curve generated by Vmax software.

As shown in Table 5.2, all MAbs show low I_{50} values (0.056 - 24.7 ng/mL) and good slopes from 0.8 to 1.6. These data indicate that these MAbs can be used to develop sensitive ELISAs. MAb G2 I shows an I_{50} of 0.056 ng/mL. To our knowledge, this is the lowest I_{50} among MAbs that have ever been reported for pesticide detection. With MAb G2 I, thiamethoxam residues could be detected at part per trillion level, which makes sample preparation much simple and fast. MAb A11 III and E6 VI have similar affinities to thiamethoxam with I_{50} of 0.24 and 0.13, respectively. I_{50} values of MAb D4 VIII and B12 XIII were 24.7 and 1.21 ng/mL, respectively.

Although all MAbs appear to be good candidates for assay development, MAb G2 I was selected for the cross-reactivity test due to its lower I_{50} . G2 I MAb supernatant was purified with a protein G affinity column. It is known that dcELISA is more sensitive than icELISA. Prior to optimization, double coated dcELISA was used with purified G2 I MAb. However, an I_{50} of dcELISA was five-fold higher than that of icELISA, indicating assay optimization is necessary. Cross-reactivities of G2 I to other neonicotinoids was tested with dcELISA. Table 5.3 shows cross reactivity of G2 I to four neonicotinoids insecticides. As shown in Table 5.3, G2 I is very specific to thiamethoxam. It shows no recognition to acetamiprid, dinotefuran, and imidacloprid, and little recognition to clothianidin with 0.16% cross reactivity.

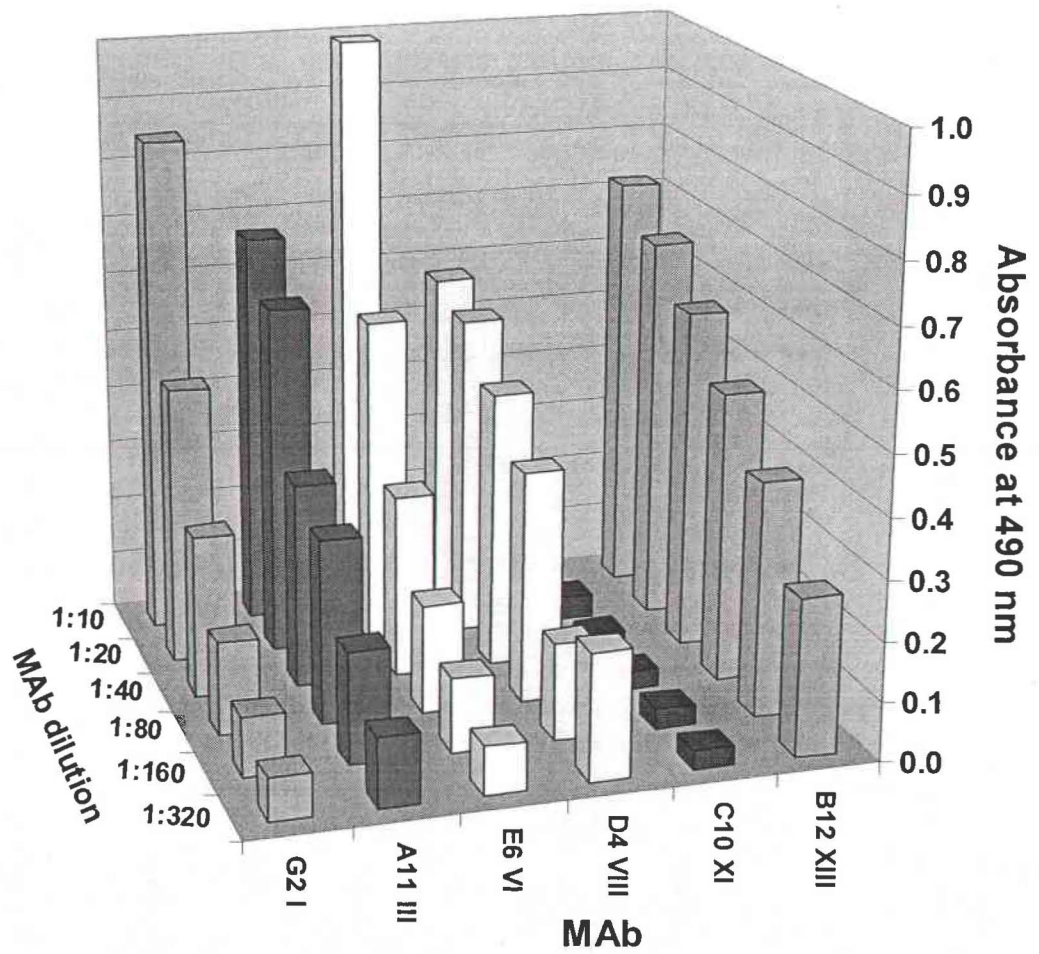


Figure 5.1. Titers of six MAbs. Plates were coated with 5 ng of coating antigen per well.

Table 5.2. Summary of icELISA^a

MAB	A _{max} (A) ^b	Slope (B)	I ₅₀ (ng/mL), (C)	A _{min} (D) ^c
G2 I	0.507	0.851	0.056	0.00206
A11 III	0.711	1.38	0.24	0.000571
E6 VI	0.631	0.801	0.130	0.00831
D4 VIII	0.826	1.02	24.7	0.00202
B12 XIII	1.15	1.6	1.21	0.0124

^a assay conditions: 5 ng of coating antigen per well, 1:20 dilution of each MAb. Data shown are the means of quadruplicate. ^bMaximum absorbance. ^cMinimum absorbance.

Table 5.3. Cross-reactivity of MAb G2 I to four neonicotinoids

Compound	I ₅₀ (ng/mL)	Cross-reactivity, %
Thiamethoxam	0.275	100
Clothianidin	174	0.16
Imidacloprid	NC ^a	
Dinotefuran	NC	
Acetamiprid	NC	

^ano competition up to 5,000 ng/mL

Chapter 6

SUMMARY, POTENTIAL APPLICATION OF ASSAYS, AND FUTURE WORK

6.1. Summary

This dissertation describes the development of ELISAs based on the antisera and MAbs for the detection of the neonicotinoid insecticides imidacloprid and thiamethoxam in environmental matrices. Imidacloprid and thiamethoxam were selected as target compounds because their world-wide application for the control of various sucking and chewing insects, concerns associated with leaching into surface or ground water due to high persistence in soil, good water solubility and high toxicity to some aquatic organisms, labor intensive work for the instrumental analysis make ELISAs very useful to detect two representative insecticides among various neonicotinoids. The researches for this purpose were described through five separate chapters.

In chapter 2, dcELISA format was utilized and assay conditions were optimized as an attempt to improve assay sensitivity. Imidacloprid hapten was synthesized again and conjugated to HRP for an enzyme tracer. The dcELISA condition was optimized for buffer concentrations and pH, solvents, and Tween 20 concentration. Comparison between I_{50s} of two ELISA formats showed that assay sensitivity was 35-fold improved with a dcELISA and a purified polyclonal antibody (designated as Ab-IIa)

Computational analysis was carried out to evaluate the correlation between structures of chemicals and antibody binding. The results suggested that the antibody specificity

primarily relate to the dihedral angles between the two rings, steric hindrance and electrostatic charges on the imidazolidinyl ring. Those parameters can not be predicted without computational analysis therefore, such information is useful for hapten design.

Chapter 3 describes the development of an *ELISA* for thiamethoxam based on the thiamethoxam specific antiserum. As shown in chapter 2, a computer modeling technique could be a powerful tool for a hapten design because it allows the prediction for the degree of dihedral angle and electron density over the molecule. To produce antisera specific to thiamethoxam, hapten was designed and synthesized by selecting the best location for a spacer arm attachment through computational analysis with three possible hapten candidates in terms of RMS deviations, dihedral angles and molecular dimensions of three candidates.

In chapter 4, development of another *icELISA* for imidacloprid was described. Two haptens were synthesized following previous procedures. One has a spacer arm on the pyridinyl ring common to metabolites of imidacloprid and the other one on the imidazolidinyl ring characteristic to imidacloprid. The first was expected to produce MAbs with broad specificities to imidacloprid and its metabolites and the second to produce imidacloprid specific MAbs. Although we were able to produce imidacloprid specific MAbs, we failed to produce MAs able to bind metabolites of imidacloprid. Our and other experiments revealed that preservation of nitroguanidine moiety ($=N-NO_2$) is important for an immune response to this compound. The equilibrium constants (K_d) and association and dissociation rate constants (k_{on} , k_{off}) for five neonicotinoids and one imidacloprid metabolite to E6F3 were determined by KinExA. Affinities ($1/K_d$) of E6F3 for acetamiprid and clothianidin were similar, but fifty-fold weaker than that of imidacloprid. MAb E6F3 had no measurable affinity for other neonicotinoids and analogs.

Chapter 5 describes the production and characterization of thiamethoxam MABs. The immunogen used the experiment in chapter 3 was injected into mice.

Table 6.1 summarizes the general performance of four ELISAs. ELISAs except for MAb G2 I were optimized for solvents, buffer pH, and ionic strengths of buffer. Ab IIa and Ab I showed the same tolerance up to 5% of acetonitrile and acetone, respectively. MAb E6F3 was tolerated up to 20% of MeOH and 15% of acetone. Although solvent effect on MAb G2 I performance was not estimated, MAb E6F3 showed higher tolerance against tested solvents than antisera. This does not mean that MABs are always more tolerant to solvents than antisera. Various pHs (4-9) and ionic strengths (1-10X PBST) negligibly influenced assay sensitivities for three ELISAs. This indicates that sample extracts can be simply diluted to adjust ionic strength. Under optimized conditions, ELISAs showed I_{50} values from 0.06 to 9 ng/mL. MAb G2 I gave the lowest I_{50} of 0.06 ng/mL which is the lowest sensitivity among ELISAs for such small molecules that have been known.

All of the antibodies were specific to target compounds with low overall cross-reactivity $\leq 9\%$ to structurally related chemicals. Ab IIa gave 85-110% recovery when tap water was analyzed. 70-120% recoveries were observed with MA E6F3 for spiked tap and stream water and cucumber samples. Ab I showed 90-105% recoveries for spiked tap and water samples. Although recovery test was not conducted with MAb G2I, very low I_{50} value indicates that this MAB could accurately determine thiamethoxam residue in more complex environmental matrices because interferences can be effectively diluted out by extensive dilution of sample extracts.

6.2. Potential application and future work

Several ELISAs from our and other laboratories have been developed for imidacloprid, thiamethoxam and acetamiprid. In general, the assays are well developed and are suitable for uses in environmental and biological monitoring. They have been preliminarily evaluated for groundwater and surface water, cucumber, green pepper, tomato and apple. These assays can be useful tools to aid the best practices of pest management using the neonicotinoids as well as to protect human and environmental health and safety from these insecticides. In addition, because of the systemic nature of neonicotinoids, these assays can be a powerful tool to study insect population dynamics and the evolution and spread of insect resistance to neonicotinoids by detecting the total residues in insects. Such studies may provide researchers with an ability of designing refuge crops for effective control of insects, particularly for the genetically modified crops.

Although these ELISAs appear to be promising in detecting individual insecticide in environmental samples, these can not be used to detect metabolites of neonicotinoids due to the compound specificities of developed antibodies. Given that neonicotinoids are quickly metabolized into several compounds and some of metabolites are more toxic than their parent compounds, a class specific ELISA may be more useful to monitor neonicotinoids. In relation to this, careful redesign of hapten with computer modeling technique may enable a developer to produce antibodies with broad specificities. However, when the practical options for hapten synthesis are so limited, that it may not be possible to derive additional antibodies with the desired selectivities by conventional immunization and hybridoma technology. Antibody engineering could be a good tool to

make customized antibody. DNA sequences of selected antibodies could be determined, and the amino acid sequences deduced. Concurrent advances in computational molecular modeling made it possible to visualize the ligand binding site and identify the bonding interactions between the ligand and amino acid side chains on the antibody. Methods of in vitro mutagenesis could be used to test hypotheses based on the structure model and attempt to create new recognition, solvent tolerance, and other properties.

Table 6.1. Performance of ELISAs in this dissertation

Compound	Antisera or MABs	Chapter	ELISA	I ₅₀ (ng/mL)	C.R. ^a , %	Solvent tolerance	pH (4-9)	Ionic Strength (1-10 X)	Sample analysis and percent recoveries
Imidacloprid	Ab IIa (antiserum)	2	dcELISA	1	≤ 9	5% ACT ^b 5% ACN ^c	NE ^e	NE	Tap water (85-110)
	E6F3 (MAB)	4	icELISA	0.8	≤ 4	20% MeOH ^d 15% ACT	NE	NE	Tap and stream water Cucumber (70-120)
Thiamethoxam	Ab I (antiserum)	3	dcELISA	9	< 2	5% ACT 5% ACN	NE	NE	Tap and stream water (90-105)
	G2 I (MAB)	5	icELISA	0.06	< 0.16	NT ^f	NT	NT	NT

^aC.R.=cross reactivity of antibodies to other neonicotinoids; ^bACT=acetone; ^cACN=acetonitrile; ^dMeOH=methanol; ^eNE=negligible effect; ^fNT=not tested

Appendix 1

Supplementary Data for the Determination of K_d and k_{on} for MAb E6F3 with KinExA.

The procedures for the determination of K_d and k_{on} for E6F3 against imidacloprid and several neonicotinoids were briefly described in chapter 4. Several graphs and error analysis curves related to kinetics for imidacloprid were exemplified in Appendix 1.

K_d value for MAb E6F3 was calculated by quantifying unbound antibody at equilibrium. Ten mixtures of a constant amount of antibody with varying concentrations of imidacloprid were allowed to reach equilibrium by 1-2 h incubation. Buffer only and the solution with antibody only were used as 0% and 100% signal, respectively. These solutions were introduced through the sample ports, and passed through hapten-BSA coated beads, followed by injection of Cy-5 conjugated goat anti-mouse IgG (1 $\mu\text{g}/\text{mL}$) through injection port to detect captured E6F3. Difference in fluorescent signals between samples and background refers to amount of free primary antibody captured to beads (Figure A.1).

Concentration of active binding sites (AbC) was measured following the procedure for K_d determination described above except that concentration of MAb for AbC determination was fifty-fold higher than that for K_d determination. Determination of AbC is essential to measure on-rate because long-term storage, chemical modification, or purification change percentage of active binding sites in the antibody stock solution, which significantly influence the results of kinetic measurement. This step ensures

accurate determination of kinetics. The graph and error analysis curve in Figure A.2 indicates that running the K_d experiment in fifty-fold higher concentration of MAb than that for K_d measurement in Figure 1 generates an accurate active-site concentration of MAB E6F3. AbC value of E6F3 was 1.886×10^{-9} M. Although accurate AbC value was obtained with KinExA, percentage of active antibody in purified E6F3 solution was unknown because we did not measure total concentration of E6F3. Prototype KinExA required a user to measure the total concentration of antibody and construct a Scatchard plot with ratio of bound/free active sites. However, this step is not necessary for KinExA 3000 that we used in this experiment.

k_{on} was determined with a fixed amount of antibody and each of twelve different concentrations of imidacloprid diluted in PBST simultaneously injected through injection and sample ports, respectively. The K_d , molar concentration of active antibody, and the time allowed for the antigen and antibody to react must be known prior to on-rate experiment. In contrast to K_d and AbC determination, the concentration of antibody is not an important factor in k_{on} determination. Results of the k_{on} analysis for imidacloprid are shown in Figure 3.

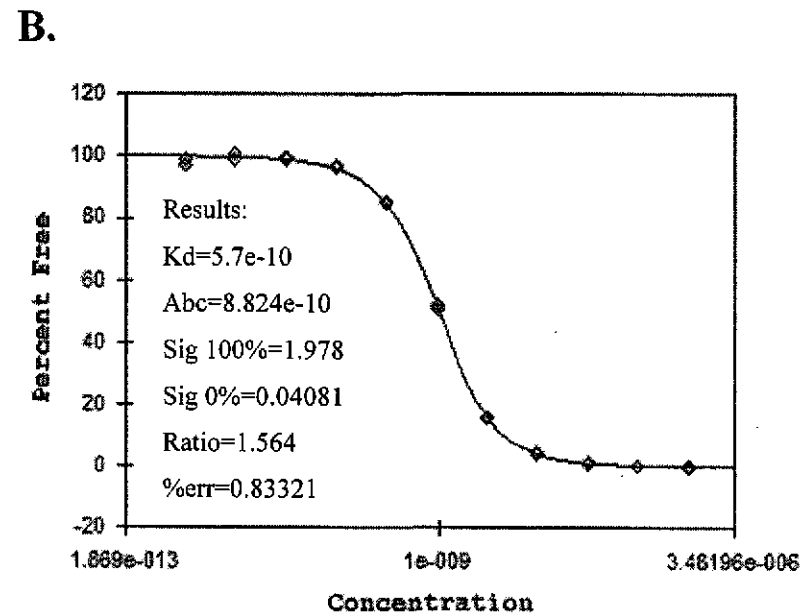
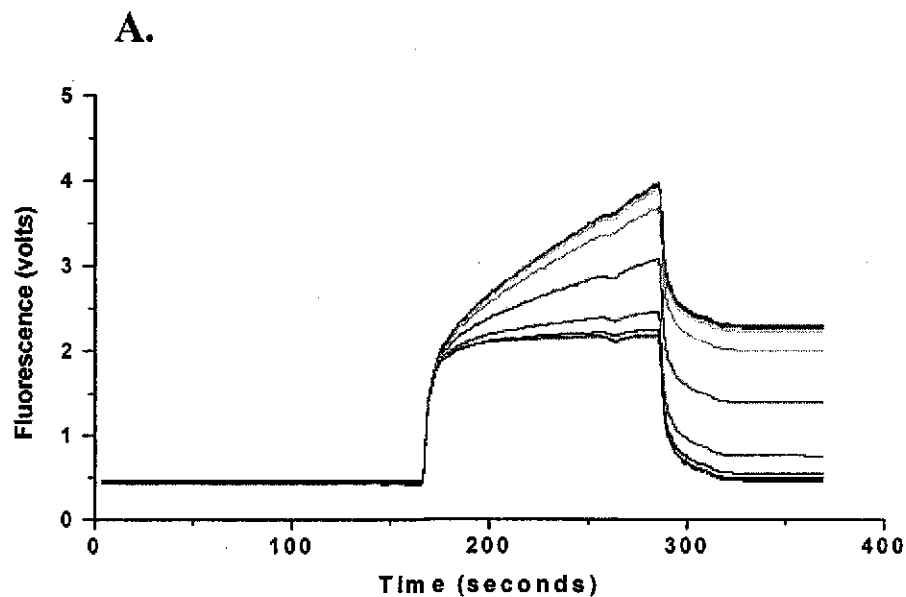
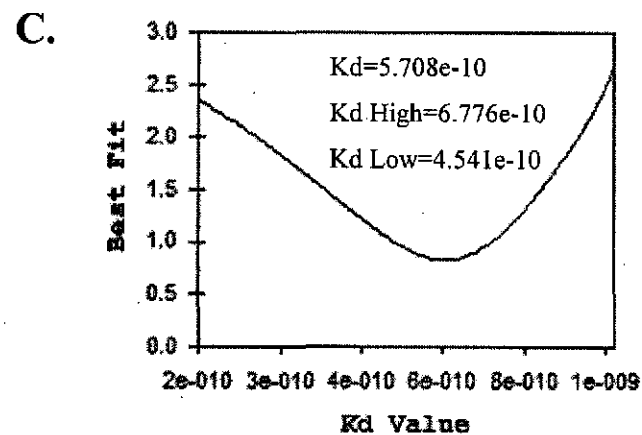


Figure A.1. Determination of K_d for MAb E6F3 against imidacloprid. A: Binding of probe to captured MAb was recorded in real time course. Signal is inversely proportional to concentration of analytes. B: KinExA software calculates K_d using input data (fluorescent signal and analyte concentrations). C: Error analyses show how well the data fit to different values of K_d .



A

Results:

$K_d=1.882e-12$

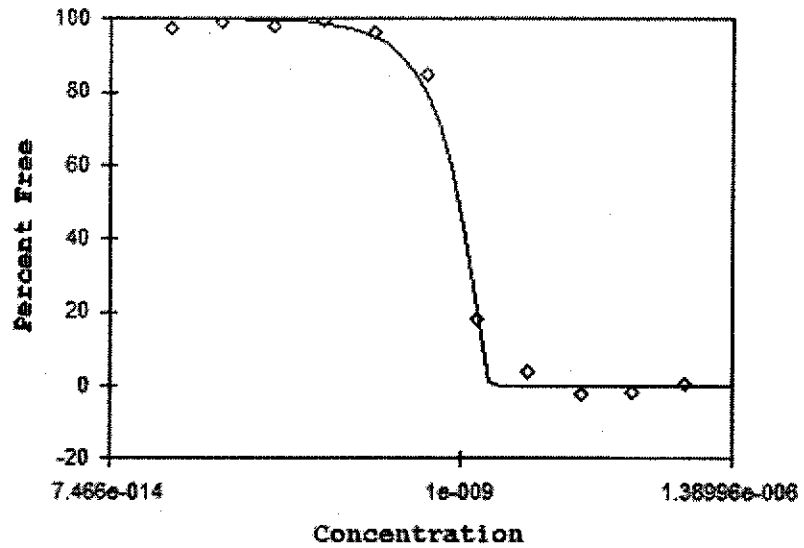
$AbC=1.909e-9$

Sig 100%=7.307

Sig 0%=0.2524

Ratio=1014

%err=2.275



B

$AbC=1.886e-9$

$AbC\ High=2.075e-9$

$AbC\ Low=1.5e-9$

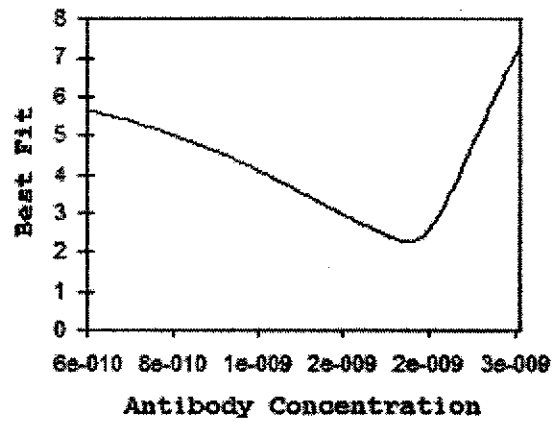
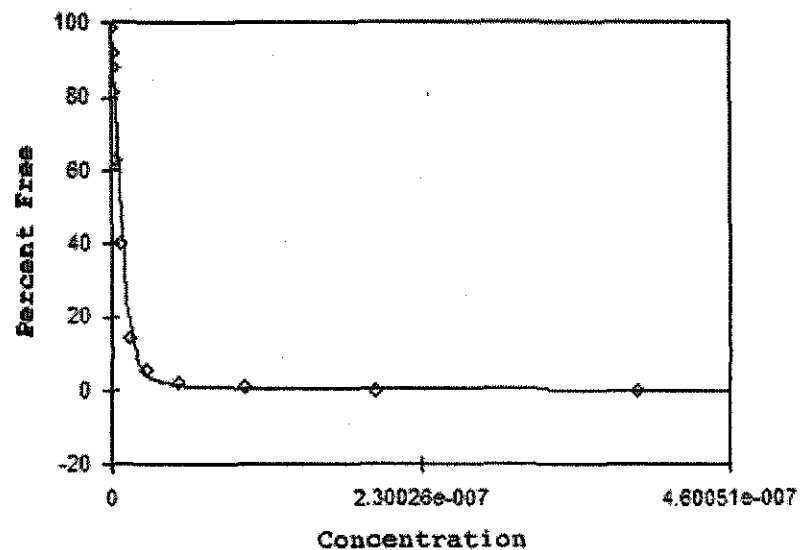


Figure A.2. Determination of active binding sites of MAb E6F3. A: Procedures were similar to that for K_d measurement except that fifty-fold concentrated MAb was added. B: Error analysis show how much accurately the AbC value was calculated.

A

Concentration (M)	Signal (V)	Given:	Calculated results:
3.912e-7	0.1131	$K_d=7.167e-10$	$k_{on}=1.051e+007$
1.956e-7	0.1057	$AbC=1.455e-9$	$k_{off}=0.007532$
9.78e-8	0.1302	Time=17.5	Sig 100%=2.391
4.89e-8	0.1532		Sig 0%=0.1098
2.445e-8	0.228		% err=1.228
1.223e-8	0.435		
6.115e-9	1.023		
3.057e-9	1.534		
1.529e-9	1.968		
7.645e-10	2.214		
3.823e-10	2.209		
0	2.354		



B

$k_{on}=1.051e+7$
 $k_{on} \text{ High} = 1.178e+7$
 $k_{on} \text{ Low} = 9.738e+7$

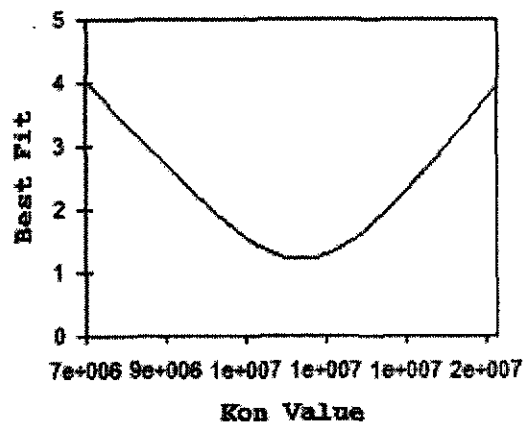


Figure A.3. Determination of on-rate for MAb E6F3. A: Twelve different concentrations of imidacloprid was reacted with a constant amount of E6F3 for 17.5 seconds. Pre-determined K_d , AbC , and time were input for on-rate determination. Upper right side shows calculated results. B: An error analysis shows how well the data can fit to different values of k_{on} .