Applications of Immunoassay to Paraquat and Other Pesticides

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The enzyme-linked immunosorbent assay (ELISA) is a rapid immunochromical procedure which can be used for trace analysis. We have applied the procedure to paraquat, and other compounds difficult to analyze by the more classical methods. The immunoassay for paraquat shows the practicality of the method for fortified and actual residue samples, and is being compared with a gas chromatography procedure. Our work with the ELISA illustrates that the immunochromical technique can be used to solve problems encountered in pesticide residue analysis.

It has been stated that progress in pesticide analysis will no longer be made in search of the proverbial zero residue level of detectability, but rather will lie in devising methods of greater selectivity for the positive identification of nanogram quantities of pesticide residues (1). We could add to this statement the need for assays of greatly reduced cost and increased speed. Many residue procedures are too expensive to be used routinely in regulatory procedures or, perhaps of greater importance, to be employed effectively in optimizing pesticide usage and monitoring worker health and safety. Immunochromical methods of analysis offer many advantages, including sensitivity, specificity, and speed of analysis (2). Compounds which are more difficult to analyze by classical procedures due to high polarity or low volatility are frequently amenable to analysis by immunochromatography. We are now investigating enzyme-linked immunosorbent assays (ELISAs) rather than radioimmunoassays (RIA) for pesticide residue work. ELISAs are cheaper, cheaper, and safer than RIAs as radioactivity is not used. However, as all immunoassays function on the principle of mass action, the same immunochromical tools can be used to devise a number of different assay procedures.

General ELISA Methodology

Immunoassays are physical rather than biological assays; they possess the specificity and sensitivity of biossays with the speed

0007-5656/85/0376-0075$00.00 © 1985 American Chemical Society
and precision of physical assays. Specific antibodies are raised in experimental animals in response to a large foreign molecule (antigen). Most pesticide molecules are not large enough to stimulate the immune system, and must be conjugated to a large molecule such as a protein. Antibodies against the pesticide are then obtained using this pesticide-protein conjugate. A small molecule which becomes immunoactive after attachment to the large carrier molecule is termed a hapten. The pesticide may already have a functionality, such as -OH, -SH, -C0OH, or -NH2, useful for conjugation to the carrier, but frequently a derivatization of the pesticide possessing such functionality must first be synthesized before conjugation can occur. In either case antibodies can be obtained which are directed against the original pesticide.

Some antibodies are heterogeneous molecules of varying antigen-specificity and affinity. Within this polyclonal population, there are probably antibodies present which recognize the carrier protein, but the contribution of these antibodies to assay binding can be eliminated by using a different carrier (2). Monoclonal antibody technology could be used to select the clone with the highest recognition to hapten. Although polyclonal antibodies are adequate for most ELISAs, monoclonal antibodies could be developed against pesticide haptens yielding an analytical reagent that is physically, chemically, and immunologically homogeneous. In monoclonal antibodies would be in a virtually unlimited supply, ELISAs could be easily standardized as several laboratories would be using the same antibody clone.

Although a very small amount of antibody is needed per ELISA, having a large supply of monoclonals could alleviate fears of eventually exhausting one's supply. Hamock and Muma (2) discuss some of the advantages of hybridoma technology. Yet, it is important to consider that, in some cases, polyclonal will be superior to monoclonal antibodies. Unless one is developing a sophisticated system for avoiding the separation step in peptide immunoassay, it will be a rare situation where provision of monoclonal antibodies for peptide residue analysis can be justified on a purely scientific basis. However, as immunoassays of pesticide small molecules become more prevalent, there will be compelling administrative and legal pressures to employ monoclonal antibodies. For these reasons they may dominate the field in a few years.

The ELISA is based on the fact that antigen or antibody can be attached to a solid-phase support while retaining immunological activity, and that either antigen or antibody can be linked to an enzyme with the complex retaining both immunological and enzymatic activity. A variety of enzymes, including alkaline phosphatase, horseradish peroxidase, and glucose oxidase have been linked to antibodies and antigens. This method has been used successfully for detection of either antigen or antibody (2), and it has been used as for the detection of mycotoxins, insecticides, surfactants and many other compounds.

To perform a microplate ELISA for the measurement of antigen, plates sensitized with the specific antigen are incubated with a mixture of reference antibody and the test sample. If antigen is present in the test solution, it combines with the reference antibody within the sensitizer plate. The amount of antibody attached to the solid phase is then indicated by
an enzyme labelled anti-immunoglobulin conjugate and enzyme substrate. There is a proportional relationship between the amount of inhibition of substrate converted to products in the test sample and to the amount of antigen in the test system. We routinely run samples in plastic plates containing 50 wells with which all of the procedures can be executed very rapidly. The end point of the assay is the bright yellow color of p-nitrophenol, an end product from the alkaline phosphatase-mediated hydrolysis of p-nitrophenyl phosphate; this product can be visually estimated for semi-quantitative answers or rapidly and precisely measured is an inexpensive colorimeter for quantitation.

Immunassays can be designed to analyze parent compounds and metabolites separately or as a group. Brogden (3) obtained antibodies against parathion which also detected its metabolite p-nitrophenol. Other workers were successful in raising antibodies to DDT and malathion metabolites (4,5). Although the specificity of immunassays are usually very high there is so guarantee against cross-reactivity. Just as good chromatographic techniques require controls, so do immunassays. Fortunately immunassays are quickly and easily performed so that the necessary controls can be run to check for interferences. The sensitivity and selectivity of immunassays can also greatly reduce the cost of analysis by minimizing the amount of sample preparation.

Examples of Pesticide Immunassays

We will first review several of the immunassays developed in this laboratory as they illustrate some of the advantages of immunassay in pesticide residue analysis. Then we will move on to a more detailed discussion of our recent analytical work with the herbicide paraquat.

Diflubenzuron. The benzoylphenyl urea insect growth regulator, for example, pose a formidable residue analysis problem. The compounds are nonvolatile and thus must be derivatized for analysis in a rather arduous chemical procedure. The immunosassay developed in this laboratory is much more sensitive and reproducible as a fraction of the cost and can be used to analyze the same difficult matrices such as milk. For example, a sensitivity of 1 ng is routinely obtained when milk is added directly to the assay (5). A series of partition steps can also be added to further clean diflubenzuron milk extracts yielding a sensitivity in the low ppt range (6). However, the increase in sensitivity may not be needed when methods in current use provide a detection limit of only 10-50 ppb.

A feature of interest of immunosassay is their specificity. One immunosassay for diflubenzuron can distinguish it from the very closely related DIF in addition to a variety of other closely related materials (7). High resolution HPLC columns can resolve these compounds, but the analysis is slow and expensive. The ELISA can distinguish these materials when applied directly, or less specific assays can be used as a highly selective detector when used as an adjunct to HPLC.
**Triton X and N.** Surfactants have many industrial applications and are found in many diverse products as pesticide formulations and cosmetic. These nonionic compounds are difficult to extract, clean up, and analyze and, consequently, no sensitive method existed for their analysis. An ELISA was developed in this laboratory which distinguishes between the monode surfactants Triton X and Triton N (11). These compounds, mixtures of ethoxylates of varying length of 6-(1,1,3,3-tetramethylbutyl)phenol and 4-nonylphenol, give numerous overlapping peaks upon chromatographic analysis and have almost identical UV and IR spectra. ELISA has been developed for class selective detection of the Triton X series; the antibody detects the 6-(1,1,3,3-tetramethylbutyl)phenol moiety and does not distinguish among molecules with etherified side chains of varying lengths.

![Chemical structure](image)

**S-Bioalatrin.** The pyrethroid S-bioalatrin ([R],3,4,4') and its inactive isomer ([1S],3,4,4') can be readily distinguished by another ELISA procedure, illustrating the assay's ability to determine chirality at the residue level (Figure 1). Antibodies were raised against S-bioalatrin using the alatrin benzisocoumar conjugated to various proteins (11-12).

![Chemical structure](image)

**Paraquat.** Paraquat, as well as some carbamate and organophosphate insecticides, are marketed as liquid mixtures, each liquid having a different degree of activity. It can be expected that environmental degradation and metabolism will occur preferentially with some isomers. Thus the ability to distinguish between optical isomers at the residue level may be of critical importance in monitoring the safety of treated substances.

**Paraquat**

During a recent year, over 950,000 pounds of paraquat dichloride
Figure 1. Standard curves for optical isomers of S-bioallethrin using ELISA.
(1,4'-dimethyl-2,3'-pyridinium dihydroiodide) was used in California agriculture (11). Much of this herbicide is used on cotton, primarily as a harvest aid. The value of this cotton production is estimated to exceed one billion dollars (13). Although it is extensively used, little is known regarding the long-term chronic effects of parquat exposure.

Parquat has a number of biological effects, but its main biochemical action is apparently a potent redox uncoupler. Regardless of the route of administration, symptoms of parquat poisoning are centered in the lungs leading to fibrosis and pneumonia (15). Substantial quantities of parquat deposited in the lungs can cause fibrotic lesions (16) which could lead to asthma and emphysema symptoms in chronically exposed individuals (17). Unfortunately, the conventional methods of analysis of parquat are too laborious and/or insensitive to handle the large sample load generated by rigorous studies needed to evaluate human exposure.

Immunochronical procedures were developed to overcome this difficulty. Fosler and Hunter (18) reported a radioimmunoassay for parquat, and Hiemola et al., (14) reported an ELISA for estimating parquat in serum. This ELISA is similar to ours but uses a different enzyme, incubation temperature and time, and shows a high degree of cross-reactivity with ethyl parquat. Methods similar to those previously reported were used to generate parquat hapten for our study. S-methyl-4-(6-pyridyl)pyridinium bromide was reacted with ethyl 5-mercapto-5-carboxylic acid to form a parquat analogue capable of conjugating with a protein. New Zealand white rabbits were injected with a parquat-protein conjugate and antibodies usable of recognizing parquat were obtained. The antibodies are quite selective showing no cross-reactivity to compounds structurally related to parquat (e.g. ethyl parquat, N,N'-dimethyl-4,4'-dihydroxydipiperidines) or compounds used in conjunction with parquat (e.g. diquat).

Using the selective antibody for parquat, environmental samples can be analyzed with little or no cleanup. Sample throughput can be assured in samples per hour rather than days per sample. This was a remarkable discovery as parquat is notorious for requiring extensive sample preparation. The limit of detection (2 μg/mL) in the ELISA procedure is also much lower than the colorimetric procedure, which has a sensitivity of 200 μg/mL (20), and lower than the GC procedure (Figure 2) (21). Perhaps the greatest advantage is the speed of the ELISA enabling large numbers of samples to be analyzed without requiring an extensive cleanup procedure, thus reducing analytical time.

Gas-liquid chromatography following reduction of parquat to the mono- and dimethylated derivatives (21) is of adequate sensitivity for most work when m-selective detectors are employed. Sieber and Wacks (22) have described a modification for preserving parquat in air samples. The method is time consuming and labor intensive, involving acid extraction and mann concentration and evaporation steps. The maximum sample output per analyst per day is 4× with no duplicates. The reported recovery efficiency was 75% (22), although an efficiency closer to 50% is frequently encountered in practice. A modified acid extraction combined with analysis by the ELISA provides recoveries of 75% (Figure 3). This
Figure 2. Standard curves for paraquat using ELISA (top), GC (middle), and colorimetry (bottom).
Figure 3. Comparison of sample preparation steps for analysis of paraquat on air filters using ELISA and gas chromatography.
illustrates the successful adaptation of an extraction technique intended for use with GC determination to an immunochromatographic method of analysis. Alternatively, the antibody itself can be used to extract paracetamol from a glass fiber filter, eliminating the time-intensive extraction procedure. Using the specific antibodies for extraction with subsequent detection by ELISA, 50 samples can be analyzed in triplicate in one day with a routine extraction efficiency of 60% (Figure 3). However, this method uses a rather large amount of antibody making the modified acid extraction procedure preferable for analyzing a large sample load.

This rapid sample-processing capability makes it feasible to measure exposure of field workers, literally on the same day the exposure occurs. In order to test this capability, air sampling was conducted in the San Joaquin valley during a paracetamol application on cotton, and the samples are now being analyzed using both ELISA and GC methods to compare the two techniques in terms of speed, precision (by calculating the percent coefficient of variation), and accuracy (by comparing results from fortified samples using both techniques). Preliminary results indicate that the ELISA compares well with GC literature values in these three parameters and additionally has the anticipated greater sample throughput capability.

Conclusions

It is probable that in certain situations immunochromatographic methods will provide distinct advantages over conventional analytical methods. However, it is unlikely that immunochromatographic methods will completely replace current, established analytical methods of pesticide analysis (5). This is in spite of the fact that chemical classes currently assayed by immunochromatographic techniques in clinical analytical labs contain the same type of functional groups as many pesticides. ELISA could potentially be used advantageously in many types of exposure and monitoring situations, for paracetamol and other pesticides amenable to ELISA analysis. An obvious use of ELISA is the detection of pesticide residue levels in plant and animal tissues, or for water. Biological materials such as plasma and urine currently analyzed by IEM seem particularly amenable to analysis by ELISA. Portable field kits could be developed to determine safe worker re-entry times into treated fields, environmental samples such as soil, water, and air, can be analyzed by the ELISA. Pesticide conjugates have been proposed for assisting in the testing of individuals suspected of sensitivity to pesticides (5); the ELISA could be used to detect specific antibodies in these individuals and aid in exposure studies.

Antibodies have been raised against representative compounds from the major classes of pesticides. Although the ELISA will be useful for individual analysis of a wide variety of compounds, if one needed to analyze several different pesticides simultaneously in one matrix immunoassay may not be the method of choice, due to the large amount of controls and standards needed. However, it could be successfully used for the rapid screening of a large number of samples for the presence of specific types of pesticides and for confirmatory tests (5). The work reported here with paracetamol,
allatrin, diflubenzuron, tritron X and Triton X provides evidence of the EPA's ability to distinguish closely related compounds. The EMA proposes to be a good supplement to current methods of residue analysis.

Literature Cited


Received November 8, 1984