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Development of Immunoassays for the Detection of the Fungicide Penconazole and Its Urinary Metabolite

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Monoclonal antibodies were raised to haptens containing moieties common to both the triazole fungicide penconazole and its proposed primary urinary metabolite (4-(2,4-dichlorophenyl)-5-(*H*-1,2,4-triazol-1-yl)pentoic acid). The monoclonal antibody 2E4 was used to develop competitive ELISA assays where binding of antibody to immobilized haptens conjugated to BSA competed with penconazole or its metabolite in solution. At pH 4.0 and pH 8.0, penconazole was detected with an IC50 of 1.0−1.2 μg/L respectively and at pH 4 penconazole metabolite was detected with an IC50 of 0.9 μg/L. These assays were specific for penconazole and/or its metabolite compared to other triazole fungicides. The immunoassay conditions optimal for penconazole metabolite (pH 4.0) were used and applied to the analysis of spiked human urine, and following sample extraction using a C18 SPE column, could detect 0.5 μg/L metabolite. This is the first report of an immunoassay to the urinary metabolite of penconazole, an assay with application in the monitoring of occupational and non-occupational exposure to this commonly used pesticide.

KEYWORDS: Penconazole; hapten; monoclonal antibody; ELISA; metabolite; SPE; urine

INTRODUCTION

The compound penconazole is an example of a 1,2,4-triazole ring containing conazole fungicide. It is used for the control of powdery mildew, scab and other pathogenic ascomycetes, basidiomycetes and deuteromycetes in a wide variety of soft fruit, vegetable, legume and grain crops by both pre- and postharvest spraying as an aqueous solution. The mode of action of penconazole is in the interference of fungal cell wall formation via inhibition of ergosterol biosynthesis. Ergosterol is thought to be important in maintaining membrane microviscosity and therefore has influence on a range of processes such as cell wall synthesis, modulation of chitin synthetase and the activity of many other membrane bound enzymes (*1*). The toxicology of penconazole has been documented, with pesticide metabolites being rapidly excreted via urine and feces over a 24 h period in all species tested (rat, chicken and goat) (*2*). There is increasing concern over human exposure to this class of fungicides, which block sterol biosynthesis, commonly targeting the C14 demethylation step during the conversion of lanosterol to ergosterol. Penconazole is therefore classified as a steroid demethylation inhibitor. Due to their mode of action, members of this class of fungicide have been implicated in potentially being able to cause endocrine-related side effects in a wide variety of vertebrates (*3*). Sterol 14α demethylase, a target for penconazole inhibition, is highly conserved at the amino acid level and is expressed in many different tissues. The substrate for sterol 14α demethylase is derived from the common precursor squalene and is converted by the enzyme into basic sterol structures by complex cyclization reactions in a wide variety of organisms from fungi to human (*4*). In mammals this step is important in the biosynthesis of cholesterol, and from there many further sterols, including the sex steroid hormones (*3*).

The widespread use of penconazole on a variety of foodstuffs has meant that the levels of permissible residue that can be found on produce is regulated nationally and internationally with maximum residue levels (MRLs) being set for each food type. The UK MRLs set for penconazole range from 0.2 mg/kg (apples and pears) to 0.05 mg/kg (cranberries and blueberries). The analysis and recording of the levels of pesticide residue in foods is of high priority, driven by both public and government concerns over safety of foodstuffs. This has historically been carried out by sensitive analytical techniques such as GC or LC mass spectrometry. Methods describing the detection and quantitation of penconazole by chromatographic methods have been restricted to the parental compound and have been applied for residue monitoring of foods and also on personal protective clothing. To illustrate the sensitivity of these assays, Oliva et al. (*5*) have described an assay to penconazole with an assay LOD of 7.6 μg/L for monitoring penconazole in grapes and wine, while Goumenou and Machera (*6*) have described another assay to penconazole with an assay LOD of 0.84 μg/L, for use in occupational exposure monitoring, by analysis of personal protective equipment.

Recent developments in the field of immunodetection have yielded highly sensitive assays to various pesticide residues. ELISA tests serve as cost-effective, high throughput alternatives to instrumental analysis. The advantages of this assay format are that they can have similar sensitivities to mass spectrometry

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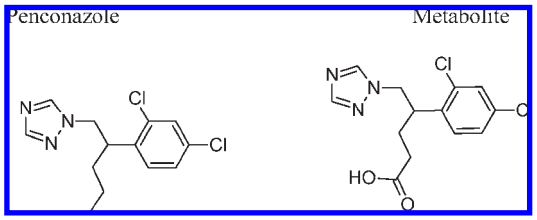


Figure 1. Structure of penconazole and the metabolite of penconazole, 4-(2,4-dichlorophenyl)-5-(*H*-1,2,4-triazol-1-yl)pentoic acid.

methodologies, can be carried out with a minimum of specialist equipment, are suitable for large sample numbers and lend themselves to automation. To date there are several reports of immunoassays developed to triazole fungicides. Manclus et al. (*7*) described the isolation of monoclonal antibodies and the development of a high sensitivity, class specific immunoassay to triazole fungicides with IC50s in the range of 0.5−2.5 μg/L (0.7 μg/L for penconazole), and a specific hexaconazole immunoassay with an IC50 of 1.3 μg/L. Additionally, a low sensitivity immunoassay has also been developed against the triazole fungicide myclobutanil (*8*), which used a polyclonal reagent and had an IC50 of 4.9 mg/L. Forlani et al. (*9*) developed a relatively low sensitivity triazole group specific immunoassay for a range of commercially important triazole fungicides with assay IC50s of 100 μg/L for tetraconazole and 120 μg/L for penconazole. High sensitivity immunoassays specific for penconazole are desirable for the rapid assay of pesticide in food produce in order to police maximum residue levels. Immunoassays could also be useful in monitoring the environmental fate of this pesticide, and in addition an assay which could measure biomarkers of exposure to penconazole, for example a specific metabolite of penconazole, would have application for the monitoring of both occupational and non-occupational low level exposure to this compound.

The aim of the present study was to produce immunoassays to detect low levels of the fungicide penconazole, and the primary metabolite of penconazole which is excreted in urine. Murine immune responses were generated against haptens with similar chemical structures to the major urinary metabolite of penconazole, 4-(2,4-dichlorophenyl)-5-(*H*-1,2,4-triazol-1-yl)pentoic acid (**Figure 1**). A resulting monoclonal antibody, 2E4, was used to develop assays that facilitated the high sensitivity detection of penconazole and its urinary metabolite. The assays developed could detect penconazole with an IC50 of around 1.0 μg/L, with an LOD of 0.3 μg/L in buffer, while the assay to metabolite could detect metabolite with an IC50 of 0.9 μg/L and an LOD of 0.4 μg/L. The assay was used for the analysis of penconazole metabolite spiked healthy urine, which demonstrated that the assay could detect penconazole metabolite at levels of 0.5 μg/L.

MATERIALS AND METHODS

Reagents. Bovine serum albumin—fraction V (BSA), analytical grade penconazole, isobutyl chloroformate, pNPP alkaline phosphatase substrate and the competing pesticide compounds were purchased from Sigma-Aldrich (Poole U.K.). Imject mariculture keyhole limpet hemocyanin (KLH), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from PerBio. Goat antimouse secondary AP conjugate (Zymed) was purchased from Invitrogen. All microtiter plates used in the described experiments were Nunc Maxisorp 96-well flat bottom plates.

Haptens. Immunizing and screening haptens (**Figure 2**) were a gift from Dr. Joanne Kilgour (Syngenta) and comprised a structural analogue of the metabolite of penconazole with an additional one or two carbons on the side chain containing the carboxylic acid, compared to the authentic urinary metabolite (**Figure 1**). The penconazole metabolite,

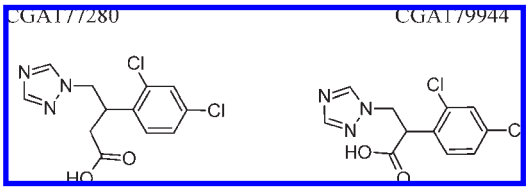


Figure 2. Haptens used for conjugation and immunization.

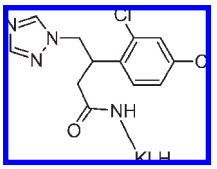


Figure 3. Proposed conjugate structure based on CGA177280 coupled via an amide bond to the carrier KLH.

4-(2,4-dichlorophenyl)-5-(*H*-1,2,4-triazol-1-yl)pentoic acid, was a gift from Kate Jones (Health and Safety Laboratories, Buxton, U.K.).

Hapten−Carrier Protein Conjugation. For immunization purposes, both haptens, CGA177280 and CGA179944, were conjugated to KLH using the carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Conjugation reactions were set up (following recommended Perbio methodology) as follows: 2 mg of each hapten was weighed out and dissolved in 200 μL of DMSO, and this was added to 2 mg of KLH protein dissolved in a final volume of 450 μL of conjugation buffer (0.2 M MES, 0.15 M NaCl pH 4.8). Two milligrams of EDC powder was then added to the reaction and immediately mixed by gently pipetting the solution. Conjugations were allowed to proceed at room temperature for two hours. The resulting KLH conjugates were then dialyzed into PBS, aliquotted and stored frozen at −20 °C. The conjugate structure, whereby hapten is linked to carrier protein by an amide bond, is depicted in **Figure 3**.

For screening purposes, and to avoid any antibody cross-reactivity with EDC coupling side reactions (*10*), haptens 1 and 2 were conjugated to BSA using the mixed anhydride reaction. Briefly, 6 mg of each hapten was dissolved in 5 mL of dioxane, and to this 11 μL of tributylamine and 6 μL of isobutyl chloroformate were added. The reactants were left at 10 °C while being stirred continually. After one hour a protein solution of BSA (50 mg BSA in 5 mL of sodium borate pH 9.0) was added dropwise to the hapten solution. The reaction was allowed to proceed for a further 2 h, and then the product was dialyzed into PBS. Aliquots of this protein−hapten conjugate were frozen at −20 °C and were used for screening both test bleeds and hybridomas.

Immunizations. Protein conjugates of haptens CGA177280 and CGA179944 were mixed together in equal proportion and used to immunize 5 balb-C female mice. This was carried out by Harlan UK (Loughborough, U.K.), using an immunization regime that consisted of a primary subcutaneous 100 μg injection using Freund's complete adjuvant, followed by 3 additional boosts using 100 μg of conjugate in Freund's incomplete adjuvant. The final boost, 2 days before termination and removal of the spleen, consisted of an intravenous injection of 100 μg of conjugate in the absence of any adjuvants. Test bleed samples were collected three weeks after the initial immunizations and at termination. Those animals producing the highest titer of circulating antibody were selected for splenocyte fusion. Animals selected for splenocyte fusion were sacrificed at 36 and 65 days post initial immunization. All animal procedures were carried out to the highest possible animal welfare standard in accordance with UK home office guidance.

Production of Monoclonal Antibodies. Two separate fusions were carried out. Splenocytes isolated from the extracted spleens were fused to NS0 myeloma cells (which had been maintained in the presence of 8-azaguanine). Fusion of splenocytes and myeloma cells was carried out at a ratio of 1:1 splenocytes to NS0 cells using Hybrimax PEG solution (Sigma-Aldrich). Fused splenocytes isolated from a whole spleen were plated into ten 96-well microwell plates (Nunclon) in the presence of RPMI-HAT media (Sigma) with 15% fetal calf serum, supplemented with L-glutamine, penicillin and streptomycin.

Screening was carried out ten days postfusion by transferring spent culture supernatant from the fusion to the wells of 96-well plates that had been coated overnight with a mixture of 10 mg/L BSA-CGA177280 and BSA-CGA179944 conjugates. Plates were then washed three times with PBS buffer and then blocked for 1 h using 3% skimmed milk powder in PBS. Cell culture supernatants were incubated overnight at 4 °C and plates washed three times with PBS-0.5% Tween 20 (PBS-t). Bound antibody was probed using an antimouse IgG alkaline phosphatase conjugate (Zymed) at 1/2000 dilution in PBS-t for one hour and then washed three times with PBS-t. The anti-IgG secondary antibody was used in the screening steps to ensure that only IgG antibodies were isolated. Wells were developed by the addition of 100 μL of pNPP substrate solution (Sigma). Wells producing signals over five times the background (wells containing just culture medium alone) were retested within 24 h by competition ELISA competing with a mix of both haptens (CGA177280 and CGA179944) at a fixed concentration of 100 mg/L. Colonies in wells producing positive signals which could be competed out by free hapten resulting in a drop of ELISA signal by > 50% compared to replicate wells in the absence of free hapten were identified, and the cells were then cloned by limiting dilution. The isotype of the cloned hybridomas was determined using the Mouse MonoAb-ID Kit (Zymed). Antibodies were grown up in 50 mL volumes in culture flasks until late culture, then filtered through 0.45 μm sterile cellulose acetate syringe filters and stored at 4 °C.

Development of Competitive ELISA Assays. Colorimetric competition ELISAs were optimized by careful titration of both the antigen coating concentration and antibody concentration in a checkerboard arrangement in order to determine concentrations where the amount of antibody was limiting. Conditions (in standard PBS buffer) were chosen where absorbance at 405 nm was about 1.0, and where further dilution of antibody resulted in a large reduction in signal (i.e., the antibody concentration was limiting). At the optimized coating and antibody concentrations, small amounts of hapten or penconazole competitor were added into the ELISA to produce a standard competition curve. Other parameters which were optimized were the pH and composition of the assay buffer, and an assessment of which hapten (CGA177280 or CGA179944) immobilized on the plate was most suitable for the sensitive detection of either penconazole or its metabolite. Optimization of the competitive ELISA assay was carried out by competing binding with haptens CGA177280 and CGA179944 due to limited supply of the authentic metabolite. However, when conditions had been optimized with these compounds, evaluation of the assay by competition with the authentic metabolite was carried out.

ELISA plates were coated overnight at 20 °C with the assay conjugate at 20 mg/L in PBS containing 8 M urea. The urea was included to denature the protein conjugate, which tended to aggregate; its inclusion improved the reproducibility of the signals in the immunoassays. Plates were washed and blocked as described above. Antibody at an appropriate dilution was diluted in assay buffer (either PBS, or citrate pH 4.0) and then added to a dilution of competitor (penconazole metabolite, haptens CGA177280 and CGA179944, penconazole or other compounds) to achieve a concentration in the ELISA well of between 40 μg/L and 0.00015 μg/L. The diluted antibody/hapten mix was then added to the ELISA plate in triplicate wells and incubated for one hour at 20 °C. Bound antibody was detected with antimouse AP conjugate (Zymed) at a final concentration of 1/2000 and pNPP substrate (Sigma-Aldrich). Plates were read at 405 nm with reference filter at 620 nm after 1 h incubation at room temperature.

Generation of Standard Curves. The standards were used to generate a semilog graph, plotted and fitted to a four-parameter logistic equation (eq 1) using Sigmaplot, which allowed accurate estimation of assay IC50 values (the concentration of competitor that caused a 50% drop in signal). A theoretical limit of detection (LOD) for the assay was also determined as the concentration of competitor that produced an absorbance which was three standard deviations below the mean absorbance produced with samples containing no competitor (*11*).

$$y = y_0 + A/1 + (x/x_0)^b \quad (1)$$

where: A = max y, x_0 = x50 (x,y,0.5), y_0 = min y, y = A405, and b = slope factor.

Calculation of Antibody Cross-Reactivity. Compounds were assayed by competitive ELISA, and standard curves were generated based on the 4-parameter logistic equation. The IC50 value for each analyte was then compared to the IC50 value of either penconazole or its metabolite, depending on which assay pH (4 or 8) was being used, and based on eq 2.

$$\text{CR} = \text{IC50 penconazole or metabolite } (\mu\text{g/L})/\text{IC50 analyte } (\mu\text{g/L}) \times 100\% \quad (2)$$

Urine Extraction. Urine was collected from healthy volunteers and processed directly or spiked with penconazole metabolite (diluted from a 1 mg/mL stock in methanol) to produce final concentrations of 0, 0.5, 1, 2, 5, and 10 μg/L. Metabolite was extracted from urine (1 mL) using C18 SPE Bond Elut columns (Varian) as briefly described. Columns were prepared by washing with 2 mL of methanol and then conditioning by passing through 4 mL of 0.1% acetic acid. The urine sample was loaded under vacuum at a rate of approximately 1 mL per minute, and then was washed by the addition of 4 mL of distilled water. The columns were then allowed to dry under vacuum. Adsorbed metabolites were eluted using a wash of 1 mL of 100% methanol. Samples were then dried and the residue was resuspended in citrate pH 4 buffer for use in the metabolite competition ELISA. Extracted urine samples were assayed by the metabolite ELISA, using a standard curve of metabolite in buffer. For each metabolite concentration, the average concentration of metabolite measured from 5 spiked samples was calculated.

Data Analysis. Competition standard curves were produced by plotting the absorbance values against the log of the concentration of competing analyte. Sigmoidal plots were produced by fitting this data to the four-parameter logistic equation using SigmaPlot software. The metabolite concentration in urine was calculated by interpolation of the mean ELISA absorbance values on a standard curve run on the same ELISA plate.

RESULTS AND DISCUSSION

Production of Antibodies to Hapten Conjugates. The excreted metabolite of penconazole has not been determined in human; however, analysis in the chicken, rat and goat all yield the same main excreted metabolite, 4-(2,4-dichlorophenyl)-5-(*H*-1,2,4-triazol-1-yl)pentoic acid (*2*). It is therefore assumed that a similar metabolism of penconazole occurs in human yielding 4-(2,4-dichlorophenyl)-5-(*H*-1,2,4-triazol-1-yl)pentoic acid (**Figure 1**) as the major metabolite. The immunizing haptens used in this study both contained structural motifs common with the pesticide penconazole and its metabolite 4-(2,4-dichlorophenyl)-5-(*H*-1,2,4-triazol-1-yl)pentoic acid (**Figures 1** and **2**). Mice were immunized with a mix of the two hapten conjugates (CGA177280 and CGA179944), a method that has previously been used to generate immune responses to multiple hapten conjugates (*12*). Responses against both haptens gave similar titers (data not shown), and those animals having the highest titers of circulating antibodies were selected for splenocyte fusion.

All antibodies that were isolated were of the IgG1 class. In total, 6 monoclonal cell lines were isolated and stabilized from 2 fusions (**Table 1**). These antibodies were optimized in competition ELISA with both the metabolite mimic CGA177280 and also the parental pesticide, the characterization of one of these antibodies, 2E4, is described and the optimization of competition ELISA assays is detailed.

Immunoassay Development for Penconazole and Penconazole Metabolite. For competition with CGA177280, antibody 2E4 displayed no difference in the IC50 of the immunoassay whichever of the two haptens was immobilized on the plate (IC50s of 4 μg/L). This trend was also seen when competition was carried out with penconazole, producing IC50s of around 1 μg/L when tested (data not shown). The IC50 value for the detection of hapten CGA177280 was higher than that for penconazole, and in order to try and increase assay sensitivity, the effect of pH on the 2E4 assay was investigated. Four different pH values in 4 separate buffer compositions were used (**Table 2**) and pH was shown to

have a profound effect on the assay, with the lowest IC50 for the detection of CGA177280 being at pH 4.0. Detection of metabolite at pH 4.0 was around 10-fold more sensitive than at pH 6.0 and pH 8.0, and the hapten was not detected at pH 10. This data demonstrated that, for 2E4, the sensitivity of the assay to the metabolite mimic could be improved by a reduction in the pH of the assay buffer. The antibody could be used for the sensitive detection of penconazole between pH 4.0 and pH 8.0, and detection of the penconazole metabolite is most sensitive at pH 4.0. It can be speculated as to why the competition with CGA177280 hapten was improved at a reduced pH. It is possible that at a lower pH the charge on the carboxylic acid residue is changed, allowing better recognition of free hapten in solution, by more closely resembling the immunogen that would not have had this charge due to conjugation through this residue (**Figure 3**). In terms of assay reproducibility there were smaller standard deviations for the points in the standard curve when hapten CGA177280 was immobilized rather than CGA179944 (data not shown), therefore the former conjugate was used as the coating hapten for all further experiments.

Table 1. Hybridoma Fusions with Splenocytes from Mice Immunized with Penconazole Moieties

			no. of wells with specificity for free hapten in solution (competition ELISA)		
fusion	wells seeded[a]	positive wells in a noncompetitive ELISA[b]	penconazole	CGA177280	no. of cloned hybridomas
1	960	9	1	2	3
2	768	100	10	3	3

[a] Number of wells in 96-well microtiter culture plates that were seeded with cells after splenocyte fusion. [b] Positive wells are the number of wells giving signals at least 5 times greater than background at one hour.

Table 2. The Effect of pH on 2E4 Competition with Metabolite Mimic CGA177280

	IC50 (μg/L)	
pH	CGA177280 coating[a]	CGA179944 coating[a]
4[b]	0.4	0.2
6	4.4	2.7
8	2.9	6.5
10	antibody not functional	

[a] Hapten−BSA conjugates were immobilized onto ELISA plates. [b] Buffers were as follows: pH 4 = citrate buffer; pH 6 = MES buffer; pH 8 = PBS; pH 10 = sodium bicarbonate buffer.

2E4-Immunoassay Detection of Penconazole and Penconazole Metabolite. An example of the assay for penconazole is depicted in **Figure 4A**. The IC50 of this assay was shown to be 1.2 μg/L in a buffered system (mean of 8 analyses). The LOD for this assay has been calculated as 0.3 μg/L, where this is defined as the lowest concentration of analyte giving an absorbance value separated from A_0 (the absorbance value at zero competitor) by 3 × SD where SD was the standard deviation of the absorbance value for the samples containing no competitor (*11*). This IC50 and theoretical detection limit (in buffer) is to date one of the most sensitive ELISA based assays that have been described for penconazole and for any of the triazole group of fungicides. At pH 8.0 this assay showed 41−109% cross-reactivity with the immunizing haptens, and 57% cross-reactivity to penconazole metabolite; however, the assay displayed minimal reactivity to other conazole fungicides tested (hexaconazole, imazalil and tebuconazole; **Table 3**). Of note is that the IC50 for the detection of penconazole was very similar at pH 4.0 and pH 8.0 (**Table 3**). However at lower pH (pH 4.0) while the assay detects penconazole at a similar level to that at pH 8.0, the cross-reactivity to several other similar compounds increases at lower pH (figures not shown). For the detection of penconazole, it would be recommended that this assay be carried out at the higher pH where the % cross-reactivities seen were lower. This immunoassay is readily applicable to the monitoring of MRL levels permitted in fruit and vegetables.

A representative curve of the penconazole metabolite assay is depicted in **Figure 4B**. This assay at pH 4.0 in citrate buffer has a mean IC50 of 0.9 μg/L (mean of 42 analyses). This assay has a theoretical detection limit (in buffer) of 0.4 μg/L. This assay shows high sensitivity for metabolite; however, there is also a high cross-reactivity (91%) with the parental compound and, as expected, there is high cross-reactivity with the immunizing haptens; however, similarly structured fungicides are not recognized to any great extent (**Table 3**). This is the first immunoassay

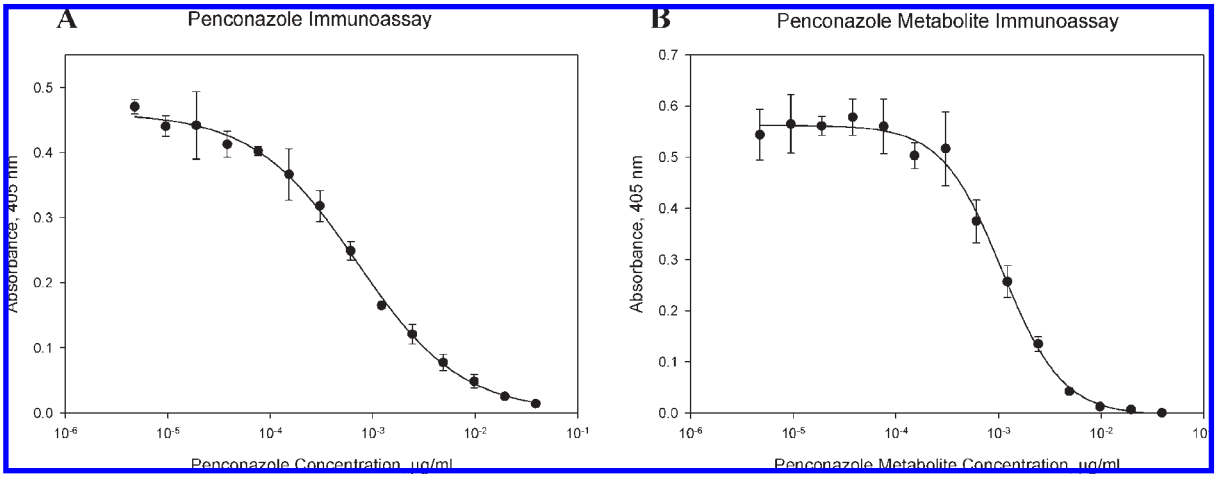
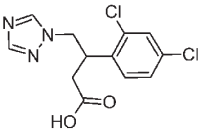
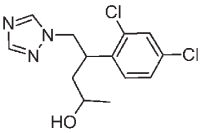
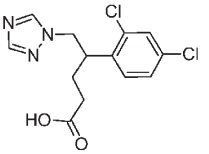
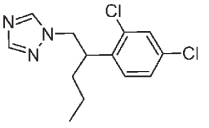
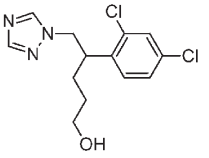
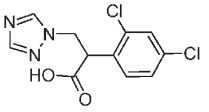
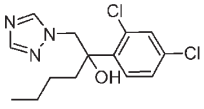
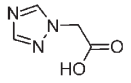
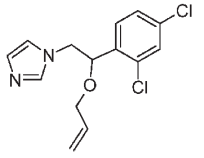
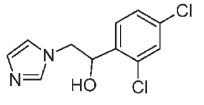
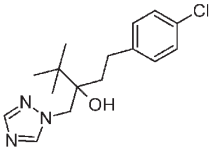
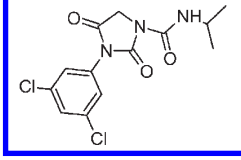


Figure 4. (**A**) Representative standard curve for penconazole, obtained under optimized conditions: assay hapten CGA177280 at a coating of 20 μg/L, and monoclonal antibody dilution of 1/500. The assay buffer was composed of PBS pH 8.0. The IC50 of this assay has been determined to be 1.2 μg/L at pH 8.0 (mean n = 8 assays). (**B**) Representative standard curve for penconazole metabolite, obtained under optimized conditions: assay hapten CGA177280 at a coating of 20 μg/L, and monoclonal antibody 2E4 dilution of 1/500. The assay buffer was citrate pH 4.0. The IC50 of this assay at the optimal pH 4.0 was determined to be 0.9 μg/L (mean n = 42 assays).

Table 3. Specificity of Immunoassay at pH 4.0 and pH 8.0[a]

Chemical Structure	Compound	2E4 ELISA at pH 4.0 IC50 (μg/L)	2E4 ELISA at pH 4.0 % CR[1]	2E4 ELISA at pH 8.0 IC50 (μg/L)	2E4 ELISA at pH 8.0 % CR
	CGA177280	0.3	303	2.9	41
	CGA132465	0.5	182	1.1	109
	Penconazole metabolite	0.9	100	2.1	57
	Penconazole	1.0	91	1.2	100
	CGA127841	3.8	23	8.7	14
	CGA179944	70	1.3	52	0.02
	Hexaconazole	330	0.27	1340	0.09
	CGA146741	NC[2]	-	NC	-
	Imazalil	NC	-	390	0.3
	Imazalil metabolite	NC	-	3.1	38
	Tebuconazole	NC	-	NC	-
	Iprodione	NC	-	NC	-

[a] [1]Cross-reactivity (CR) is expressed as (IC50 penconazole metabolite or penconazole/ IC50 compound tested) × 100. CR at pH 4.0 is cross-reactivity to penconazole metabolite (where this assay has the best sensitivity to metabolite), and at pH 8.0 this is cross-reactivity measured against the parental penconazole (the pH recommended for the detection of penconazole). [2]NC, no competition

Table 4. Analysis of Penconazole Metabolite Spiked Urine Samples

urine[a] spike concn: (μg/L) metabolite	level detected[b] (μg/L) metabolite	% recovery	% CV
0.5	0.7	140	57
1.0	0.9	90	48
2.5	1.6	80	25
5.0	3.9	78	13
10.0	10.6	106	24

[a] Urine consisted of pooled urine from healthy volunteers. Urine was extracted using C18 column chromatography, and applied to the immunoassay at pH 4.0 (for the most sensitive detection of the metabolite) as a 1 in 2 dilution of extracted urine (in order to relieve matrix effects). The levels of the metabolite measurable in urine were calculated using a standard curve and the 4-parameter logistic equation. [b] The mean levels recorded were derived from spiking, extraction and analysis of 5 separate samples. The calculated figure for the level of spike detected has been adjusted to remove the background signal of unspiked urine extract (0.3 ppb mean, $n = 5$).

describing a test for a metabolite of penconazole; the rationale for isolating a monoclonal antibody to this compound was to develop a method of measuring either occupational or low level exposure to penconazole via urine analysis. The high cross-reactivity with penconazole is not anticipated to be a problem for such an application as animal model studies have shown that only very small quantities of penconazole (less that 1%) are excreted in urine as nonmetabolized pesticide (*2*).

Detection of Penconazole Metabolite in Spiked Urine. The analysis of spiked urine samples for penconazole metabolite was carried out to define the methodology for the analysis of urine and to determine the assay parameters. The urine used for these tests was provided by healthy volunteers and used as a pool and with no knowledge of prior exposure to penconazole above that for the general population. The immunoassay was sensitive to urine resulting in complete inhibition of the antibody binding, and this effect could not be overcome by simple dilution of the matrix (data not shown) as reported for other immunoassays (*13*). As an alternative strategy to overcome the matrix effects of urine, Shackleford et al. (*14*) successfully applied C18 column chromatography to extract metabolite of the organophosphate chlorpyrifos (3,5,6-TCP) from urine. Here, this methodology was applied to the extraction of the penconazole metabolite from spiked urine samples. The analysis of spiked urine was carried out using multiple extracts at the same spike concentration, the extracted samples were assayed, and the metabolite concentration was calculated from the standard curve. The results of these extractions (recovered spike) are detailed in **Table 4**, and a percentage recovery has been calculated at each spike level. The assay is able to detect the presence of 0.5 μg/L of penconazole metabolite in urine; however, this must be near to the LOD of this assay in this matrix as the % CV of this assay is high at 57%, and the data shows that reproducible, quantitative determination of metabolite in urine would be achievable in the region of 1−2.5 μg/L. This compares well with the sensitivities of instrumental analyses of urine to monitor pesticide exposure in population screening (*15*). The analysis of unspiked urine did show a low mean value of 0.3 μg/L penconazole metabolite being present. This value is below the LOD of the assay and as such cannot be represented as a background level of penconazole in this urine. For the purpose of this study, this value was subtracted from the calculated values of metabolite found at each spike level. The described immunoassay can detect levels of penconazole metabolite in urine at levels which should be appropriate for monitoring human population exposure to low levels of penconazole such as through diet. The assay is also wholly applicable for monitoring those occupationally exposed during either the manufacture or the application of this pesticide. Within the animal models, the excretion of 4-(2,4-dichlorophenyl)-5-(*H*-1,2,4-triazol-1-yl)pentoic acid is not thought to be associated with glucuronide conjugates (*2*); therefore the extraction and analysis of the metabolite would not require treatment with glucuronidase or by acid hydrolysis.

The present report describes a novel quantitative immunoassay which was able to detect penconazole with the best specificity at pH 8.0 and with an assay IC50 of 1.2 ppb (1.2 μg/L). This assay has obvious application for the monitoring of foodstuffs for contamination with penconazole and compliance with the MRL, as well as for possibly studying pesticide fate in the environment. When the assay was performed at pH 4.0, it detected penconazole metabolite with an assay IC50 in buffer of 0.9 μg/L and an LOD of 0.4 μg/L. When spikes of metabolite were added to urine, extracted by C18 column chromatography and assayed at pH 4.0, spike present at a level of 0.5 ppb (0.5 μg/L) could be detected, with reliable quantitative detection above 1 μg/L. The analysis of urine with this assay has application in describing human exposure to this commonly used pesticide from occupational and non-occupational contact.

ABBREVIATIONS USED

BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ELISA, enzyme linked immunosorbant assay; MRL, maximum residue limit; IC50, concentration of competitor giving 50% inhibition of maximum signal; LOD, limit of detection; PBS, phosphate buffered saline; PBS-t, phosphate buffered saline including 0.05% Tween 20.

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