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Utility of immunoassay in pesticide trace analysis

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Abstract - Immunoassays are one of several powerful biotechnologies immediately applicable to pesticide chemistry. The power of immunochemical technology to solve analytical problems in the pesticide field is becoming widely appreciated. This chapter reviews some of the applications of immunochemical technology to problems in the pesticide field.

INTRODUCTION

Immunochemical methods of analysis are widely used in biochemistry, endocrinology, and clinical chemistry, but they rarely have been used in environmental chemistry. The past failure of environmental chemists to apply this powerful technology largely is due to historical precedent rather than to inadequacies in the technology. In 1980 Hammock and Mumma (ref. 1) reviewed the potential of immunochemistry in pesticide analysis and urged its acceptance. Six years later the interest of many academic, governmental, and industrial laboratories indicates that the potential of the technology finally is beginning to be realized (see Mumma, this volume). During the last decade the application of immunochemical technology to problems in environmental chemistry has been explored in this and other laboratories. Our conclusion is that the technology is not a panacea, but that it complements classical analytical procedures in many applications, it offers dramatic advantages over more classical techniques for some problems, and may be the only viable technology for still other areas.

In this chapter no attempt will be made to describe how one develops an immunoassay since this is well documented in other reviews. However, the status of immunochemistry in pesticide analysis will be detailed in light of the relative advantages and disadvantages of this technology. Some of the past and current research from this laboratory will be reviewed on pesticide analysis by immunoassay to illustrate the advantages and limitations of immunochemical approaches as well as some of the special applications of immunoassay in pesticide analysis. Analysis of products of research in biotechnology represents a major challenge for environmental chemists, and the application of immunochemical technology to this problem will be discussed. Immunoassay often is thought to be simply an inexpensive alternative to classical technology. As outlined below, the availability of inexpensive analytical data is likely to impact the pesticide field in a variety of novel ways far outweighing the impact of the technology in classical uses. Finally, recommendations for the implementation of this technology in the pesticide field will be presented.

ADVANTAGES OF IMMUNOCHEMICAL TECHNOLOGY

Immunoassays are generally applicable to pesticide chemistry. In addition they are highly sensitive, specific, and precise which leads to rapid, cost effective assays. In addition the technology is very adaptable to a variety of analytical problems.

Applicability to pesticide chemicals

Immunochemical assays represent an immediately practical application of biotechnology. However, these assays are physical assays based on the law of mass action and are not bioassays. They owe their great sensitivity and specificity to biological systems which can produce the reagent antibodies which will bind with high affinity to compounds of interest. Immunoassays probably can be applied to more diverse structures than any other analytical technology. Since the affinity of the antibody for the compound to be analyzed depends upon the summation of a variety of noncovalent interactions, it is difficult to prepare antibodies to exceptionally small molecules. Fortunately, there appears to be no upper limit on the size of the compounds that can be analyzed. As our field moves toward the utilization of increasingly complex synthetic molecules (ie diflubenzuron and chlorsulfuron), (refs. 2-5), fermentation products such as avermectin, and even proteins such as the toxins of *Bacillus thuringiensis* (ref.6), it will become increasingly important to have accepted techniques for the analysis of such large molecules.

Immunoassays are usually performed in aqueous solution, thus the molecule to be analyzed should be at least moderately stable in water. Surprisingly, solubility in water is only rarely a problem since even very lipophilic compounds often are soluble at the pico or femtomolar concentrations used in an immunoassay. Even highly insoluble compounds often can be presented to the antibody in micelles or with water soluble cosolvents. Thus, problems associated with the analysis of highly lipophilic compounds are more commonly associated with removing them from an oily matrix than with absolute solubility (ref. 7). As a general rule immunoassays are most applicable to molecules difficult to analyze by gas liquid chromatography, which makes immunoassays a very attractive complementary technology. Although it is easiest to devise assays for water soluble compounds, with sufficient imagination, immunochemical technology can be applied to the analysis of almost any compound. With little difficulty, the technology certainly can be applied successfully to most current pesticides, and it is likely to be even more appropriate for the next generation of compounds.

Balancing the advantages of immunoassay

In 1974 we initiated studies to explore the possibility of using immunochemical technology for the analysis of pesticides and other chemicals. Much of our research has been designed to evaluate the advantages and limitations of immunochemical technology in general to the field of environmental chemistry. Some of these advantages and limitations are indicated in Table 1. These criteria are not firm rules, for one can

TABLE 1. Advantages and disadvantages of immunochemical technology

Advantages	Disadvantages
Generally applicable	New technology in environmental labs
Highly sensitive	Too sensitive
Highly specific	Hard to apply to multianalyte problems
Highly precise	Cross reactivity and interference
Very rapid	Reagents not available
Cost effective	Confusing terminology
Highly adaptable	Large sample load required

overcome many of the limitations of immunoassays with innovative applications of this very adaptable technology. However, these criteria can be used to indicate the likelihood of a successful project. It also is critical to remember that immunoassays are physical assays sadly lacking in magic. With a working knowledge of the technology, one often can balance the advantages and limitations to obtain an assay of the desired properties. However, one cannot have all of the advantages of an immunoassay simultaneously. For instance it is possible to design very inexpensive immunoassays of moderate sensitivity that can be performed in the field with no specialized equipment or highly trained personnel. One cannot expect these assays to be highly sensitive and precise as well. Although immunoassays excel as single analyte methods, it is possible to design an immunoassay capable of detecting a class of compounds (acyl urea insecticides), (refs. 2-4) or a mixture of compounds (nonionic surfactants), (ref. 8). However it is not reasonable to expect these same assays to be highly specific.

Many years of experience in clinical chemistry and increasing experience in environmental chemistry serve to verify the tremendous advantages of immunoassay. The high sensitivity of immunoassays is based upon the reversible but tight binding of an antibody to the compound to be analyzed. Since this binding is based on the sum of many noncovalent interactions (especially weak molecular interactions that are highly dependent upon the proximity of interacting groups), the interaction is highly specific. Specificity and sensitivity often permit one to carry out an analysis directly in a crude biological matrix as shown in Fig. 1A for paraquat in serum. This advantage often allows one to avoid some extraction and/or clean up steps leading to rapid, inexpensive assays of increased precision. This was demonstrated with an acyl urea insecticide where immunoassay allowed 100 times as many milk samples to be processed per worker day with a reduction in cost and an increase in precision and sensitivity (ref. 3).

As more effective compounds are developed such as the pyrethroid and acyl urea insecticides or sulfonyl urea herbicides, it will be important to develop more sensitive assays to detect toxicologically relevant residues. The tremendous sensitivity possible with some immunoassays with minimal clean up is shown for the acyl urea diflubenzuron in Fig. 1B.

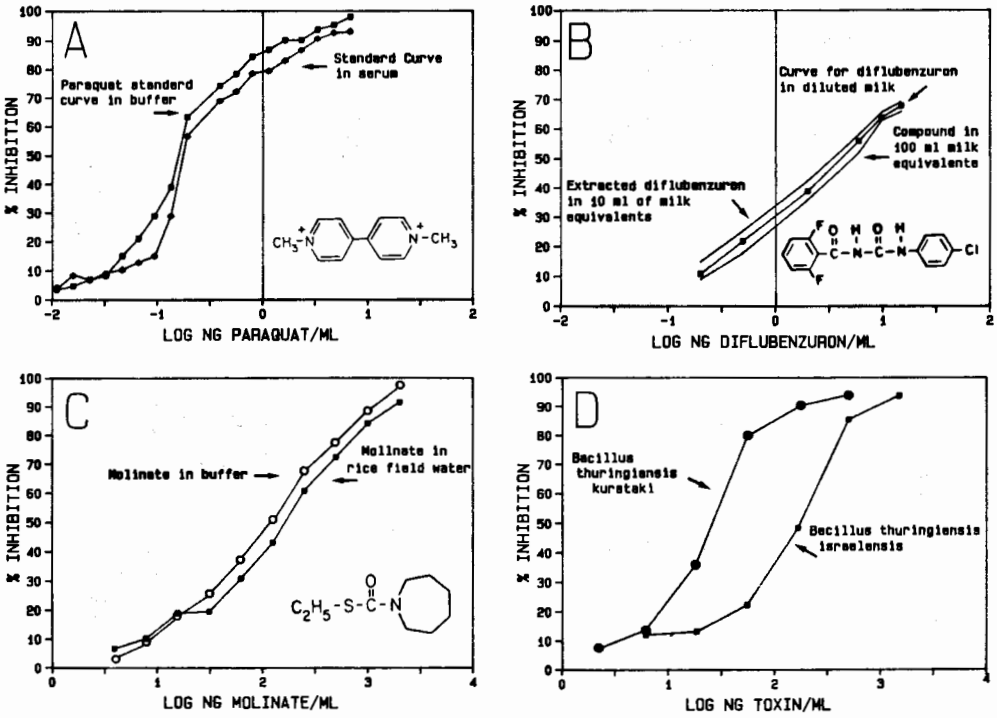

Figure 1. Some examples of applications for immunoassay in pesticide analysis. Percent inhibition in a competitive ELISA was plotted against log ng analyte/ml. Panels A-C show a standard curve of the analyte run in buffer solution vs one or more curves of either compound run directly in the environmental matrix or extracts of that matrix. A. Analysis of paraquat (1,1'-dimethyl-4,4'-bipyridinium) in sheep serum. These data demonstrate that up to 33 percent serum (♦,or lymph) can be analyzed for paraquat without shifting the standard curve. Larger amounts of serum can be analyzed by running a standard curve in serum, or paraquat can be extracted from the serum. The coating antigen concentration was 1 μg/ml and the antibody dilution was 1/4000. B. Analysis of diflubenzuron (Dimilin, N-[[(4-chlorophenyl)amino]-carbonyl]-2,6-difluorobenzamide) in whole milk (■) and extracts of whole milk (solid lines). Whole milk was extracted with ethyl acetate using the AOAC approved method. These data indicate that the antibody is able to extract diflubenzuron from micelles in the milk by mass action. Simple extraction procedures followed by adequate presentation to the antibody can result in highly sensitive assays. Up to 50% milk was added with no statistical difference in the standard curves. Within run variation was 3%, between run variation was 5% over several months. Antibody dilution was 1/1200. C. Analysis of molinate in rice field water samples. A field water sample (♦) was buffered with a concentrated PBS solution and spiked with molinate. No significant effect on the standard curve was observed when the final concentration of field water in the assay was 50%. Increased sensitivity may be obtained by extracting water samples into ethyl acetate or toluene. Extracts are concentrated, the molinate exchanged into propylene glycol, and added to antibody. Up to 5% propylene glycol may be used in the ELISA. The coating antigen concentration was 2 μg/ml and the antibody dilution was 1/4000. D. Standard curves for the purified crystal toxins of *Bacillus thuringiensis israelensis* (■) and *Bacillus thuringiensis kurstaki* (♦) run in formulated material. These data indicate that immunoassays can be used for the quantitative analysis of the proteins which are active ingredients of biological pesticides and genetically engineered material. These assays can be used to supplement bioassay for the monitoring of production and for quality control of formulations. Coating antigen concentration was 1 μg/ml. Antibody dilutions were 1/4000 for *Bacillus thuringiensis israelensis* and 1/800 for *Bacillus thuringiensis kurstaki*.

However, of the many advantages of immunoassay, the one most feared is sensitivity. There is the possibility that immunoassays can be employed to reduce further the levels of a compound that can be detected. Certainly this possibility is true, but the same fear applies to all physical assays. Fantastically sensitive immunoassays can only be developed with the loss of speed, precision, and economy. The true advantage of the method is in offering toxicologically relevant levels of sensitivity at a tremendous savings. In fact, the developer of an immunoassay has more control over the ultimate sensitivity of that assay than the developer of any other physical assay.

All immunoassays are based on the law of mass action and depend upon measuring the antibody bound to a compound and/or the free antibody and compound. However, they offer the advantage of being highly adaptable to the problem at hand. Using the same antibody one can develop a rapid field assay, a highly precise laboratory assay, or an assay to monitor production in real time. Many physical methods are used for the analysis of bound vs free material including scintillation counting, turbidity, polarization of light, visible, ultraviolet and electron spin resonance spectrophotometry, and a host of other methods. Each of these methods has numerous variations. Even if we address one major approach, that of the enzyme linked immunosorbant assay (ELISA), there are many subclasses of ELISA and the same basic assay is renamed (Western blot, dot blot) when it is applied to different matricies or when it is packaged for sale. Thus, the great advantage of phenomenal adaptability has led to excessively confusing terminology for a basically simple technology. Once one is familiar with one immunoassay format, the mysticism falls from the other formats as well. However, the terminology still serves to intimidate many analytical chemists. All of the variations in immunoassay format rely on a reversible antibody-compound interaction. Given an excellent antibody, almost any of the formats can be used to design a qualitative or quantitative immunoassay. In approaching this technology, the analytical chemist already has mastered the difficult concepts of sampling, sample handling, sample presentation, and the basic concepts of analysis. With the three working definitions of the terms below, immunochemistry easily can be added to an analysts' repertoire.

ANTIBODY-One of a class of serum proteins which binds to an antigen.

ANTIGEN-A molecule (usually a protein) which will elicit the production of antibodies and bind to them.

HAPTEN-A compound (usually a small molecule) which will bind with antibodies but will not alone cause the production of antibodies

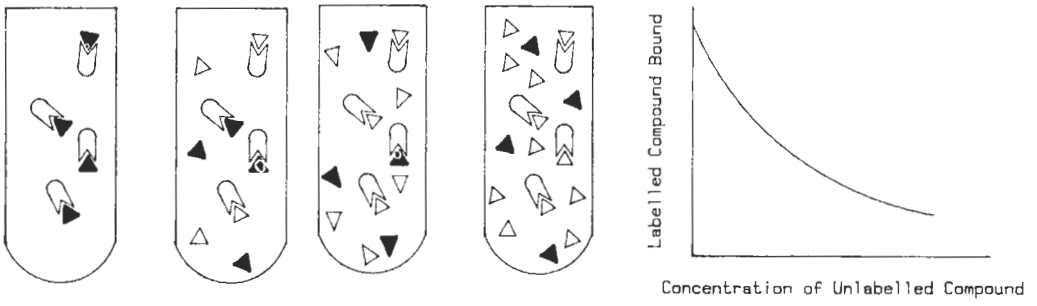

Figure 2. All immunoassays depend upon the reversible interaction of an antibody (⊂≺) with a compound (△). All assay formats determine the amount of compound free vs bound. A common way to carry out this procedure is to label small, constant amounts of the compound (▲) as shown above for a competition radioimmunoassay. Following separation, the antibody bound to radiolabeled compound is related to concentration to yield a standard curve useful for the determination of compound in unknown samples.

APPLICATIONS TO PESTICIDE CHEMISTRY

The advantages and limitations of immunochemical technology listed in Table 1 translate into some immediate applications to pesticide chemistry listed below. Some of the work from this and other laboratories is reviewed below in the context of these applications.

Analysis of compounds difficult to analyze by classical methods

The most obvious application of immunochemistry is for compounds difficult to analyze by classical methods. Gas liquid chromatography is most applicable to compounds that are

volatile (this usually means hydrophobic and small), heat stable, and which have functionalities easily detected by available selective detectors. High performance liquid chromatography is less restrictive, but still depends upon specific functionalities for detection. The above criteria are not necessary for a successful immunoassay. Since there is a trend indicating that immunoassays are most easily developed to large hydrophilic compounds, immunoassays are complementary to existing technology and can even be used as HPLC detectors. Since it is human nature to only try a new technology when an existing technology fails, immunoassays unfortunately will first be applied to apparently intractable analytical problems. In most cases immunoassay will excel in this role as savior. However, if immunoassays are introduced into a laboratory by untrained personnel for the analysis of certain "nightmare" compounds (highly reactive, very small, very lipophilic, present in very low levels, immunosupressive), the entire technology may fail to be accepted.

We have applied immunochemical technology to a variety of compounds difficult to analyze by classical methods. The pyrethroid allethrin lacked a functionality easily recognized by GLC or HPLC; the acyl urea insecticides and paraquat both required a multistep work up procedure before GLC could be performed and HPLC procedures were too insensitive, while Triton X was nonvolatile, hard to extract, and yielded multiple peaks on HPLC (refs. 2-4 and 9-11). The structure and high activity of the sulfonylureas indicates that immunoassays also will be very applicable to those structures (ref. 5).

Discrimination of chirality and closely related compounds

Due to the increasing cost of starting materials and environmental concerns one can anticipate that an increasing number of compounds will be sold as optically rich or pure materials. Other compounds are racemates which may be degraded differently in the environment. Thus, the ability to distinguish among optical isomers at a residue level becomes important. The insecticide allethrin consists of 8 optical and geometrical isomers with most of the biological activity in the 1R, 3R, 4'S isomer. We developed an immunoassay which was very selective for this single isomer (refs. 9, 10) illustrating the potential of this technology to deal with chirality.

In the very competitive area of pesticide chemistry, compounds are often developed which have almost identical physical properties. This is the case for Dimilin and a number of other active compounds such as BAY SIR 8514. A very high efficiency HPLC column is needed to separate these compounds while we developed immunoassays which were able to detect the class of compounds and others that could distinguish clearly between Dimilin and a host of related compounds including some active compounds whose structures were released after the development of the assay (refs. 3, 4).

Development of immunoassays is most cost effective shortly after a compound class is discovered. Such class specific immunoassays could be very useful in the early development and screening of a new class of insecticides.

Analysis of human body fluids and as biomarkers

To evaluate human exposure it often is necessary to analyze human body fluids such as urine and blood. As we move toward relating exposure to toxicity, analysis of parent compounds, metabolites, and indicators of toxicity in occupationally and environmentally exposed individuals is of increasing importance. Immunoassays have been optimized in the clinical area just for this task, and highly sensitive immunoassays often require only trivial amounts of biological material. For instance immunoassays for paraquat are used for clinical diagnosis of poisoning (refs. 12-14), and we have found that crude blood, urine and lymph can be analyzed directly in an immunoassay for paraquat with greater sensitivity than other published techniques (Fig. 1A). Such rapid assays make it possible to carry out the pharmacokinetic studies needed to evaluate the significance of occupational exposure to pesticides.

Analysis of large numbers of samples

Immunoassay is so easy and inexpensive to automate that it is ideal for analyzing very large numbers of repetitive samples. This attribute makes immunoassays perfect for registration procedures, quality control work, and residue procedures where one major compound is suspect. Even if classical assays are used for confirmation, immunoassays are useful for eliminating negatives and ranking samples in terms of concentration for subsequent analysis. In this regard we have developed an immunoassay for the thiocarbamate herbicide molinate. The compound is very valuable in rice culture, but if released prematurely into drains it can result in fish kills. An assay has been developed which can handle the tremendous numbers of samples generated in an attempt to evaluate the dynamics of molinate in rice culture. Fig. 1C indicates that water from rice fields can be added directly to an immunoassay while retaining adequate sensitivity for the determination of release times for the water. These data also indicate that it is possible to develop assays for small, volatile, water unstable molecules. The assay can be adapted following extraction to a highly sensitive analytical tool for trace analysis. In order to integrate pesticide usage with social concerns it will be increasingly important to develop such

markers for realistic levels of environmental contamination. Assays for relatively water soluble herbicides such as the thiocarbamates and 2,4-D and 2,4,5-T (ref. 15) are likely to be valuable in a surface water monitoring program while rapid assays for triazines and acetanalides (ref. 16) could be very valuable in monitoring of ground water.

Rapid and/or field analysis

In the above applications immunoassay simply replaced classical methods when it was more appropriate. However, there are many applications where immunoassay represents the only viable technique. For instance the molinate assay is being configured to allow data to be collected very rapidly in the field using little or no equipment. This assay will allow farmers and county agricultural personnel to test a field before draining water. Similarly assays can be used to check a field for toxic chemicals before reentry or to monitor drift. Such applications will be very important with highly toxic compounds such as paraquat (ref. 11) and parathion (refs. 17-20). Such assays also can be used to monitor a field before planting for phytotoxic herbicide residues. This has been important in the use of some triazine herbicides and will likely be important in the use of some sulfonylureas. The same basic technology could be used by a farmer to monitor veterinary drugs and pathogens, plant pathogens, and pesticides. This represents an entirely new concept in residue analysis by placing the technology in the field where the contamination occurs and by making the analytical technology available to the individual who needs it. This technology will be critical to wholesalers interested in insuring that residues are low in key crops and to pesticide companies in stewardship programs.

Analysis of products from research in biotechnology

Until recently most compounds introduced into the environment were, for economic reasons, relatively small molecules of simple structure. Biotechnology will certainly change this situation. For instance fermentation technology has already given us avermectin and ivermectin while a complex exotoxin from *Bacillus thuringiensis* is not allowed in current formulations and has been considered for development itself. These materials are far more complex than the compounds normally analyzed by GLC and HPLC. Certainly their analysis can be approached by classical means, but immunoassay seems particularly advantageous with these molecules. We can anticipate the development of more such complex molecules, and society needs the analytical technology to deal with them.

The major public concern in the immediate future will not be over fermentation products but over genetically engineered materials. It is certain that the vast majority of products from this type of research will be peptides and proteins. For such materials immunochemical methods are unsurpassed. In the last year the protein toxin of *Bacillus thuringiensis kurstaki* has been cloned into and expressed in plants, and Monsanto is still trying to test the same toxin in the field following expression in an alternate species of bacteria. In this regard we have developed immunoassays for the crystal toxin of the *kurstaki* and *israeliensis* strains (Fig. 1D, refs. 21-24). These assays are valuable for monitoring production, quality control, and may be adapted to actual residue procedures. In situations when foreign genes are released into the environment, immunochemical procedures are necessary to monitor expression as well as the products of expression in the environment. Thus, immunoassays become complementary to the genetic probes which will be necessary to monitor the actual gene. Fortunately, most companies will have developed both immunoassays and genetic probes in the research leading to a successful genetically engineered product. Thus, the analytical tools will be available, but regulatory agencies must have the foresight how to use them in environmental chemistry and how to obtain administrative and legal acceptance of the procedures. Thus, this technology has come the full circle. Not only do immunoassays represent a biotechnology themselves, but they may well be the best way to analyze for products of biotechnology research.

RECOMMENDATIONS

Although immunochemical technology is not a panacea, it is very unfortunate that it has not been used extensively in environmental chemistry for the last 20 years. It is important that the technology be implemented in both the public and private sector. Numerous small companies are capitalizing on the technology and will serve a major role in introducing and perfecting the technology in the pesticide area. However, if it is to be used effectively, both the primary producers of pesticides and regulatory agencies must have expertise on the technology in-house. Hopefully the trend toward increased use of immunoassay in analytical chemistry will continue (refs. 25, 26).

Validation of immunoassays

For immunoassays to gain acceptance as an analytical technique they should first be treated as any other analytical technique and compared against an established method. For such work it is important that an assay format be used which will give quantitative rather than qualitative data. The extensive comparisons needed for such validation have not yet been reported for any compound in the pesticide field. However, Wie and Hammock (ref. 2) have reported an extensive study on within, and between run variation for an ELISA for

diflubenzuron (3 and 5% respectively) and the influence of the biological matrix on the immunoassay. Comparisons of immunochemical and classical procedures are becoming more prevalent in the literature. For instance, in the fungicide area, such data are available for benomyl, metalaxyl, and triadimefon (refs. 27-29). Van Emon et al. (ref. 11) found that GLC and colorimetric procedures for paraquat compared favorably with immunoassay in the analysis of samples generated in a worker exposure study, while Schwalbe et al. reported a good correlation between an enzyme immunoassay and fluoroimmunoassay for the herbicide diclofop-methyl (ref. 30).

For initial studies, immunoassays should be compared with classical techniques as if they were simply a new GLC procedure. However, immunoassays do have their own unique properties and regulatory agencies should move toward the establishment of validation and quality control protocols to address these properties. Such protocols will be of even greater importance as immunoassays are applied to the analysis of biological and genetically engineered pesticides for which no physical assay exists.

Introduction of immunoassays into the analytical laboratory

Both regulatory agencies and primary producers of pesticide chemicals must have in-house expertise on immunochemistry if they are to evaluate critically, analytical procedures developed in academia and the private sector. In developing in-house expertise it is important that a team approach be followed. It often is ignored that the most critical members of the team already are present. These individuals are the ones who appreciate the analytical and regulatory problems faced with a compound. Another very important member of the team often overlooked in the press for biotechnology is the chemist. Some toxins are easily coupled to proteins for analysis, while others will require innovative and difficult chemistry for attachment. There also is the temptation to mask key recognition sites for antibody specificity by using them for attachment. Very expensive approaches involving the screening of numerous antisera or clones then are needed to develop a useful assay when inexpensive chemistry should have solved the entire problem. There may be a long delay in the development of immunoassays due to the design and synthesis of an appropriate hapten. If this development is initiated in the early stages of compound development, appropriate haptens are likely to exist as synthetic intermediates, structural analogs or metabolite standards.

The antibody is the critical component of the immunoassay regardless of the format. Surprisingly, in the early phases of this program the production of antibodies is the task most easily contracted. If hapten and antigen preparation is careful, the production of antibodies is straightforward and can be done by numerous immunochemical companies with no loss of quality. As discussed earlier by Hammock and Mumma (ref. 1), polyclonal antibodies will be adequate or even preferable to monoclonal antibodies for many projects (ref. 31). In the past, rabbit polyclonal antibodies often have yielded assays more sensitive than the corresponding murine monoclonal antibodies. There also is no guarantee that monoclonal antibodies will yield a more specific assay. The success of an initial project should not be sacrificed simply to use monoclonal antibodies for the sake of apparent timeliness.

There is no doubt that hybridoma technology will dominate the immunoassay field in a few years for both commercial and scientific reasons. There are problems such as the analysis of multiple peptides responsible for the toxicity of *Bacillus thuringiensis israelensis* (ref. 32) where hybridoma technology is indispensible. The cost of production of monoclonal antibodies is dropping rapidly, especially in laboratories able to take advantage of economies of scale. The use of multiple, genetically diverse strains of mice for initial immunization followed by screening of crude serum before fusion is resulting, in some cases, in monoclonal antibodies of greater sensitivity than polyclonal antibodies.

Before undertaking such a project, the researcher should determine that a monoclonal antibody is needed and should develop a rapid screening procedure for isolating exactly the clones desired and eliminating those not desired. The researcher also should realize that the true strength of hybridoma to technology is in having a library of well characterized antisera. Thus, at some stage it will be important to acquire expertise in hybridoma technology.

Haptens, ligands, and antibodies are the reagents for an immunoassay, but they must be combined into an analytical method. There are numerous recipe procedures, but forging an assay is a craft, and the expertise to do this must be in-house. The assay must then be applied to real analytical problems. In both the development and application one can draw on expertise in the clinical area, but the matrices and ultimate analytical goals are much different. Thus, one needs an environmental chemist trained in immunoassay or a close collaboration between two individuals.

The sampling strategies, clean up procedures, data handling and a myriad of other tasks in analytical chemistry are identical regardless of whether one is relying on GLC-MS or immunoassay. The major expertise needed for immunoassays to be successful is already in-house in the form of good analytical chemists.

Selection of initial compounds for analysis

In introducing any new technology, it is important that the initial in-house application of the technology is successful. Thus, selection of the first problem to be addressed with immunochemical technology becomes critical. This selection process will vary with the company or agency but several general criteria can be considered.

The compound should be at least of moderate water solubility and should not be notorious for adhering to surfaces or the matrix for analysis. The compound should be nonvolatile and stable in water. The chemistry and physical properties should be familiar to the personnel involved, and at least one matrix of interest for analysis should be easy to handle.

Certainly the compound should be of interest to the research group, but it need not be the worst problem the group faces. The number of analyses performed on the compound should be high. If the analytical data can first be used in-house, frustrations with delays in acceptance by outside agencies can be avoided. There should be good classical procedures for the compound for validation studies. Thus, the decision making process regarding which compound to target is not complex, but it is essential that a good decision be made if the technology is to be accepted quickly. Fortunately, such sound decisions now are being made in a variety of industrial and regulatory laboratories.

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