Development of an Enzyme-Linked Immunosorbent Assay for a Broad Spectrum Triazole Fungicide: Hexaconazole

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Polyclonal antibodies were produced against 5-(2,4-dichlorophenyl)-5-hydroxy-6-(1*H*-1,2,4-triazol-1-yl)hexanoic acid (hexaconazole-hapten) conjugated to bovine serum albumin. Using these antibodies, an enzyme-linked immunosorbent assay was developed for detecting and quantifying hexaconazole and its metabolites. This assay was sensitive and had a linear range from 1 to 60 ng/mL. The IC_{50} was 8 ng/mL and the detection limit 0.1 ng/mL. The polyclonal antibodies recognized hexaconazole and its acid and alcohol metabolites. They cross-reacted with penconazole (46%) and propiconazole (43%), but not with diclobutrazol, imazalil, paclobutrazol, or triadimefon. Using this assay, the concentration of hexaconazole in a commercial fungicide was determined.

Keywords: *Hexaconazole; antibodies; enzyme-linked immunosorbent assay*

INTRODUCTION

Triazole fungicides, including hexaconazole, are registered agrochemicals that have been extensively used on crops worldwide. Hexaconazole is sold in 18 countries under the trademarks ANVIL and PLANETE (Smith, 1991). The forest products industry uses several hexaconazole formulations to control staining and decay fungi, as well as mold. The toxicity of hexaconazole by oral, inhalation, or contact routes is low to moderate to man, mammals, fish, and birds. The Canadian Pest Management Regulatory Agency has completed a toxicological evaluation of hexaconazole that established an acceptable daily intake of 0.005 mg/kg in drinking water. The LD_{50} for male rats is 2189 mg/kg (Technical Profile: ICI Chipman). It is also of low mobility in soil.

The antifungal agent hexaconazole, 2-(2,4-dichlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol, was discovered by ICI Agrochemicals in the early 1980s. The compound is highly active and has a broad spectrum of action against basidiomycetes and ascomycetes (Worthington, 1991). Hexaconazole belongs to the triazole fungicide family and, like the imidazole fungicides, interferes with fungal sterol synthesis. Both types of chemicals seem to inhibit the C-14-demethylase step between 24-methylenedihydrolanosterol and 4,4-dimethylergosta-8,14,24(28)-trienol (Vanden Bossche, 1988). This sterol inhibition strategy has been very successful for controlling plant and animal fungal pathogens.

Environmental and biological monitoring and quality control programs require the development of precise, specific, and sensitive techniques for the rapid detection and identification of hexaconazole in crops, wood, water, and soil. Since traditional GC or HPLC methods are complex and time-consuming, there is increasing interest in using immunoassays for trace chemical analysis (Chen *et al.*, 1995; Li *et al.*, 1994; Spinks *et al.*, 1993; Deschamps and Hall, 1991; Harrison *et al.*, 1991; Giersch and Hock, 1990; Jung *et al.*, 1989; Hammock and Mumma, 1980). An enzyme-linked immunosorbent assay (ELISA) for triazole fungicides was developed by Forlani *et al.* (1992). However, their polyclonal antibodies were more specific to 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol, tetraconazole, and penconazole than to hexaconazole or propiconazole.

In the present work, we report the production of anti-hexaconazole polyclonal antibodies, the partial characterization of the antibodies, and the development of an ELISA for the quantification of trace amounts of hexaconazole and its acid or alcohol metabolites.

MATERIALS AND METHODS

Reagents. Hexaconazole, 5-(2,4-dichlorophenyl)-5-hydroxy-6-(1*H*-1,2,4-triazol-1-yl)hexanoic acid (hexaconazole-hapten), hexaconazole alcohol metabolites, diclobutrazol, penconazole, propiconazole, imazalil, paclobutrazol, triadimefon, and other triazole compounds were obtained from ZENECA Agrochemicals. ANVIL was obtained from Forintek. Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants, carbonate/bicarbonate buffer capsules, and 3,3′,5,5′-tetramethylbenzidine blue (TMB) tablets were purchased from Sigma (St. Louis, MO). Isobutyl chloroformate and tributylamine were purchased from Aldrich (Milwaukee, WI). The anti-rabbit IgG−horseradish peroxidase was obtained from Caltag Laboratories (San Francisco, CA). Bacto dehydrated skim milk was obtained from Difco Laboratories (Detroit, MI). All solvents were of HPLC grade, and the deionized water was purified by the Nanopure ultrapure water system (Barnstead-Thermolyne, Dubuque, IA).

Equipment. Microtitration plates (Immulon 4, Dynatech Laboratories, Inc., Chantilly, VA) and a THERMOmax microplate reader (Molecular Devices Corp., Meulo Park, CA) were used for the ELISA.

Immunogen Design and Synthesis. A hexaconazole-hapten with a carboxylic group at the end of the propyl chain, 5-(2,4-dichlorophenyl)-5-hydroxy-6-(1*H*-1,2,4-triazol-1-yl)hexanoic acid, was chosen to allow conjugation with different carrier proteins. The hapten and the corresponding immunogen and coating antigen synthesis are shown in Figure 1. The hexaconazole-hapten (21.3 mg, 0.062 mmol) was dissolved into 1 mL of dry dioxane. Tri-*n*-butylamine (17.9 μL, 0.074 mmol) and isobutyl chloroformate (9.7 μL, 0.074 mmol) were added, and the resulting mixture was stirred at room temperature for 30 min. This mixture was added dropwise to 15

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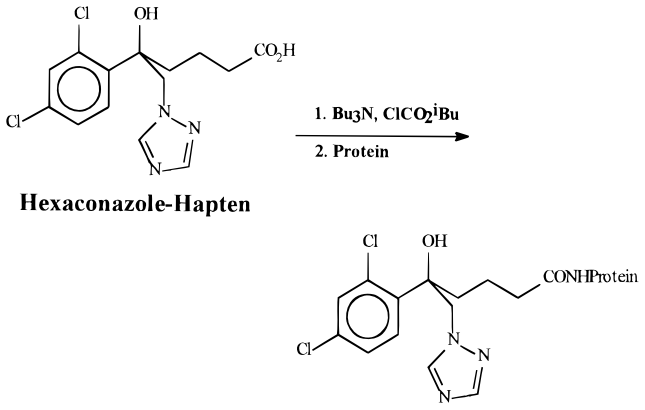


Figure 1. Synthesis of the hexaconazole immunogen and coating antigen. The carrier protein for the immunogen is BSA and for the coating antigen, OVA.

mL of solution containing 50 mg of protein in 0.2 M borate buffer, pH 8.7. The solution was stirred gently overnight at room temperature. OVA was used to prepare the coating antigen and BSA to prepare the immunizing antigen. The conjugates were dialyzed for 24 h against water and then lyophilized.

Producing and Screening the Polyclonal Antibodies. Two New Zealand female white rabbits weighing between 2 and 4 kg were immunized intradermally. Each rabbit received 1 mL of an emulsion (1:1) containing 1 mg/mL hexaconazole-hapten−BSA in saline and complete Freund's adjuvant. The rabbits were boosted five times with 2 week resting intervals, using the same amount of antigen emulsified in incomplete Freund's adjuvant. The rabbits were bled and the serum separated by centrifugation and stored at −70 °C. The titer of the anti-hexaconazole serum was determined by ELISA.

Indirect ELISA and Competitive Inhibition ELISA. Microtitration plates were coated with 100 μL of hexaconazole-hapten−OVA in $NaHCO_3$, pH 9.6, and dried overnight at 37 °C. The plates were washed four times with PBS, blocked with 200 μL/well of 2% milk in PBS for 1 h at 37 °C, and then washed four times with PBS. Diluted hexaconazole polyclonal serum in 0.1% milk in PBS was added to the wells and incubated at 37 °C for 2 h. After the plate was washed four times with PBS, a secondary antibody conjugated to an enzyme, the anti-rabbit IgG−horseradish peroxidase diluted 1:3000 into 0.1% milk in PBS, was added. Both the diluted serum and the antibody−enzyme conjugate were applied at 100 μL/well. The enzymatic reaction was carried out at room temperature, in the dark, using 3,3′,5,5′-tetramethylbenzidine (TMB, 100 μL/well). The reaction was stopped after 18 min with 25 μL of 2.5 M H_2SO_4. The absorbance was measured at 450 nm.

A competitive inhibition ELISA was used for assessing the specificity of the antibodies to free hexaconazole and their cross-reactivity with related compounds. Stock solutions of 1000 ppm of hexaconazole, related chemicals, or formulation of hexaconazole were prepared in methanol. Appropriate concentrations of the chemical, mixed with the hexaconazole polyclonal antiserum diluted 4000 times into 0.1% milk in PBS, were then added to a plate that had been coated with hexaconazole-hapten−OVA and blocked with 2% milk in PBS. The rest was carried out as described for the indirect ELISA procedure.

RESULTS AND DISCUSSION

Immunogen Design. Hexaconazole was attached to a carrier protein to elicit the production of antibodies in the rabbits. For a conjugation process that may involve more than one step, the choice and design of the hapten are critical for the production of highly selective antibodies (Harrison *et al.*, 1991). To avoid altering the important structural features of hexaconazole, i.e. tertiary hydroxyl, dichlorophenyl, and triazolyl groups, a hexaconazole-hapten with a carboxyl group at the end of the propyl chain, 5-(2,4-dichlorophenyl)-5-hydroxy-6-(1*H*-1,2,4-triazol-1-yl)hexanoic acid, was synthesized to link to the carrier proteins. This propyl chain is the simplest component of the molecule and is far from the hydroxyl center, which is linked with both the phenyl and triazole rings. In addition, the alkyl chain serves as a spacer arm for maximizing the exposure of the analyte to the immune system. It is unlikely that the alkyl chain would be an important determinant in the production of highly specific antibodies.

Table 1. Solvent Effect on the Reactivity of the Antiserum Polyclonal Antibodies[a]

solvent	amount (% in solution)	% residual activity
ethanol	0.1	91 ± 5
	0.5	84 ± 5
methanol	5	99 ± 5
	6	91 ± 5
DMSO	0.1	56 ± 3

[a] The ELISA conditions are described in the text.

Effects of Solvents on the Antibody. Prior to the detailed characterization of the assay, we determined the relative effect of different solvents, ethanol, DMSO, and methanol, on the reactivity of the hexaconazole antibodies (Table 1). The hexaconazole antibodies retained their immunoreactivity in the presence of 5% (by volume) methanol. However, ethanol and DMSO at concentrations equal to or greater than 0.5% reduced the reactivity of the antibodies by 16−44%. These organic solvents might alter the binding properties of the antibodies. Solvent effects on immunoassays are complex and depend on the antibody and application conditions. Some immunoassays can tolerate high concentrations of solvents up to 10% (Krämer *et al.*, 1994; Lucas *et al.*, 1993), while others are severely affected by them (Bekheit *et al.*, 1993; Wittmann and Hock, 1993; Harrison *et al.*, 1991). Consequently, in all of the ELISAs described in this work the final methanol concentration was less than 0.4% (by volume).

Titration and Inhibition Curve. Since hexaconazole-hapten−BSA was used as the immunogen, the antisera were screened by indirect ELISA(s) using hexaconazole-hapten−OVA as the coating antigen. The results of a titration curve for the serum of one of the rabbits is shown in Figure 2. The other rabbit showed a similar profile. Increasing the coating antigen above 100 ng/well did not significantly increase the overall reaction. The end point titration was reached for an antiserum dilution of 1:16000. The results showed that in order to be in the linear portion of the response curve, which requires an absorbance reading of about 1, a coating antigen concentration of 50 ng/well and an antiserum dilution of 1:4000 were needed. These conditions were used for the rest of the experimental work.

To determine whether the antibodies were specific and recognized free hexaconazole, we used an indirect competitive ciELISA. An inhibition curve was obtained for hexaconazole concentrations ranging from 0.025 to 4000 ng/mL (Figure 3). The curve was linear from 1 to 60 ng/mL with a correlation coefficient of 0.998. The IC_{50} value for this assay was 8 ng/mL, and the detection limit was 0.1 ng/mL. The detection limit was defined as the lowest concentration of hexaconazole giving an absorbance value separated from A_0 by 3 × SD, where SD was the standard deviation of the blank absorbance value (Fleeker, 1987).

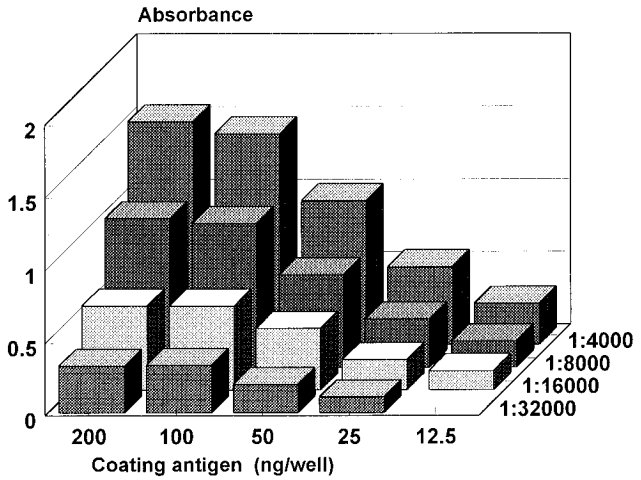


Figure 2. Titer determination for the anti-hexaconazole polyclonal serum. The indirect ELISA was done as described under Materials and Methods. The substrate used was TMB. The serum dilutions were between 4000 and 32 000.

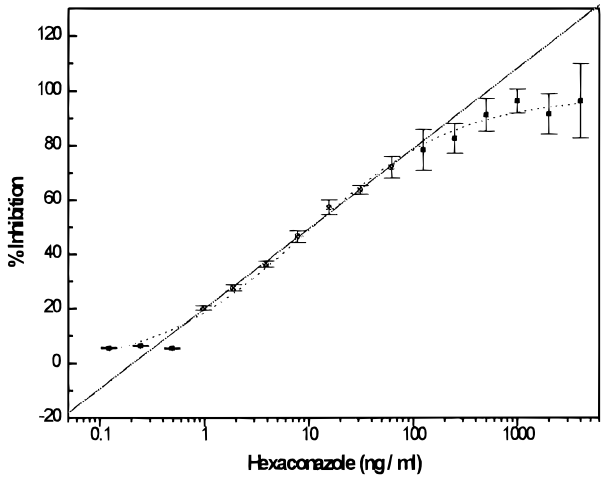


Figure 3. Standard inhibition curve for the anti-hexaconazole polyclonal antibodies using free hexaconazole. The ciELISA was done as described under Materials and Methods using a serum dilution of 1:4000 and a coating antigen concentration of 50 ng/well. Percent inhibition was calculated as 100 [1 − ($A_{490}^{(x)}/A_{490}^{(ctl)}$)],where x and ctl refer to unknown and control, respectively. The linear range is 1−60 ng/mL, with a correlation coefficient of 0.998. The data represent the mean obtained from four wells, and the error bars, the standard error of the mean.

Specificity and Sensitivity of the Polyclonal Antibodies. A series of chemicals containing structures similar to the hydroxyl, phenyl, and triazolyl functions of hexaconazole were tested to determine their cross-reactivities with the anti-hexaconazole antibodies. To differentiate the different potential epitopes, and for convenience, we treated the alkyl group as part of the parent skeleton and designated the other three structural groups A, B, and C, as shown in Figure 4.

Since the tested compounds include only part of the hexaconazole structure, the cross-reactivities were considerably lower than for the original parent molecule. Consequently, these data should only be used as an index of the relative ease with which the antibodies recognized the different epitopes of the hexaconazole molecule. The percentage cross-reactivity for each compound, which is listed to the right of the compound's structural diagram, was calculated as

$$[\%\ \text{inhibition}_{\text{compound}}/\%\ \text{inhibition}_{\text{hexaconazole}}] \times 100$$

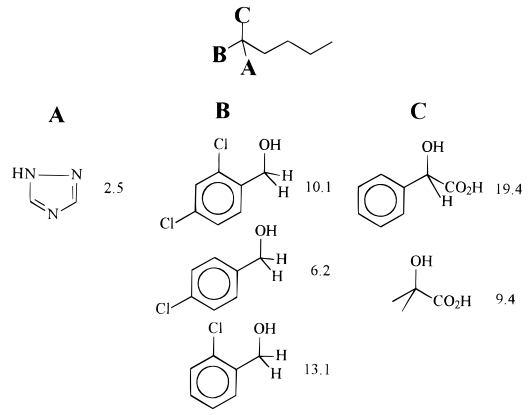


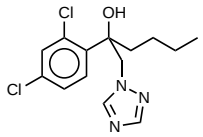
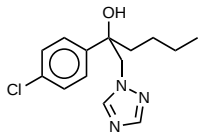
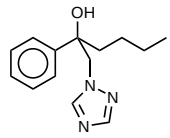
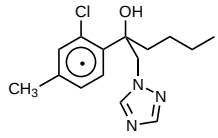
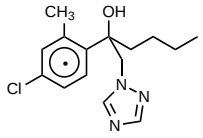
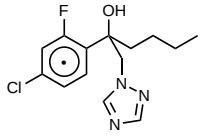
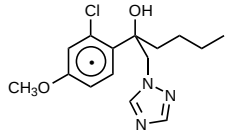
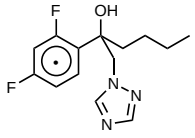
Figure 4. Cross-reactivity of the anti-hexaconazole antibodies with different compounds having partial structural similarity with hexaconazole. The compound concentration used was 8 ng/mL, and TMB was used as substrate. The detailed conditions are listed in the text. The molecular weights were 69 for A, 176 and 142 for B, and 156 and 104 for C.

Figure 4 suggests that the hydroxyl group and the phenyl ring were more critical than the triazole ring in the recognition of hexaconazole by the antibodies. It is important to note that the molar concentration of the triazole ring was 2−3 times that of the phenyl ring. This reinforced the assumption that the triazole ring was not involved in the interaction of the antibody and the antigen. Similar cross-reactivities were observed for 2,4-dichloro and 2-chlorobenzyl alcohols. However, 4-chlorobenzyl alcohol showed only half the cross-reactivity of 2-chlorobenzyl alcohol. This suggested that the substituents and their position on the phenyl ring play an important role in determining the specificity of the polyclonal antiserum.

To confirm this initial observation, different substituted derivatives of hexaconazole were tested. The results are summarized in Table 2. When the chloro groups were absent (entry 3), the antibodies did not cross-react with the compound. For the analogue monochlorinated at the para position on the phenyl ring, the cross-reactivity decreased by 77% (entry 2). To determine whether this recognition depended primarily on size or on the electronic character of the substituents on the phenyl ring, we measured the antibodies' cross-reactivities with a set of samples bearing only different substituents on the phenyl ring. Replacing the chloro groups by methyl groups, which have a similar size but a different electronic character, preserved most of the cross-reactivity (entries 4 and 5). Replacing chlorines with fluorines, which have a similar electronic character but are smaller, was more detrimental to the cross-reactivity (entries 6 and 8). Furthermore, replacing both chlorines by hydrogens, the smallest possible substituent, resulted in no cross-reactivity (entry 3). Therefore, it seems that size is a more important factor than the electronic distribution across the phenyl ring for the recognition of hexaconazole by the polyclonal serum.

The cross-reactivity of the anti-hexaconazole serum to other triazoles or related compounds was also investigated. The results are shown in Table 3. The acid or alcohol derivatives of hexaconazole cross-reacted strongly with the polyclonal antibodies. Although the results suggested that the alkyl chain was not the principal determinant of antibody recognition, the introduction of a secondary carbon atom did lead to some reduction in cross-reactivity. Penconazole and propiconazole also showed high cross-reactivity with the antiserum, whereas

Table 2. Correlation between the Percentage Cross-Reactivity and Substituent Effect on the Phenyl Ring of the Hexaconazole Derivatives[a]

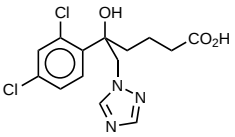
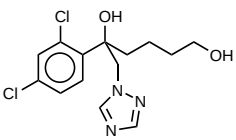
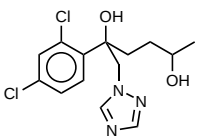
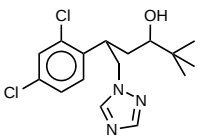
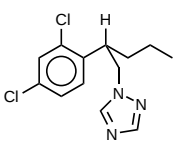
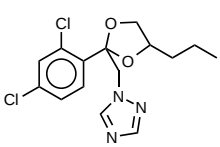
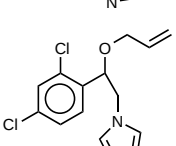
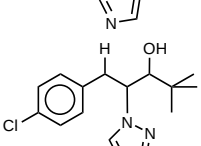
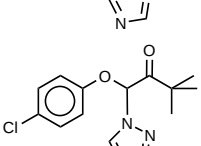
entry	sample	structure	% cross-reactivity
1	hexaconazole		100
2			23.0
3			1.2
4			62.0
5			53.1
6			40.4
7			31.4
8			12.6

[a] Coating antigen: hexaconazole−OVA, 50 ng/well; polyclonal serum dilution, 1:4000; TMB as substrate and concentration of compounds, 8 ng/mL. IUPAC chemical names for the entries are (1) (*RS*)-2-(2,4-dichlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol; (2) (*RS*)-2-(4-chlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol; (3) (*RS*)-2-phenyl-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol; (4) (*RS*)-2-(2-chloro-4-methylphenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol; (5) (*RS*)-2-(4-chlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol; (6) (*RS*)-2-(2-fluoro-4-chlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol; (7) (*RS*)-2-(2-chloro-4-methoxyphenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol; (8) (*RS*)-2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol.

diclobutrazol, imazalil, paclobutrazol, and triadimefon showed little or no cross-reaction. Taken in conjunction with the data presented in Table 2, these results suggested that the minimum structural requirements for "high" cross-reactivity comprise the following: a 2,4-disubstitued phenyl ring, bearing groups approximatively isosteric with the chlorine substituents; and a tertiary benzylic carbon (the hydroxyl group does not appear to be essential) bearing the triazole function.

Quantification of Hexaconazole in a Commercial Product Using a ciELISA. ciELISA was used to analyze hexaconazole in a commercial product, ANVIL. The commercial product was diluted to give a range of concentrations varying from 2 to 40 ng/mL. The concentrations of hexaconazole present in the different solutions were measured by ciELISA. The ciELISA included a standard inhibition curve for pure hexaconazole, which corresponded to the linear part of Figure 3. Figure 5 shows good agreement between the concentrations determined by the immunoassay and the values reported by the manufacturer. The other components present in the formulation did not affect the immunoassay.

Table 3. Cross-Reactivity of Some Triazole Compounds in the ELISA with Respect to Hexaconazole[a]

sample	structure	% cross-reactivity
hexaconazole-hapten (523-02)		74 ± 5
523-03		95 ± 9
ICIA0523/04/08		63 ± 4
diclobutrazol (ICIA0296)		6 ± 6
penconazole (C181261)		46 ± 2
propiconazole (C145618)		43 ± 7
imazalil (R87665)		4 ± 4
paclobutrazol (ICIA0333)		0
triadimefon (R85827)		0

[a] Conditions were the same as in Table 2.

Conclusion. Polyclonal antibodies were raised against hexaconazole, using a hexaconazole-hapten linked to BSA through the ω-carboxylic group at the end of the alkyl chain. The antibodies reacted with hexaconazole and with its acid and alcohol metabolites. The assay has a linear range of 1−60 ng/mL, a lower detection limit of 0.1 ng/mL, and a 50% inhibition of 8

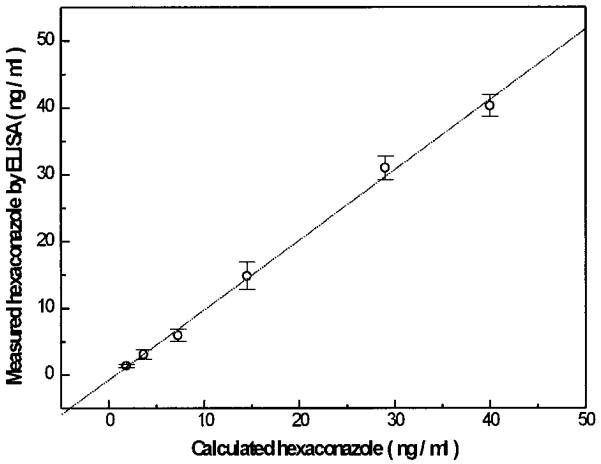


Figure 5. Analysis of hexaconazole in a commercial product, ANVIL (PP523), using the anti-hexaconazole serum in a competitive ELISA. Anvil contains 50 g/L hexaconazole in oil-miscible liquid. The slope was 1.05 ± 0.03, and the correlation coefficient was 0.99. The experiment was repeated three times with five replicate wells per concentration per assay.

ng/mL. The recognition of the antibodies to hexaconazole is more dependent on the dichlorophenyl ring than on the triazole ring, and the size of chlorine atoms on the phenyl ring appears to be more important than their effect on the electron distribution at the ring. The antiserum cross-reacts with two related fungicides, penconazole and propiconazole, but not with imazalil, paclobutrazol, or triadimefon. For the commercial product, a good correlation was obtained between the values reported by the manufacturer and the values measured by the ciELISA.

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