Detection of Pesticides and Pesticide Metabolites Using the Cross Reactivity of Enzyme Immunoassays

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Enzyme immunoassay is an important environmental analysis method that may be used to identify many pesticide analytes in water samples. Because of similarities in chemical structure between various members of a pesticide class, there often may be an unwanted response that is characterized by a percentage of cross reactivity. Also, there may be cross reactivity caused by degradation products of the target analyte that may be present in the sample. In this paper, the concept of cross reactivity caused by degradation products or by nontarget analytes is explored as a tool for identification of metabolites or structurally similar compounds not previously known to be present in water samples. Two examples are examined in this paper from various water quality studies. They are alachlor and its metabolite, alachlor ethane sulfonic acid, and atrazine and its class members, prometryn and propazine. A method for using cross reactivity for the detection of these compounds is explained in this paper.

The use of immunochemical techniques in environmental chemistry was first proposed in 1971 by Ercegovich (1), who suggested the use of immunological screening methods for the rapid detection of pesticide residues and for confirming results of conventional analyses. A radio-immunooassay (RIA) for the insecticides aldrin and dieldrin was the first reported immunoassay (IA) for an environmental contaminant (2). Although RIAs still exist in the medical field, they are seldom used in environmental and food analyses because of the need for special handling and disposal of radioactive materials.

The development of IA for environmental applications was rather slow, in spite of the concept existing in the literature for more than 20 years. In the late 1980s and early 1990s, the scientific community and regulatory agencies recognized the strength and advantages that IAs can offer (3–6). Currently, there are at least 11 commercially available IA kits that have been validated by the U.S. Environmental Protection Agency to be included in SW-846 methods, and this list will continue to grow as more kits prove useful and reliable for the particular matrix of concern.

One of the concerns about the usefulness of IA is its specificity for the target analyte, such as a specific pesticide. In general, the more specific the antibody is, the less the cross reactivity will be for other compounds in the class or for degradation products of the parent compound. This paper will examine the concept that the cross-reacting species, the nontarget analyte, may sometimes be an unknown metabolite of the parent compound or another nontarget compound. Comparison of IA concentrations with another standard method, such as gas chromatography/mass spectrometry (GC/MS), may be used to indicate these new metabolites or compounds; furthermore, more detailed analysis may reveal the structure of these degradation products in water samples. Two examples will be discussed. The compounds in the examples were discovered by comparison of enzyme-linked immunoabsorbent assay (ELISA) results with GC/MS analysis for pesticides in ground-water samples. The first example involves the herbicide alachlor and the discovery of alachlor ethane sulfonic acid (alachlor ESA) as a major surface-water and ground-water contaminant (7–11). The ESA metabolite results from the degradation of alachlor in soil (9). This ESA metabolite has been shown to be a major class of sulfonic acid metabolites that form from other chloroacetanilide herbicides, such as acetochlor, metolachlor, and dimethanamide. Thus, the importance of IA in the discovery of this metabolite in the environment has been significant, and there have been numerous studies on the detection and transport of these products in the aquatic environment (7–12).

The second example involves the monitoring for atrazine in playa lake samples in West Texas and the discovery of prometryn and propazine, instead of atrazine. Thus, a nontarget analyte of the triazine class was accidentally discovered while screening for atrazine. The importance of these compounds as herbicides used on cotton and sorghum is also discussed in this paper.
Experimental

Reagents

Methanol (Burdick and Jackson, Muskegon, MI) and ethyl acetate (Fisher, Springfield, NJ) were HPLC grade reagents. Alachlor and alachlor ESA were obtained from Monsanto Agricultural Company (St. Louis, MO). Atrazine, alachlor, and prometryn were obtained from Speciality Chemicals (Philadelphia, PA). The SPE cartridges that were used (Sep-Pak from Waters-Millipore, Milford, MA) contained 360 mg 40 μM C18 bonded silica. Phenathrene-d10 (EPA, Cincinnati, OH) was used as the external standard for GC/MS quantitation. Stock solutions were prepared in methanol.

ELISA Analysis

The alachlor and atrazine IAs used in this study included the EnviroGard assay (Millipore, Bedford, MA), which had antibodies coated on the wells of the microtiter plate, and RaPID assay (Ohmicron Corp., Newton, PA), which had antibodies coated on magnetic particles. These 2 assays are not available currently from these companies. However, both alachlor and atrazine IAs may be obtained from Strategic Diagnostics, Inc. (SDI, Newark, DE) in the magnetic particle format that is identical to the RaPID assay (Ohmicron Corp.). For the EnviroGard assay used for both alachlor and atrazine, 80 μL sample and 80 μL hapten–enzyme conjugate were mixed in the microtiter plate well, and the mixture was incubated at 30°C in an orbital shaker (200 rpm). After 1 h, the plate was rinsed 5 times with deionized water, and the excess water was removed. A color reagent (160 μL of a 1:1 mixture of 0.02% hydrogen peroxide and 3,3,5,5-tetramethyl-benzidine) was added. The color was allowed to develop for 30 min, and the stop solution (40 μL 2M sulfonic acid) was then added. Optical densities were read in a Vmax microplate reader using Softmax software (Molecular Devices, Menlo Park, CA) at a wavelength of 450 nm.

For the RaPID assay used for both alachlor and alachlor ESA, 250 μL sample was mixed with 250 μL hapten–enzyme conjugate and 500 μL paramagnetic particles. The mixture was incubated for 30 min at room temperature, and then a strong magnetic field was applied to separate unbound analytes from the antibody-bound analytes. The excess reagent was removed. A 500 μL aliquot of color reagent was added next, and the mixture was incubated for 20 min at room temperature, followed by addition of the stop solution (500 μL sulfuric acid). The optical densities were read using the RPA-I RaPID photometric analyzer (Ohmicron Corp.) at 450 nm.

GC/MS Analysis

GC/MS analysis of alachlor, atrazine, and prometryn was performed on a Hewlett-Packard Model 5890A GC (Palo Alto, CA) and a 5970 mass-selective detector (MSD). Operating conditions were: carrier gas, helium; flow rate, 1 mL/min; and head pressure, 35 kPa. The samples were injected in the splitless mode by auto injection. A 12 m, HP-1 capillary column (Hewlett-Packard) made of cross-linked methylsilicone with a film thickness of 0.33 μm and 0.2 mm id was used for the separation. The column temperature was held at 60°C for 1 min, then increased at 6°C/min to 250°C, and held at this temperature for 10 min. Injection temperature was 280°C. The MSD detector was operated in a selective-ion monitoring (SIM) mode, and confirmation was based on the presence of the molecular ion peak, 2 confirming ions (with area counts ±20%), and a retention time match of ±0.2% relative to the retention of the phenanthrene-d10 internal standard (13).

Solid-Phase Extraction

The solid-phase extraction (SPE) procedure was automated with a Millipore Workstation (Waters, Milford, MA) as described previously (13). The C18 cartridges used for SPE were washed sequentially with 2 mL methanol, 6 mL ethyl acetate, 2 mL methanol, and 2 mL distilled water. A 100 mL aliquot of sample was passed through the cartridge at a flow rate of 10 mL/min. The cartridge was eluted with 3 mL ethyl acetate, followed by a transfer step to remove the ethyl acetate (top layer) in the eluate. The top layer (ethyl acetate) contains alachlor, atrazine, and prometryn. Then, the cartridge was eluted with methanol to remove the alachlor ESA, which was collected in a separate test tube. Both ethyl acetate and methanol eluates were evaporated to dryness under nitrogen at 45°C using a TurboVap (Zymark, Palo Alto, CA). The ethyl acetate layer was transferred to a vial and analyzed by the GC/MS method described. The methanol eluate was reconstituted in 100 μL 10mM phosphate buffer–methanol (20 + 80) mixture for analysis by high-performance liquid chromatography with diode array detection (HPLC–DAD).

HPLC/MS Analysis

The HPLC analysis of the methanol extracts for the determination of alachlor ESA was performed on an HP model 1100 LC/MS with MSD detector (Hewlett Packard). The HPLC was equipped with two 5 μm Phenomenex columns (C18) coupled to a 3 μm Phenomenex column (C18) in series (14). The smaller particle size (3 μm) C18 column was placed last to maintain a constant head pressure. The flow rate was 0.3 mL/min using a mobile phase of 0.1% acetic acid, 40% methanol, 55% distilled H2O, and 5% acetonitrile. The interface was operated in electrospray negative mode, and the presence of alachlor ESA was identified by a single ion at m/z 280, held at this temperature for 10 min. Injection temperature was 280°C. The MSD detector was operated in a selective-ion monitoring (SIM) mode, and confirmation was based on the presence of the molecular ion peak, 2 confirming ions (with area counts ±20%), and a retention time match of ±0.2% relative to the retention of the phenanthrene-d10 internal standard (13).

Results and Discussion

Alachlor and Alachlor ESA

The importance of cross reactivity in an immunoassay has been discussed extensively (15). Because interfering substances may also respond in immunoassay, incorrect results can occur, and this fact makes it sometimes difficult to apply
IA to regulatory issues of pesticides in water. The 2 alachlor IAs discussed in this paper react to the structure of alachlor (Figure 1) with a detection limit sufficient to detect alachlor in ground water at 0.1 µg/L (8). Figure 1 not only shows the structure of alachlor, but also the structure of the other common chloroacetanilide herbicide used in the United States at the time of this study (1992), which is metolachlor. Since 1993–94, dimethenamid and acetochlor (not shown in Figure 1), respectively, have been added as parent corn herbicides. The cross reactivity of alachlor and metolachlor is also shown in Figure 1 as the IC50. Note that metolachlor has a low cross reactivity for the alachlor antibody, with an IC50 of 109 µg/L using the EnviroGuard assay and 27 µg/L using the RaPID assay. The IC50 is the concentration required to give a 50% absorbance reading in the colorimetric immunoassay and is a good indicator of the percentage of cross reactivity that may occur.

Because of the selectivity of the alachlor antibody, the alachlor IA was marketed as a reliable assay for ground-water studies by both Millipore (Milford, MA) and Immunosystem (Scarborough, ME). However, papers published by Macomber et al. (7) and Baker et al. (16) found that a metabolite of alachlor, alachlor ESA, also cross reacts with the antibody giving a response that subsequently caused a number of false positives in ground-water surveys of Ohio, Indiana, and Illinois. Baker et al. (16) reported that approximately 100 false positives were reported in ground-water surveys of Ohio, Indiana, and Illinois. Baker et al. (16) reported that approximately 100 false positives were reported in ground-water samples collected from these states as part of a survey of water quality.

Subsequently, Aga et al. (8) used the cross reactivity of the alachlor antibody toward alachlor ESA to develop an analytical method for detecting the alachlor metabolite in water. In this method, the alachlor ESA is separated from the parent alachlor by selective elution of the SPE cartridge. Furthermore, Aga et al. (8) examined the cross reactivity of many other metabolites of alachlor to the alachlor antibody (Figure 2). Figure 2 shows that, for the 2 IA antibodies tested, the IC50s were 5.4 and 1.7 µg/L for alachlor ESA. None of the other metabolites examined gave this type of sensitivity to the alachlor antibody. Since the first publication by Macomber et al. (7), a number of studies (8–12, 16) have been published on the importance of the ESA metabolite of alachlor and its related analogs.

For example, not only has alachlor ESA been found in surface water and ground water since its discovery, but also acetochlor and metolachlor ESA metabolites have been reported in surface water (10) and in ground water (11) using HPLC/MS methods. Table 1 shows the concentrations of alachlor ESA, metolachlor ESA, and acetochlor ESA in surface water of Iowa. The results of the study (10) indicate that not only was alachlor ESA a common metabolite of chloracetanilide herbicides but also that metolachlor ESA and acetochlor ESA were commonly found. Surprisingly, the parent compound was not detected by GC/MS analysis at a detection limit of 0.05 µg/L. This result shows the importance of tracking down unknown cross reactivity of an ELISA analysis to discover new metabolites of herbicides in water. A second example of how cross reactivity has led to the detection of new compounds in water samples is shown in the following section on the detection of the cotton herbicide prometryn, and the sorghum herbicide propazine.

**Triazine Immunoassay**

The second example discussed in this paper is a different application of the question of cross reactivity. In this example, a metabolite is not the issue for cross reactivity, rather other triazine parent compounds are the cause for the response. In this example, a surface-water survey was completed at playa lakes in West Texas. The original survey was performed on 30 playa lakes to look for atrazine in the lakes. The playa lakes are natural impoundments that are created by rainfall in parts of West Texas. The playa lakes collect runoff from the surrounding fields rather than from streams (17).

To evaluate the effect of atrazine on the playa lakes, 30 water samples were collected from the playa lakes and analyzed by IA. The GC/MS analysis data were compared with the IA data for atrazine; there was a poor correlation (Figure 3). Figure 3A shows that the correlation coefficient was 0.39 for the 30 samples. This compares with a study in the corn belt of the upper midwestern United States, which had a correlation coefficient of 0.92 for 7000 assays of atrazine in rainfall and a 0.87 correlation coefficient for 150 atrazine assays in samples of surface water (18). Also, there were many false positives for atrazine in the playa lake study (6 of 30 samples or 20%), which was surprising.

With such a poor correlation by GC/MS analysis (intercept of 1.47 and slope = 1.8, Figure 3A), the cross reactivity of atrazine metabolites was considered (Table 2), but there was no response from the common atrazine metabolites of deethylatrazine and desisopropylatrazine IA (IC50 = 3.2 and...
Table 1. Concentrations of chloroacetanilide herbicides and their metabolites in selected surface-water samples from Iowa

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alachlor</th>
<th>Alachlor ESA</th>
<th>Acetochlor ESA</th>
<th>Metolachlor ESA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iowa River</td>
<td>&lt;0.05</td>
<td>1.3</td>
<td>0.3</td>
<td>5.0</td>
</tr>
<tr>
<td>Old Man’s Creek, Iowa</td>
<td>&lt;0.05</td>
<td>1.3</td>
<td>0.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Wolf Creek, Iowa</td>
<td>&lt;0.05</td>
<td>0.8</td>
<td>0.2</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Typical of concentrations found in the study cited (10).
217 μg/L, respectively. Therefore, other triazines that cross react with the atrazine IA were examined. Table 2 also shows the response of each of the triazines that are used in the United States. Note that the cross-reacting species (IC50s) were the triazines, ametryn (0.39 μg/L), prometryn (0.64 μg/L), propazine (0.74 μg/L), and simazine (4.9 μg/L).

The cross-reactivity data in Table 2 indicate that structural similarities are important to cross reactivity. For example, prometryn and propazine (Table 2) have a cross reactivity nearly equal to atrazine, whereas cyanazine and simazine have less cross reactivity.

Prometryn and propazine have isopropyl groups present on the amino groups of the triazine herbicide. Thus, both are similar in structure to atrazine. The major difference in structure lies in the thiomethyl group of prometryn versus the chloro group in propazine. The most likely reason that prometryn has a slightly higher cross reactivity than propazine is that the linker for the tracer was through a surface atom at the 2 position in the triazine ring. Because of the sulfur linkage, ametryn also has an important cross reactivity of 0.39 μg/L, which is nearly twice as sensitive, and prometryn is 1.5 times as sensitive as atrazine.

When GC/MS analysis was completed on the playa lakes samples, atrazine was detected in all the samples and prometryn and propazine were also frequently detected. The ELISA analyses indicated positive hits in all samples, while the GC/MS showed 5 false positives compared with ELISA. When atrazine ELISA is correlated to atrazine GC/MS, the correlation coefficient is 0.39 (Figure 3A), which is a poor correlation. If prometryn, propazine, and atrazine were added together as a GC/MS response rather than atrazine, it was found that the correlation with atrazine ELISA improved substantially from 0.39 to 0.96, with a slope of 1.08. These results indicate that the entire IA response is being accounted for by response of these 3 analytes and the importance of the cotton herbicide prometryn in the playa lakes area of West Texas.

Further investigation found that 0.47 million pounds of prometryn were applied annually to cotton in Texas in 1992 (17). The occurrence of propazine in the playa lake samples was a surprise because this herbicide has been off the market since 1991 and has a half life of approximately 60–120 days. Apparently, the propazine detections indicate that although propazine had been removed from the market in 1991, it was still being used on sorghum in West Texas (probably as stockpiles). The importance of this compound, however, should shrink as the existing reserves are used up.

Finally, these results indicate that one of the ways to use cross reactivity is to detect, and sometimes to discover, analytes in the water that were not the target analyte. Prometryn and propazine, as used in the playa region of West Texas, are examples of the detection of these nontarget analytes because of cross reactivity.

### Table 2. IC50 values determined for triazine herbicides and their metabolites using the atrazine RaPID immunoassay

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine</td>
<td>0.72</td>
</tr>
<tr>
<td>Ametryn</td>
<td>0.39</td>
</tr>
<tr>
<td>Prometryn</td>
<td>0.64</td>
</tr>
<tr>
<td>Propazine</td>
<td>0.74</td>
</tr>
<tr>
<td>Prometon</td>
<td>2.2</td>
</tr>
<tr>
<td>Simazine</td>
<td>4.9</td>
</tr>
<tr>
<td>Terbutryn</td>
<td>5.5</td>
</tr>
<tr>
<td>Hydroxyatrazine</td>
<td>148</td>
</tr>
<tr>
<td>Deethylatrazine</td>
<td>3.2</td>
</tr>
<tr>
<td>Deisopropylatrazine</td>
<td>217</td>
</tr>
<tr>
<td>Cyanazine</td>
<td>&gt;10 000</td>
</tr>
</tbody>
</table>

Figure 3. Correlation of ELISA response in μg/L in 30 playa lakes with the concentration of atrazine (A) and atrazine plus prometryn and propazine (B) in μg/L.
Conclusions

In conclusion, 2 examples are presented in this paper that show that cross reactivity of IAs may be used to detect or to discover metabolites that were not previously known. Also, cross reactivity may be used to detect other parent compounds that were not monitored for or were not suspected in the original water quality survey. Thus, when using IAs for water quality surveys, it is important to realize that when there is a greater response from the IA than from the verifying method (i.e., GC/MS or LC/MS), one should consider a careful examination of the sample for degradation products of the target analyte or for other parent compounds of similar structure.

References
