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Laboratory and Field Enzyme-Immunoassays for Diazinon and Their Application to Residue Analysis in Lanolin, Water, and Fruit Juice

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Diazinon (O,O-diethyl-O-2-isopropyl-6-methylpyrimidin-4-yl-phosphorothioate) is an organophosphate insecticide widely used on turf, crops such as rice and citrus, and for the control of external parasites. When applied as a dip to control insect pests of sheep, diazinon can accumulate in lanolin, the greasy secretion that protects the wool fibers, which is used as a base for many facial creams and pharmaceutical ointments (Moody and Nadeau 1994). Monitoring of lanolin and lanolin-based products is necessary to allay possible human health concerns, and diazinon use on food crops also has the potential to lead to residues. As a consequence of its solubility and widespread use, diazinon residues have been found to enter surface water through runoff (Fushiwaki et al. 1993) with its persistence in estuarine conditions influenced by water temperature and pH (Lacorte et al. 1995) and sorption to particulates (Villarosa et al. 1994). In some degradation studies, diazinon has been one of the more persistent organophosphorus pesticides noted (Frank et al. 1991a). Pollution from diazinon may have effects on aquatic eco-systems through toxicity to and bioconcentration in microorganisms and fish (Tsuda et al. 1989, Alam and Maughan 1993), while bird deaths have been reported following turf treatments (Frank et al. 1991b). Analytical methods typically use liquid-liquid or solid-phase extraction, concentration and analysis by gas chromatography (GC) (Sasaki et al. 1987, Edgell et al. 1991, Hsu et al. 1991). These methods can be expensive and require skilled analysts and well equipped laboratories. In this report, we describe the development of laboratory (96-well microplate) and field (polystyrene tube) competitive enzyme-immunoassay (EIA) formats, based on polyclonal antibodies. These provide an alternate method of diazinon quantitation, and require little or no cleanup or sample concentration for detection of residues in well water, lanolin and fruit juice.

MATERIALS AND METHODS

The development of a diazinon hapten required a different approach from that used for other organophosphates (Skerritt and Lee 1996), because diazinon was hydrolyzed at both low and high pH. Thus the route used by us for other organophosphate- spacer arm haptens was not possible, as it involved either deprotection of a *tert*-butyl ester using trifluoroacetic acid or of a trimethylsilyl ester (McAdam and Skerritt 1993). The chemical route followed instead used a neutral 3-aminopropanol spacer arm, and involved four steps: 1. Diazinon (18 g, 59 mmol) was hydrolyzed by refluxing for 4 h in 115 mL of 70 % ethanol in water, containing 10 g (16.5 mmol) KOH. The mixture was neutralized and a

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pyrimidone product (90 % yield) was extracted using chloroform, and recrystallized from 10 % chloroform in petroleum ether. 2. The sodium salt was formed by treatment of 150 mg (1 mmol) of the pyrimidone with 60 mg (1.1 mmol) of sodium hydride by stirring for 15 min at 20 °C in 5 mL toluene, then ethyl dichlorothiophosphate (132 µL, 1 mmol) was added and stirred 3 d at 20 °C. The product was isolated by chromatography in chloroform : petroleum ether, 1 : 1 with a 59 % yield. 3. Displacement of the chlorine by the amino substituent of 3-aminopropanol; 1 g (3.4 mmol) of the product from the previous step, *O*-ethyl *O'*-[6-methyl-2-(1-methylethyl)-pyrimidinyl] phosphoroamido-chloridothioate, was stirred with 0.51 g (6.6. mmol) of 3-aminopropanol and 0.75 g (9 mmol) $NaHCO_3$, dissolved in 25 mL acetonitrile, for 16 h at 0° C, filtered, and chromatographed on silica in ethyl acetate: petroleum ether, 60:40, to yield the alcohol, *O*-ethyl *O'*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] *N*- [3-hydroxypropyl] phosphoro-amidothioate, 87 % yield. The structures of intermediates in reactions 2 and 3 were confirmed by ^{1}H-NMR 4. Formation of the imidazole ester of the alcohol; by stirring 70 mg (0.21 mmol) of the alcohol with 41 mg (0.25 mmol) of carbonyldiimidazole (CDI) in 2 mL acetonitrile, for either 48 h at 18-23°C (method 1) or 24 h at 4°C (method 2). Thin layer chromatography on silica (ethyl acetate: petroleum ether, 1:1) indicated that the reactions had yielded 40-60 % and 20-40 % product, respectively. Attempts to isolate these products led to hydrolysis and thus failed.

Haptens were then coupled to the following carrier proteins: ovalbumin (OA), chicken IgG (termed IgY) or keyhole limpet hemocyanin (KLH) for use as immunogens, or coupled to horseradish peroxidase (HRP) for use as an enzymatic detector. A 40 molar excess of CDI derivative (16 mg/mL in N,N'-dimethylformamide) was incubated with protein (4 mg/mL) in potassium phosphate, pH 9.1. Rabbit antisera were only raised to haptens produced using from method 1 (coupled to OA [antibody l] or KLH [antibody 2]) and method 2 (coupled to IgY [antibody 3]), and antibody purified as previously described (McAdam et al. 1992). The immunogens had the following coupling ratios: 1.8, 2.3 and 2.8 mol hapten/mol protein, while hapten from method-1-HRP had a coupling ratio of only 0.5 mol hapten/ mol protein, determined using the method of Plapp et al. (1971).

Antibodies were assessed in a solid-phase antigen assay using Maxisorp 96-well plates (Nunc, Roskilde, Denmark) coated overnight with 200 µL/well of 0.5 µg/mL of hapten-IgY (for antibodies to KLH or OA conjugates) or hapten-OA (for antibody to IgY conjugate), in 50 mM sodium carbonate, pH 9.6. Plates were washed 3 times with water, then active sites blocked 60 min with 150 µL of 1 % (w/v) bovine serum albumin (BSA) in 50 mM sodium phosphate-0.9% NaCl (PBS). Antibodies were added (100 µL) to microwells after diluting serially in 1% BSA-PBS-0.05 % Tween 20 and incubated for 60 min. After 4 washes with PBS-0.05 % Tween, 200 µL/well of HRP-goat-anti-rabbit immunoglobulins (Dako, Carpinteria, CA) at 3.25 µg/L in 1 % BSA-PBS-Tween were added and incubated 30 min. After 4 washes, 150 µL of H_2O_2-3,3',5,5'-tetramethylbenzidine (Hill et al. 1991) were incubated 30 mm, and after addition of 50 µL/well 1.25 M H_2SO_4, to stop the reaction the absorbance was read at 450 nm. Solid-phase antibody competitive assays were also performed on plates coated with 100 µL/well of 10 ug/mL antibody in 0.05 M sodium carbonate buffer, pH 9.6. After overnight incubation and a wash step, remaining active sites were blocked as for the solid-phase antigen assay format. Samples (100 µL) and diazinon-peroxidase (500 ng/mL, 100µL) were incubated simultaneously and the color was developed and read as for the solid-

phase antigen format for plates. An alternative version of this assay was developed based on polystyrene tubes (Sarstedt, Germany) coated with 500 µL antibody per tube (2 µg). The sample and enzyme-labeled hapten were incubated either simultaneously for 30 min or sequentially (2 x 15 min) and color read with a photometric analyser (Source Scientific, Garden Grove, CA). All incubation steps were performed at room temperature (18-23 °C), and experiments performed in triplicate. For instrumental analyses of diazinon, a Hewlett Packard (Palo Alto, CA) model 5890 gas chromatograph (GC) and a model 5921A atomic emission detector (AED, monitoring S) were used. Samples of 2 µL were injected using a splitless injection mode and a 250°C injection port temperature, into a 5% phenyl methyl silicone column (HP-5 crosslinked, 25 m x 0.32 mm x 0.17 µm film thickness), with a 100 °C to 225°C gradient over 6 min.

After characterization of the antisera and assay formats, plates and tubes were pre-coated with antibody 1, and sample analysis performed either using the solid-phase antibody competitive assay on microwells or a sequential tube assay. In this assay, diazinon standards and samples were prepared as for the laboratory assay, but 500 µL were added to the tubes. After a 30 min incubation at ambient temperature, tubes were rinsed 3 times in tap water, diazinon-peroxidase (500 µL) was added and the tubes incubated a further 30 min. Following a second washing step, substrate/chromogen (500 µL) was added and color developed for 30 min.

Well and surface water, obtained from sites near Portland, ME was fortified with 60, 75, 100, 200 and 250 ng/L diazinon (Reidel-de-Hahn, Seelze, Germany). Several pesticide-free fruit juices (under 10 ng/L by GC) were also obtained and used to test for potential matrix interference in the EIA. Diluted juices were fortified with diazinon standard at 5 concentrations from 25 to 400 ng/L. Fortified lanolin samples were prepared from 1g of commercial lanolin (Westbrook Lanolin, Bradford, UK) dissolved in 9 mL of dichloromethane then shaken orbitally (180 rpm, 10 min). Samples of lanolin containing incurred residues were obtained from stores in Bangor, ME. Rapid methods were developed for extraction of diazinon from lanolin, For EIA, 1 gram of lanolin was added to 9 mL dichloromethane, and shaken (180 rpm, 10 min) to dissolve. The extract was either a) diluted 1: 100 in distilled water, the suspension sonicated 10 min, then filtered through a 0.45 pm filter or b) diluted 1:100 in isopropanol. For GC, 1g of lanolin was dissolved in 20 mL of dichloromethane and 2 µL applied to the system without extract cleanup. For method comparisons, a more lengthy solid-phase extraction method enabled the same lanolin sample extracts to be anayzed by GC and by EIA. This involved dissolving 1g of lanolin in 20 mL of tetrahydrofuran. Tween-20 (100 µL) and 20% methanol : water (50 mL) were added to 1 mL of extract and the solution was passed through octadecylsilica cartridge (Millipore, Bedford, MA), after pre-conditioning with 5 mL methanol and 5 mL water. The column was rinsed with 25 mL 20% methanol; after drying 20 min, the column was eluted with 1 mL of isopropanol. The eluate was used for both GC and immunoassay (after diluting 1:20 in 0.1 M phosphate buffer, pH 7.2).

RESULTS AND DISCUSSION

While the method for immunogen preparation achieved low hapten-to-protein coupling ratios (1.5-2 haptens per mole), two of the three antisera provided very sensitive assays.

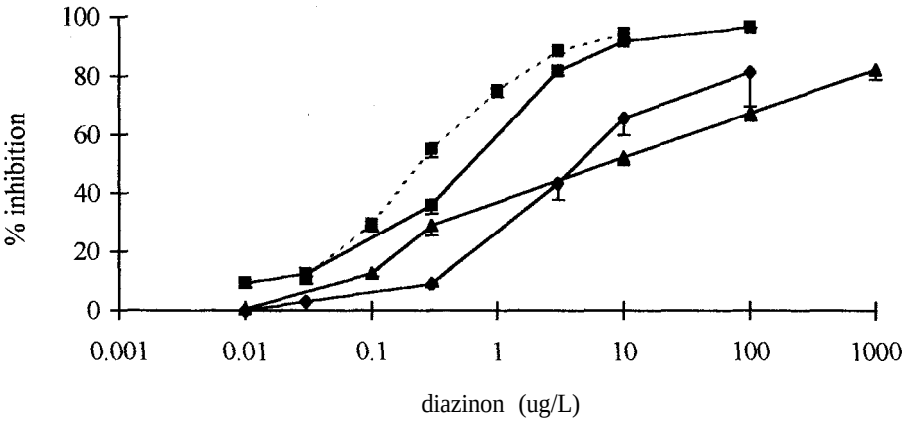

Figure 1. Standard curves for Ab1 (■), Ab2 (▲) and Ab3 (◆) in the microwell assay and Ab1 (dashed line) in the tube assay. Data means ± standard deviations from 3-10 assays.

Ab1 had IC_{50} values of 0.4 µg/L in both assay formats, while Ab2 had IC_{50} values of 3 and 7.5 µg/L in the solid-phase antigen and antibody formats respectively. In the assays using solid-phase antibody, Abl had a steep response curve, with a 39 % change in OD for a ten-fold change in pesticide concentration; Ab2 had a much less dynamic response curve (14 % change in OD). Ab3 gave a dynamic assay (38 % change in OD), but was 15 and 3 times less sensitive for diazinon than Ab1 and Ab2 respectively (Figure 1). Ab3 sera also had a very low titer, and therefore was not assessed further. The antibodies detected diazinon in the solid phase antigen and antibody formats with approximately similar sensitivities (Table 1), although the latter format has been more sensitive in other organophosphate immunoassays (McAdam et al. 1992). Using Ab1, the solid-phase antibody format was selected for routine use because of its sensitivity, linear range and since it was consistent with most of the other organophosphate assays that we have developed. Surprisingly, the rapid tube version of the assay with Ab1 was 2-3 times more sensitive than the microwell version (limit of detection, IC_{10} of 0.05 µg/L).

Ab1 and 2 were very specific for the diazinon structure in both the solid-phase antigen and antibody formats (Table 1). Cross-reaction with the oxon form of diazinon was approximately 10 %, while reaction with pirimiphos-methyl and ethyl, which share the di-substituted pyrimidine ring structure, was minor, with assay sensitivities 5,000 and 50,000 times lower, respectively. Apart from the compounds shown in Table 1, etrimfos, a tri-substituted pyrimidine organophosphate, and other compounds were not detected at 10 ppm, including insecticides (cythioate, dicapthon, DDT, dieldrin, dimethoate, fensulfothion, methidathion, paraoxon and temephos) and herbicides (atrazine, chlorthal, diuron, molinate, simazine). Like other antibodies we have developed using organophosphate haptens coupled through the phosphate ester, the hydrolysis metabolite (pyrimidinol) was not detected. The rapid tube assay, using Ab1, had a similar specificity to the microwell assay that utilized this antibody.

The performance of the microwell assay with diazinon spikes in water was assessed in two experiments. In the first, seven surface water samples were fortified with 60 and 200 ng/L diazinon; these showed mean recoveries of 98 ± 17 % and 118 ± 14 % respectively (Table 2). The performance of the microwell EIA was also assessed by analysis of residue recovery from nine well water samples, spiked at 100 ng/L. There was a 98 ± 7 % mean recovery of pesticide, and in four assays, coefficients of variation (CV, standard deviation divided by the mean) for each sample between 3 and 18 % (average 11 %) were obtained. The relationship between instrumental and microwell immunoassay data was evaluated in two experiments. In the first, analysis of 8 samples of industrial wastewater, containing 0.2-1.4µg/L diazinon by GC/MS yielded highly correlated results (diazinon (ETA, µg/L) = 0.96 x diazinon (GC/MS, µg/L) + 0.11; r = 0.91, P < 0.01). In the second, EIA analyses of 46 samples of urban drainage water from Fort Worth, TX were compared with GC data obtained using USEPA method 8140. Results were highly correlated (diazinon (EIA, µg/L) = 0.97 x diazinon (GC/MS, µg/L) + 0.03; r = 0.89, P < 0.001).

Table 1. Specificities of antibodies (Ab) 1 and 2 in different immunoassays.

Antibody:	Ab 1				Ab2			
Solid phase:	Antigen		Antibody		Antigen		Antibody	
Compound:	IC15	% X	IC15	% X	IC15	% X	IC 15	% X
diazinon	.02	100	0.05	100	0.1	100	0.2	100
diazoxon	.3	9	0.5	11	1	10	3	6
hydroxy- metabolite	nd	nd	nd	nd	500	>	100	>
pirimiphos- methyl	500	>	2750	0.002	10	0.2	500	0.3
pirimiphos- ethyl	50	0.02	55	0.02	5	1.7	10	4
fenitrothion	nd	nd	10000	>	nd	nd	nd	nd
chlorpyrifos- methyl	100	>	100	>	10000	>	nd	nd
chlorpyrifos- ethyl	10000	>	1000	>	nd	nd	nd	nd
azinphos-methyl	300	>	2000	>	nd	nd	nd	nd
fenthion	2000	>	5000	>	nd	nd	nd	nd
dichlorvos	nd	nd	10000	>	nd	nd	nd	nd
parathion-methyl	nd	nd	nd	nd	10000	>	nd	nd
fenchlorphos	nd	nd	nd	50	nd	nd	nd	nd
bromophos	nd	nd	nd	nd	10000	>	nd	nd
tetrachlorvinphos	nd	nd	nd	nd	1000	>	nd	nd
molinate	nd	nd	nd	nd	10000	>	1000	>
atrazine	nd	nd	nd	nd	10000	>	100	>

% X denotes cross-reaction = IC50 (diazinon)/ IC50 (test compound) x 100
nd = no inhibition at 10 ppm; > denotes IC50 greater than 10000 ppb

Using the tube test, no matrix interference was found when commercial pasteurized and filtered juices (including 2 apple, 2 orange, 2 cranberry, 2 pinneapple, 2 apple cider, 1 apple-strawberry and 1 raspberry, lacking detectable incurred residues by GC) were diluted 1:10 in water for analysis. Each of these juices was spiked with 25, 50, 100, 200 and 400 ng/L diazinon and analyzed four times which gave the following averaged recoveries: 59, 57, 72, 73 and 77 % respectively, with a 10 % CV in each case. Recoveries were similar in each

juice type; the incomplete recovery appeared to be due to the acidity of the juices, since diazinon hydrolyzes much more quickly at low pH (Worthing 1991).

Table 2. Mean recoveries of diazinon from fortified surface water samples. Data are means from 6 experiments, standard deviations less than 15 % of the mean.

Fortification level	60 ng/L		200 ng/L	
	ng/L recovered	% recovery	ng/L recovered	% recovery
river 1	43	72	210	105
river 2	54	90	220	110
river 3	75	125	265	133
lake	59	98	205	102
swamp 1	53	88	210	105
swamp 2	55	92	280	140

EIA analysis of lanolin extracts was performed using sequential tube assay, which had the advantage of separating the sample and enzyme conjugate incubations, allowing undiluted solvent extracts to be used. In this way sufficient sensitivity is retained yet the activity of the enzyme-conjugate is not compromised by exposure to high solvent concentrations. Although dichloromethane has very low aqueous solubility, the dichloromethane extract (selected for rapid solubilization) could then be "diluted" 1:100 in distilled water (with some simple steps to solubilise and cleanup the extract), or directly 1:100 in isopropanol before analysis by EIA. The latter approach is simpler but has the disadvantage of reducing assay sensitivity by 800-1200 fold, compared with dilution of standards in water. However, since the limit of detection of diazinon in an isopropanol propanol diluent is still under 5 µg/L, the loss in assay sensitivity can be tolerated. For comparing GC-AED and EIA methods, a solid-phase extraction procedure was used for lanolin-based products. Highly significant correlations were found between incurred diazinon residues determined by both methods (Figure 2). A further 12 samples were found to be diazinon-free by both GC-AED and EIA. One sample, a commercial handcream, was unable to be analysed by the GC-AED because of matrix interference. The precision of this assay was also high; six separate analyses of two lanolin samples spiked at 0.04 and 9.0 mg/kg diazinon gave coefficients of variation (CV) of 4.8 % and 3.3 % respectively. Assay performance was also assessed, by comparison of the microwell EIA (four experiments) and GC-AED (six experiments) for the 12 lanolin samples depicted in Figure 2. Precision results were similar for both techniques, with the microwell EIA providing CV values of 5-39 % and GC-AED providing CV values of 10-36 %.

Using the tube test, no matrix interference was found when commercial pasteurized and filtered juices (including 2 apple, 2 orange, 2 cranberry, 2 pinneapple, 2 apple cider, 1 apple-strawberry and 1 raspberry, lacking detectable incured residues by GC) were diluted 1:10 in water for analysis. Each of these juices was spiked with 25, 50, 100, 200 and 400 ng/L diazinon and analyzed four times gave the following averaged recoveries: 59, 57, 72, 73 and 77 % respectively, with a 10 % CV in each case. Recoveries were similar in each juice type; the incomplete recovery appeared to be due to the acidity of the juices, since diazinon hydrolyzes much more quickly at low pH (Worthing 1991).

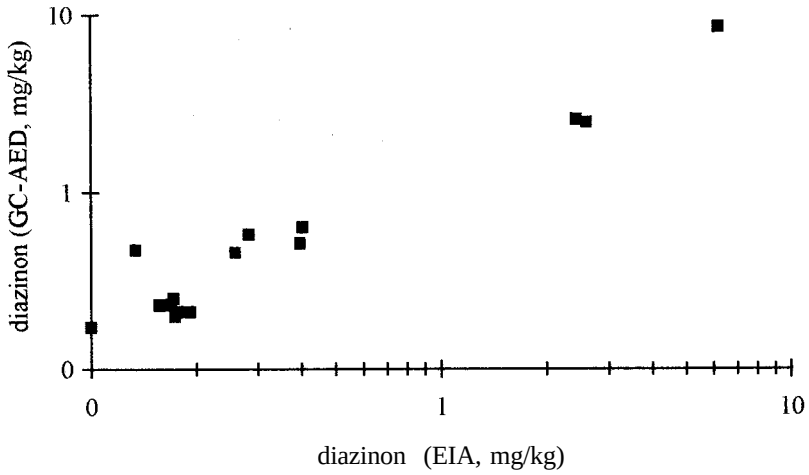

Figure 2. Relationship between GC-AED and EIA data for incured residues in lanolin and lanolin-based products. Equation for linear regression: Diazinon (GC, mg/kg) = 0.76 x Diazinon (EIA, mg/kg) + 0.022, n = 15, r = 0.99, P < 0.001

In conclusion, we have produced highly sensitive and robust immunoassays for diazinon. Of the 27 lanolin and lanolin-based samples analysed, no false positives/negatives were observed. Analysis of water and fruit juice samples did not require sample cleanup or extraction and good recoveries were obtained. The high sensitivity of the assay, presumably resulting from a high affinity antibody, enabled either suspensions of dichloromethane extracts diluted in buffer or undiluted isopropanol extracts to be successfully analysed. Other groups have also demonstrated that immunoassays can be established to haptens such as 4-aminobiphenyl (Russell et al. 1989) and progesterone (Weetall 1991) using neat organic solvents as the liquid phase. While the extraction and dilution of lanolin extracts in isopropanol reduced the lower limit of detection of this assay about one thousand-fold, this provided adequate sensitivity for the detection of incurred residues in this matrix encountered in this study. The limit of detection of the assay was well below the Codex Maximum Residue Limit for diazinon in grapes and many other fruits (500 mu g/kg) and the Australian and New Zealand Environment and Conservation Council guidelines for raw drinking water (10 µg/L).

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