DEVELOPMENT OF LABORATORY AND FIELD ELISA TESTS FOR DETECTION OF ENDOSULFAN IN WATER AND SOIL

N. LEE[1,2,3], J. H. SKERRITT[1,2], I. R. KENNEDY[3], D. P. McADAM[2], H. L. BEASLEY[1] & A. S. HILL[2]

1 - CSIRO Division of Plant Industry, North Ryde, NSW, 2113, AUSTRALIA
2 - CSIRO Division of Plant Industry, Canberra, ACT, 2601, AUSTRALIA
3 - Department of Agricultural Chemistry and Soil Science, Sydney University, NSW, AUSTRALIA

Abstract

Environmental monitoring of endosulfan residues is currently being achieved by gas chromatographic analysis, but there is a need for faster and cheaper analysis that can be conducted in the field as well as in the laboratory. To satisfy this need, two enzyme immunoassay methods, a laboratory assay based on microwell plates and a field test based on the use of small plastic tubes, have been developed for the detection of endosulfan residues in water and soil. These assays have the detection limit of 0.2 ppb of endosulfan, and the detection range of 0.2 ppb - 10 ppb for laboratory assay and 0.2 ppb - 20 ppb for field assay. The field test takes 15 minutes to complete using dropper bottles containing enzyme conjugate and colour development reagents, and can be performed at the site of possible contamination. The laboratory test takes longer to run, but the ability to analyse large numbers of samples simultaneously gives the method high throughput. Water samples can be analysed directly and soil samples are simply extracted with 90% methanol. The tests also detect endosulfan sulfate with similar sensitivity to endosulfan but are four to ten times less sensitive to endosulfan diol, and therefore can potentially determine endosulfan and endosulfan sulfate from the total endosulfan residues present in the environment.

Introduction

The most important use of endosulfan in Australia and many other countries is to control *Helicoverpa* spp., especially in cotton (Fitt, 1994). It is often considered to be a 'soft pesticide' as it is less persistent and also much less toxic to most organisms except fish, compared to other organochlorine pesticides (Goebel *et al.,* 1982). Recent studies have shown endosulfan may persist longer in field water and soil by binding to sediments and soil particles (Chandler and Scott, 1991; Peterson and Batley, 1993). Many studies have been conducted to fully investigate the fate of endosulfan in the environment and the effects of particle-bound endosulfan on aquatic organisms. Problems arising from the environmental studies are: 1) inadequate number of samples being analysed due to the cost and the time involved in analysis, and 2) a long interval between the collection and the analysis.

Endosulfan is generally analysed by instrumental methods such as gas chromatography with electron capture detection (Quintanilla-Lopez *et al.*, 1992), gas chromatography/mass spectrometry (Wilkes, 1981) or high performance liquid chromatography (Galeano *et al.*, 1992). Each of these methods requires extraction, clean-up and concentration of the sample. This is not only labour intensive, time consuming and expensive, but it is also the rate-limiting step in performing more detailed environmental studies. Therefore there is a need to develop a rapid, simple and cost-effective method of analysis for endosulfan in water and soil.

The assay was designed to detect selectively the group of compounds of endosulfan that are most toxic to fish, namely isomers of endosulfan and the metabolite, endosulfan sulfate.

Hapten synthesis, immunoassay sensitivity, specificity and performance with water and soil samples are discussed in this paper.

Materials and Methods

Hapten synthesis

Hapten I was prepared by esterifying one of the hydroxyl groups of endosulfan diol with succinic anhydride, then coupled to a carrier protein for immunisation (Dreher and Podratski, 1988). The synthetic scheme for enzyme conjugate has been modified as the published method resulted in instability of enzyme conjugate. Hapten II was synthesized by the esterification of 1-hydroxy-chlordene with succinic anhydride and the resulting acid was coupled to a carrier protein for immunisation and horseradish peroxidase (HRP) as an enzyme conjugate. The esterification of the alcohol compound of heptachlor with succinic anhydride produced hapten III.

Antibody preparation and purification

Antibody was raised by intradermal and intermuscular injections of haptens conjugated to keyhole limpet hemocyanin (KLH) and ovalbumin (OA) into white New Zealand rabbits. The blood was collected every four weeks and the antiserum was stored at -4˚C until purification.

Preparation of endosulfan standard

100 ppm endosulfan (isomer mix) was prepared in methanol as a stock solution. From this stock solution, 100 ppb was prepared by 1/1000 dilution in purified water and then serially diluted to obtain 100 ppb, 30 ppb, 10 ppb, 3 ppb, 1 ppb, 0.3 ppb and 0.1 ppb in borosilicate glass tubes for the standard curve. The standard curves for field samples were prepared in the same way, using turbid water from cotton farms and other irrigation areas in Australia, with turbidity ranging from 27 to 86 NTU (Nephelometer Turbidity Units measured by Analite 152, McVan Instruments, Australia); extract of soil (from cotton farms, at Narrabri) was diluted 1/100 in water.

Laboratory ELISA

Antibodies were diluted in 50 mM carbonate buffer, pH 9.6, to 10 µg/ml and coated on a polystyrene 96-well plate at 100 µl per well overnight at 20º C. The plate was washed twice with phosphate buffer-saline containing 0.05% (v/v) Tween 20 (PBS/T, 0.05M sodium phosphate-0.9% (w/v) NaCl, pH 7.2 with 0.05% (v/v) Tween 20) to remove any unbound antibodies, and 150 µl of PBS containing 1% bovine serum albumin (1% BSA-PBS) was incubated for one hour to block unbound sites. After three washes with PBS/T, 100 µl of endosulfan standard or sample followed by 100 ml HRP-conjugate diluted in PBS containing 0.5% (w/v) fish skin gelatin (0.5% FG/PBS), were incubated for one hour at 20˚C. Hydrogen peroxide substrate/chromogen (3,3',5,5"-tetramethylbenzidine-hydrogen peroxide in acetate buffer, pH 5.5, 150 µl) was added and incubated for 30 minutes at 20˚C. The colour

development was stopped by adding 50 µl 1.25M sulfuric acid and the plate was read at 450 nm.

Field ELISA

Antibodies (12.5 µg/ml) were immobilised at 400 µl per tube overnight at 20˚C. After washing the tube twice with PBS/T, the unbound sites were blocked by adding 500 µl per tube of 1% BSA-PBS. The tubes were then washed three times to remove excess 1% BSA-PBS. The reagents were filled into the dropper bottles, and the samples and standards were added into the tube using the application droppers. Four drops (160 µl) of sample and four drops (160 µl) of HRP-conjugate were added to a tube and incubated for 10 minutes at 20˚C. The tube was washed with tap water four times and shaken vigorously. Four drops of substrate (300 µl) then chromogen (150 µl) were added for colour development and incubated for 5 minutes. Finally, four drops (150 µl) of stopping solution (0.625M sulfuric acid) were added to stop the colour development and the absorbance read with a portable photometer.

Soil extraction

Ten grams of soil was weighed into a 100 ml conical flask, then 20 ml of 90% methanol was added. The flask was shaken overnight and allowed to stand until the particles settled. The supernatant was diluted 1/100 with water, and then directly analysed in the immunoassay.

Results and Discussion

Assay sensitivity

To improve the sensitivity and the specificity of the assays, structurally different haptens were used in the preparation of immunogen and enzyme conjugate. The optimised assay was developed by raising antibodies against hapten II and enzyme conjugate prepared from hapten III. A mean standard curve of the laboratory assay (plotted % inhibition of colour development versus concentration of endosulfan) representing data from eight curves obtained on four different days is shown in Figure 1. The standard curve prepared in purified water showed the limit of detection (taking endosulfan concentration at 15% inhibition of colour development) of 0.2 ppb of endosulfan (isomer mix) and the reliable range of quantitation of 0.2 ppb - 10 ppb. The IC_{50} (endosulfan concentration giving 50% inhibition of colour development) was 1.6 ± 0.5 ppb, and the difference in the percentage inhibition of colour development between 1 ppb and 10 ppb was 43%, indicating the slope of the curve was steep enough for quantitative analysis. The matrix effects of water and soil extract were examined by comparing the standard curve generated in purified water with those produced from either turbid water (Fig. 1) or soil extract (Fig. 2). The curves were superimposed, showing there was no significant matrix effect from the turbid water and the soil extract.

A comparison of the standard curve of the field assay and the laboratory assay of the same antibodies is shown in Figure 3. The field assay was developed by using antibody I and hapten II conjugate as the enzyme detection system. This assay had a detection limit of 0.2 ppb and the range of detection of 0.2 ppb - 20 ppb. The laboratory assay using the same antibodies and the enzyme conjugate also had the detection limit of 0.2 ppb, but the range of detection was between 0.2 ppb and 10 ppb. The laboratory assay gave 42% difference in the percentage inhibition of colour development between 1 ppb and 10 ppb and the field assay 36% (between 2 ppb and 20 ppb), thus the slope of the field assay curve was slightly flatter than that of the laboratory assay.

Assay specificity

Endosulfan sulfate, one of the major metabolites, has a toxicity to fish similar to that of the parent compound, whereas the hydrolytic product, endosulfan diol is much less toxic (Mattiessen *et al.*, 1982; Novak and Ahmad, 1989). Antibodies were selected so that the assay was specific to the toxic compounds, namely endosulfan and endosulfan sulfate. The cross reactivities of the antibody (on the plate format) for endosulfan metabolites are shown in Figure 4. The sensitivity of the assay for endosulfan diol was ten times less (calculated with IC_{50}) than those for endosulfan (both isomers) and endosulfan sulfate. Other minor metabolites, endosulfan lactone, endosulfan ether and endosulfan hydroxyether, were also detected at lower sensitivity than endosulfan.

Since antibodies were generated by haptens consisting the norbornene structure, which is common for all the cyclodiene insecticides, the cross reactivity for cyclodienes was investigated. Among the cyclodienes tested, dieldrin and endrin showed cross reactions of 100% (equivalent to endosulfan) or higher. Heptachlor also showed significant cross reaction with 50%. Aldrin, chlordane and lindane showed cross reactions below 12%. Pesticides such as synthetic pyrethroids (including alpha-cypermethrin, deltamethrin and lambda-cyhalothrin), organophosphates (including chlorpyrifos, methyl-parathion and profenofos), carbamates (thiodicarb and methomyl) and herbicides (including diuron and triclopyr) did not show any cross-reaction even at 1000 ppb. The Australian cotton industry has a history of the heavy usage of DDT until the mid 1980's. DDT and its metabolites such as DDE are still detected in soil at significant levels. DDT residues may interfere with GC analysis of endosulfan in soil and so the soil extracts routinely undergo extensive clean-up to remove such residues before GC analysis can be performed. The immunoassay did not show cross-reaction with DDT, even at 1000 ppb.

Spike and recovery studies

Water samples of varying tubidity (27 - 86 NTU) were spiked with endosulfan (isomer mix) for the recovery studies (Fig. 5). The coefficient of correlation between the concentration of endosulfan spiked and the recoveries on ELISA (laboratory assay) was 0.98 and the slope of regression line was 1.02 with the y-intercept of 0.13, showing a good recoveries were observed for water samples.

Fifteen soil samples were spiked with endosulfan between 0 to 10 ppm. The correlation coefficient obtained using the plate format was 0.99 with slope of 0.91 and y-intercept of 0.06. The soils that were determined to be free of endosulfan residues by GC analysis were also shown to be negative by ELISA. This result again validated the performance of ELISA, good recoveries being obtained even with a complex matrix such as soil.

Correlation between field assay and laboratory assay

The use of dropper bottles for reagents and the flatter slope of the standard curve for field immunoassay, compared to the laboratory assay, makes it a less-quantitative assay. However the advantage of the field assay is that it can be performed at the site of contamination. Therefore no lag time between the collection and the analysis. Water samples were analysed on the day of collection using field assay (tube format) and analysed again using the laboratory assay (plate format) 2 - 5 days after the collection. The comparison of the laboratory assay and the field assay for sixty nine water samples showed correlation coefficient of 0.85 for the regression line (Fig. 6). Possibly, some of the endosulfan was lost during freeze and thaw processes, leading to slightly lower estimation on the laboratory assay and poor agreement with some of the samples.

In conclusion, two simple and rapid enzyme immunoassays, namely a laboratory assay using 96-well plate and a field assay using polystyrene tubes, have been developed for detection of endosulfan residues in water and soil. They are sufficiently sensitive to detect endosulfan at the ecotoxicological level in water and soil. Water samples can analysed directly and soil only requires a simple extraction with 90% methanol. These assays do not detect pesticide other than endosulfan and other cyclodienes. Since the residues of typical cyclodienes such as dieldrin and heptachlor are found at 0.2 ppb or lower concentration in Australia soil, they are not of a concern for these immunoassays. These assays are specific for endosulfan and endosulfan sulfate; endosulfan diol, a major metabolite much less toxic to fish, is detected much less sensitively. The performance of the tests have validated with the spike and recovery studies and good recoveries have obtained with both water and soil. These immunoassays will be a very useful tool for regular monitoring of water and soil samples for possible contamination of endosulfan. They can also be used as a research tool for environmental studies that may require many samples needing to be analysed in a short period of time.

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Figure 1. Comparison of standard curves prepared in purified water (circle) and field water (triangle) for matrix effects.

Figure 2. Comparison of standard curves prepared in purified water (circle) and soil extract (square) for matrix effects.

Figure 3. Standard curves for field assay (circle) versus standard curve for laboratory assay (square).

Figure 4. Cross reactions for endosulfan metabolites, alpha endosulfan (closed circle), beta endosulfan (open circle), endosulfan sulfate (closed square), endosulfan diol (open square), endosulfan lactone (closed triangle), endosulfan ether (open triangle) and endosulfan hydroxyether (closed diamond).

Figure 5. Spike and recovery studies for water samples. The regression line (unbroken line) represents endosulfan recoveried = 1.02 x endosulfan spiked + 0.13 with r = 0.98.

Figure 6. Comparison of field and laboratory assays for field water samples with the regression line (unbroken line) of field assay = 1.09 x laboratory assay + 0.04 and r = 0.85.