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Automated flow fluorescent immunoassay for part per trillion detection of the neonicotinoid insecticide thiamethoxam

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Abstract

An ultra sensitive automated flow fluorescent immunoassay was developed using the KinExA™ 3000 system for quantitative analysis of the neonicotinoid insecticide thiamethoxam. Five new monoclonal antibodies were obtained and screened with a competitive ELISA. One monoclonal antibody designated as E6VI was evaluated for sensitivity, selectivity and solvent tolerance with the KinExA. Sensitivity determined from the concentration of half-maximal inhibition (IC_{50}) was obtained by plotting KinExA signals to a four-parameter sigmoidal curve as a function of analyte concentrations. For the most sensitive clone, the IC_{50} and the limit of detection were approximately 30 pg ml^{-1} and 16 pg ml^{-1}, respectively. Cross-reactivity was estimated by measuring the equilibrium constants (K_d) for four other neonicotinoid insecticides (clothianidin, imidacloprid, dinotefuran, and acetamiprid). E6VI was very specific to thiamethoxam with <0.11% cross-reactivity for tested neonicotinoids. An excellent correlation ($r^2 = 0.99$) was obtained between spiked and measured concentrations of thiamethoxam in stream and tap water, potato, cucumber, and apple samples.

Keywords: Thiamethoxam; Kinetic exclusion assay; KinExA; Immunoassay; ELISA; Insecticide

1. Introduction

Thiamethoxam, 3-[(2-chloro-5-thiazoly)methyl]tetrahydro-5-methyl-*N*-nitro-4H-1,3,5-oxadiozin-4-imine, belongs to a relatively new class of insecticides known as neonicotinoids which act as agonists of the post-synaptic nicotinic acetylcholine receptors [1,2]. Thiamethoxam and imidacloprid are representatives of two classes of neonicotinoid insecticides. Thiamethoxam is a chlorothiazoyl derivative and imidacloprid is a chloropyridinyl derivative.

Advantages of neonicotinoids including a broad spectrum of insecticide activity and low acute mammalian toxicity have led to replacement of the organophosphates, carbamates and synthetic pyrethroids that previously dominated the world market. The extensive use has led to concern about leaching of neonicotinoids into surface and ground water by run-off or wind drift [2], since these compounds show high toxicity to some aquatic organisms.

High performance liquid chromatography (HPLC) with UV detection or HPLC–mass spectrometry (HPLC–MS) are currently preferred for the determination of neonicotinoids in environmental samples [3–8]. Although these methods have provided reliable and accurate quantification of neonicotinoids, they involve tedious sample extraction and cleanup procedures. The thermolability and high polarity of neonicotinoids make them difficult to analyze by gas chromatography (GC) or GC–MS, requiring derivatization of the analytes prior to analysis [9–12].

Immunoassay has proven to be a good analytical method for rapid monitoring of agrochemicals without multiple sample cleanup steps. To date, several enzyme-linked immunosorbent assays (ELISAs) based on monoclonal and polyclonal antibodies for neoniconinoids have been developed [13–18]. These ELISAs are very simple to execute and have been demonstrated to accurately determine neonicotinoids in environmental samples. To further improve the assay of neonicotinoids, we describe the development of an automated flow fluorescent immunoassay

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with Kinetic Exclusion Assay system (KinExA™ 3000, Boise, Indiana, USA) for the quantitative analysis of thiamethoxam.

KinExA systems are used primarily to determine true liquid-phase equilibrium dissociation constants (K_d) and association rate constants (k_{on}) of antibodies by measuring the amount of antibodies in an equilibrium state with ligands in solution phase [19–21]. Limited use of KinExA was explored for quantitative analysis [22–25]. Polyclonal antibody-based ELISA was previously reported [17]. In this study, we describe the production of five new monoclonal antibodies (MAbs) specific to thiamethoxam, selection of the best one using the competitive ELISA, and an application of this MAb to develop an automated flow fluorescent KinExA assay for the determination of thiamethoxam in water and crop samples.

2. Experimental

2.1. Reagent

All reagents were of analytical grade unless specified otherwise. Reference standards of clothianidin (99.9%), acetamiprid (99.5%), imidacloprid (99.5%), thiamethoxam (99.7%), and dinotefuran (99.7%) were kindly provided by the National Institute of Agricultural Science and Technology, South Korea. Purchased from Sigma (St. Louis, MO, USA) were bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), goat anti-mouse IgG-horseradish perosidase (IgG-HRP), phosphate-citrate buffer capsules with sodium perborate, carbonate-bicarbonate buffer capsules, complete and incomplete Freund's adjuvant, polyethylene glycol 1500 (PEG), DMSO, and *o*-phenylenediamine (OPD). The monoclonal antibody isotyping kit was purchased from Pierce (Rockford, IL, USA). HT, HAT and RPMI 1640 supplements were bought from Life Technologies (Grand Island, NY, USA). Fetal bovine serum from Hyclone (Logan, UT, USA) was heat inactivated at 56 °C for 30 min prior to use. Hybridoma cloning factor (ORIGEN) was obtained from Fisher Scientific (Pittsburgh, PA, USA). Murine myeloma cell line Sp2/0Ag14 was purchased from the American Type Culture Collection (Manassas, VA, USA; ATCC catalog No. CRL-1581). The ELISAs were carried out in 96-well polystyrene microplates (MaxiSorp F96; Nalge Nunc International, Copenhagen, Denmark). The protein A affinity purification kit was purchased from Pierce. Goat anti-mouse IgG conjugated with Cy5 was purchased from Amersham Bioscience (Piscataway, NJ, USA). Polymethylmethacrylate (PMMA) beads were purchased from Sapidyne (Boise, ID, USA). Thiamethoxam MAb was purified with the protein A immunoaffinity column and dialyzed against PBS buffer (5 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 136 mM NaCl, and 2.7 mM KCl, pH 7.2).

2.2. Monoclonal antibody generation and characterization

The methods used to produce the MAbs were adopted from Shelver et al. [26]. Initial immunization with 50 μg of thiamethoxam hapten–KLH conjugate mixed with complete Freund's adjuvant was carried out by intraperitoneal (i.p.) injection of five female Balb/c mice, 6 weeks old (Jackson Labs., Bar Harbor, ME, USA). Four monthly i.p. booster immunizations were administered with 50 μg of hapten conjugate emulsified with incomplete Freund's adjuvant. The titers of the serum samples were measured. The mouse with the highest titer and strong competitive binding of thiamethoxam was selected for hybridoma production. The mouse was injected intraperitoneally with 50 μg of the hapten-KLH conjugate in PBS, and 50 μg through the tail vein 4 days prior to splenocyte harvest. Murine Sp2/0Ag14 myeloma cells were grown in RPMI 1640 supplemented with 15% fetal bovine serum, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B (designated as complete medium). Conditioned medium for replacing feeder cells in the fusion and cloning experiments was the culture medium supernatants from each Sp2/0Ag14 cell splitting. Splenocytes from the best-responding mice for thiamethoxam were used to produce hybridomas 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). The HAT selection was performed from 2 to 14 days postfusion. Two weeks postfusion, the cell culture supernatants were screened for hybridomas' ability to produce antibodies to thiamethoxam utilizing concurrent indirect non-competitive and competitive ELISAs. Hybridoma colonies were selected based on their binding ability towards thiamethoxam, continuity to secrete antibody, and ability to withstand preservation in liquid nitrogen.

2.3. Immobilization of thiamethoxam hapten–BSA conjugate to PMMA beads

An amount of 200 mg of dry PMMA beads of 98-μm diameter in a 1.5 ml Eppendorf tube was suspended with 1 ml of nanopure water and washed two times with PBS buffer by centrifugation and removal of supernatant solution. Beads were then suspended with 1 ml of coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6), 0.2 mg of hapten–BSA conjugate was added, and the tubes were rolled with an end-over-end rocker for 3 h at room temperature. After discarding the coating buffer, 1 ml of blocking buffer (1% BSA in PBS with 0.05% Tween 20) was added and the tubes rolled again for 2 h at room temperature. After blocking buffer was discarded, 1 ml of PBS was added. Bead preparations were stored at 4 °C until use. On the day of use, the contents of two tubes were transferred into the bead reservoir along with 27 ml of PBST for the constant supply of beads into the capillary flow column.

2.4. Indirect competitive ELISA

Microplate wells were coated overnight at 4 °C with thiamethoxam–BSA (2 ng in 100 μl per well in 0.05 M carbonate–bicarbonate buffer, pH 9.6). The next day, the plate was washed four times with PBS containing 0.05% Tween 20 (PBST) using a manual plate washer (Nunc International, Copenhagen, Denmark) and then blocked by incubation for 1 h at room temperature with 1% BSA in PBS (200 μl per well). After washing the plates five times, a solution of 50 μl per well