Determination of Imazethapyr Using Capillary Column Flow Injection Liposome Immunoanalysis

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A sensitive immunoanalysis system was developed for the quantitation of imazethapyr, the active ingredient in PURSUIT herbicide. Imazethapyr [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid] is one of the imidazolinone class of herbicides. The assay was based on sequential competitive binding of imazethapyr and liposomes for a limited number of antibody binding sites. A capillary tube (20 cm × 0.53 mm i.d.) with immobilized antibody was used as the immunoreactor column. Liposomes that entrap fluorescent molecules as the detectable label provide instantaneous, rather than time-dependent, enhancement, common with enzyme immunoassays. In this study, liposomes encapsulated carboxyfluorescein dye and were made antigenic by incorporating in the bilayer a phospholipid that had the analyte conjugated to its polar head group. The calibration curve for imazethapyr in Tris-buffered saline solution had a working range of 0.1−100 ng/mL. In the range between 1 and 100 ng/mL, recoveries from fortified tap and pond water samples ranged from 93 to 114%. Filtration was the only step needed for sample cleanup, and an assay could be performed in <10 min.

Keywords: *Imazethapyr; FILIA (flow injection liposome immunoanalysis); capillary immunocolumn; immunoassay; herbicide; liposomes*

INTRODUCTION

Imazethapyr [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid] (Figure 1) is the active ingredient in PURSUIT herbicide. This herbicide is being developed for use with corn, soybeans, peanuts, beans, peas, alfalfa, and other leguminous crops (Los et al., 1984). The effectiveness in weed control is due to its ability to inhibit branched-chain amino acid biosynthesis (Anderson and Hibberd, 1985; Shaner and Anderson, 1984). Generally, the imidazolinone herbicidal selectivity between weed species and crops is attributable mainly to the differential metabolic rates or, in some cases, to the absorption rate at different growth stages rather than differential sensitivity of the target site (Brown et al., 1987; Shaner and Robson, 1985). Metabolic reactions of imidazolinones in plants are aliphatic hydroxylation and glucosidation. As imidazolinone herbicides are more widely used, the demand for imidazolinone residue analysis will also increase. Therefore, it is desirable to develop a simple and rapid analytical method for quantitating imidazolinone residues to increase analytical capacity and provide a rapid turnaround time for sample analysis.

Immunoassay technology is being demonstrated as a viable alternative for and a complement to traditional analytical methods for monitoring agrochemicals (Brecht et al., 1995; Schwalbe et al., 1984; Thurman et al., 1990; Wong and Ahmed, 1992). Most assays have followed the enzyme-linked immunosorbent assay (ELISA) format, in which measurements are made of the color produced from a colorless substrate by the action of an enzyme conjugated to either an antibody or an analyte molecule. In some cases, a drawback of this approach is the long analysis time due to the required incubation step. In general, this method is carried out using microtiter plates and requires extensive pipetting, thereby increasing the possibility of experimental error. However, the major drawback is the difficulty in automating the assay. Automated ELISA is now commercially available, but the cost of the equipment is very high, which limits its use.

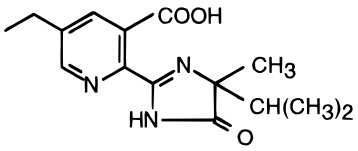

Figure 1. Structure of imazethapyr.

Liposomes that entrap fluorescent molecules as the encapsulated molecule (the marker) provide instantaneous, rather than time-dependent, enhancement. Liposomes are formed when phospholipid molecules spontaneously self-assemble in aqueous solution to form a spherical structure composed of a bilayer membrane enclosing an aqueous volume. They can be made immunogenic by incorporating a phospholipid that has an antigen or antibody associated with its polar head group. Liposomes have been successfully used in flow injection immunoanalysis (Locascio-Brown et al., 1990, 1993; Reeves et al., 1994; Rule et al., 1994). Flow injection analysis is an easily automated technique and is an attractive method to apply to immunoassays because precise control of reagent addition and reaction times offers the potential for high analytical precision.

Recent papers have suggested that capillary immunoassay might provide a number of advantages in comparison with conventional immunoassay methodology (de Frutos et al., 1993; Jiang et al., 1995; Kanneki et al., 1994). The amount of reagent required for an assay can be significantly reduced due to the small size of the capillary. Also, the analysis time can be dramatically decreased because the shortened diffusional distances to the surface reduce the reaction and amplification time. In the previous study conducted in our

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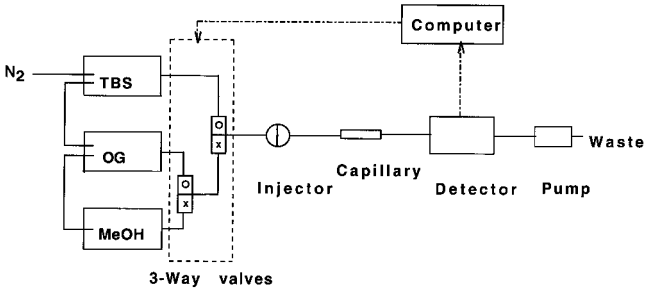

Figure 2. FILIA system. TBS, Tris-buffered saline with 0.01% sodium azide, pH 7.0; OG, 30 mM *n*-octyl β-D-glucopyranoside in TBS; MeOH, 30% methanol solution.

laboratory, the column used was packed with glass beads which were covalently bound to the antibodies. Due to the high surface-to-volume ratio, there was a considerable amount of nonspecific binding, and this nonspecific binding limited the sensitivity of the analysis as reported by Jenkins et al. (1988). An effort was therefore made to control the nonspecific binding by reducing the surface-to-volume ratio. The capillary column was derivatized to conjugate with antibody on the inside of the capillary wall. In this paper, we report the successful demonstration of capillary immunoassay in a flow injection analysis system for imazethapyr determination.

MATERIALS AND METHODS

Materials. Imazethapyr, monoclonal antibody to imazethapyr, and dipalmitoylphosphatidylethanolamine conjugate (DPPE conjugate) were kindly provided by the American Cyanamid Co. (Princeton, NJ). 5-(and 6-)-carboxyfluorescein was purchased from Molecular Probes, Inc. (Eugene, OR). Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Polycarbonate syringe filters of 3, 0.4, and 0.2 μm pore sizes were purchased from Poretics (Livermore, CA). Protein A immobilized on porous silica glass was purchased from Bioprocessing Inc. (Princeton, NJ), and dimethyl pimelimidate (DMP) was from Pierce (Rockford, IL). 3-Glycidoxypropyltrimethoxysilane (GPTMS) was purchased from Aldrich (Milwaukee, WI), undeactivated fused-silica capillary (0.53 mm i.d.) was from Alltech Associates (Deerfield, IL), and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Apparatus. A schematic diagram of the flow injection liposome immunoanalysis (FILIA) system is shown in Figure 2. The injector (Rheodyne Model 7725, Rainin, Emeryville, CA) had a 1-mL sample loop. The flow system was constructed with two three-way 12-V solenoid pinch valves (Biochem Valve Corp., East Hanover, NJ) to control the flow of reagents. All connecting poly(ether ether ketone) (PEEK) tubing (0.02 in. i.d.) was purchased from Upchurch Scientific Inc. (Oak Harbor, WA). A peristaltic pump (Rainin) at the outlet of the system was used to maintain a flow rate of 1.1 mL/min of Tris-buffered saline (TBS) with 0.01% sodium azide (pH 7.0) in conjunction with 41-kPa (6 psi) head pressure on each of the mobile phase bottles. A MacIntegrator I data analysis package, used for valve operation, data collection and integration, was purchased from Rainin. The fluorescence detector with a 3.5 μL flow cell was a Model FL-1 obtained from Rainin.

Production of Liposomes. Liposomes were formed according to the reversed-phase evaporation method (Szoka et al., 1980; O'Connell et al., 1985) from a mixture of DPPC, cholesterol, DPPG, and DPPE conjugate in a molar ratio of 5:5:0.5:0.002. This mixture was dissolved in 8 mL of a solvent mixture containing chloroform, isopropyl ether, and methanol (6:6:1, v/v). The dye solution of 200 mM carboxyfluorescein (1.4 mL) was added with swirling, and this mixture was sonicated for 5 min under a low flow of nitrogen. The organic phase was removed under vacuum on a rotary evaporator at 45 °C. An additional aliquot of the dye solution (2.6 mL) was added and sonicated for 6 min. The liposomes were then extruded through three polycarbonate filters of decreasing pore sizes of 3, 0.4, and 0.2 μm. Finally, any unencapsulated dye was removed following the method of Siebert et al. (1993). The liposomes were gel filtered on a 1.5 × 25 cm Sephadex G-50-150 column equilibrated with TBS and dialyzed overnight against TBS at 4 °C.

Purification of the Monoclonal Antibody. The monoclonal antibody was purified from ascites by passage through a protein A column. The manufacturer's instructions were followed for this procedure. The ascites was loaded onto the column after dilution with 1 M glycine/0.15 M NaCl buffer (pH 8.6). The column was then washed with the glycine buffer, and the bound monoclonal antibody was eluted with 0.1 M citrate buffer (pH 3.0). Finally, the antibody concentration was determined by using a protein test kit from Bio-Rad (Hercules, CA).

Preparation of the Capillary Column. The capillary was internally coated with protein A to which the antibody could be covalently attached. The feasibility of flow injection immunoassays using a protein A matrix for antibody immobilization has been demonstrated (Palmer et al., 1992; Evans et al., 1994; Rule et al., 1994). The advantage of such a system is that the antibody can be oriented to the matrix with minimal interference of antigen binding sites. Protein A is a protein component in the cell wall of most strains of *Staphylococcus aureus*, which interacts with many immunoglobulins of almost all mammals (Surolia et al., 1982). The binding between protein A and antibody occurs at the Fc ("fragment crystallizable" obtainable in a crystalline form) region and not in the antigen binding Fab ("fragment antigen binding") region. The fact that the antigen binding region is not affected has made protein A useful as an antibody binding protein in immunoassays. The procedure for modification of the capillary is as follows: Introduce the solutions into the capillary with a syringe. Pretreat the fused-silica capillary with 1 M NaOH overnight followed by rinsing with 1 M HCl and doubly distilled water. Fill the capillary with 10% GPTMS solution in 0.1 M acetate buffer (pH 3.5) and heat at 90 °C for 2 h for silylation. Rinse the capillary with water and treat with 10 mM sulfuric acid at 90 °C for 10 min to convert residual epoxy groups to diols. After flushing with water, treat the capillary with 20 mM sodium metaperiodate containing 2 mM potassium carbonate at room temperature for 2 h. [In this step, aldehyde groups are formed by the periodate cleavage. Protein A is then conjugated through the aldehyde groups by a modification of the Larsson (1984) and de Frutos et al. (1993) methods in which the capillary is filled with 1 mg of protein A and 0.5 mg of sodium cyanoborohydride (to reduce the Schiff base) in 200 μL of 0.1 M phosphate buffer (pH 6) and incubated overnight at room temperature.] Treat the capillary with a solution of sodium borohydride in 0.1 M phosphate buffer (pH 8) for 1 h to reduce remaining aldehyde groups to their alcohols, and rinse with doubly distilled water and 1 M glycine buffer (pH 8.6). Fill the capillary with antibody in glycine buffer and incubate at room temperature for 1 h followed by 8 h at 4 °C. After washing with glycine buffer and 0.2 M triethanolamine (TEA) buffer (pH 8.2), fill the capillary with DMP solution (6.6 mg of DMP in 1 mL of TEA buffer with the pH readjusted to pH 8.2) and incubate for 45 min (Brew et al., 1975; Schneider et al., 1982). Rinse the capillary with 0.1 M ethanolamine (pH 8.0) and incubate overnight at room temperature. Rinse the capillary sequentially with TEA buffer, 1 M NaCl, 0.1 M glycine/HCl buffer (pH 2.5), and TBS. The capillary filled with TBS can be stored at 4 °C for 1 month without any significant change in the antibody binding capacity.

FILIA. One milliliter of the imazethapyr sample in TBS was introduced onto the immunoreactor column by manual injection. This was followed by introduction of the analyte-tagged liposomes. After all unbound liposomes were eluted, a detergent solution (30 mM *n*-octyl β-D-glucopyranoside) was passed through the column. The detergent caused rupture of the liposomes, and the fluorescence of the released dye was generated and measured. Finally, a 30% methanol solution

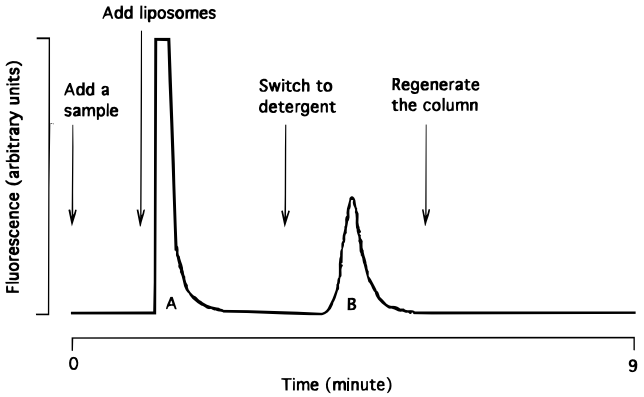

Figure 3. Trace of typical FILIA run. Response was measured as the area of peak B.

was passed through the column to regenerate the antibody binding sites by dissociating bound analyte, and the column was then reconditioned for the next analysis by returning the mobile phase to TBS. Another analytical format was investigated in which the sample and liposome solutions were mixed and injected simultaneously. The remainder of the procedure was performed as described above for the sequential injection format.

Water Sample Analysis. Pond water, provided by American Cyanamid, had been determined to be free from imazethapyr and was stored frozen until required. Tap water was obtained in the laboratory when needed. For water analysis, each 20-mL water sample was spiked with known amounts of imazethapyr to yield water concentrations of imazethapyr from 1 to 100 ppb and filtered through 0.2-μm nylon membranes. TBS buffer chemicals were then added, and the pH was adjusted to 7.0. The volume of the water samples injected was the same as that for the samples in TBS (1 mL), and concentrations were calculated from a TBS standard curve.

RESULTS AND DISCUSSION

Assay Method. Figure 3 shows a typical diagram for an analytical run. Peak A represents the signal from unbound liposomes and traces of free dye passing through the fluorescence detector. Peak B is the analytical signal produced by the lysis of bound liposomes and consequent release of the fluorescent label. Response was measured as peak area. A 30-μL aliquot containing ca. 10^{10} liposomes tagged with the analyte was injected to give the fluorescence signal of about 10 (arbitrary units) in the absence of imazethapyr. The fluorescence signal from this amount of liposomes was within an optimal range of the detector having the highest reproducibility. When imazethapyr is introduced onto the column as a sample, it binds to the antibody sites in proportion to its concentration. After 1 min, when the analyte-tagged liposomes are injected, they bind to unoccupied antibody sites. Since the binding of liposomes depends on the number of remaining antibody sites available, the area of peak B is inversely proportional to the amount of imazethapyr present in the sample.

The immobilized antibody showed strong affinity for imazethapyr. To demonstrate this, after the injection of an imazethapyr sample, TBS solution was passed through the column for 30 min. Liposomes were then injected, and the response measured did not show any significant change in comparison with the usual analysis format of 1-min TBS flow. This result indicates that the antibody−imazethapyr dissociation rate (i.e., antigen off-rate) is slow, which is in agreement with the study reported by Stenberg and Nygren (1988) in which

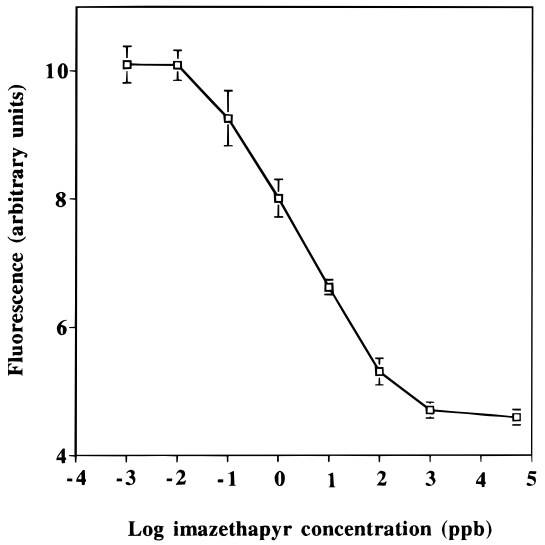

Figure 4. Dose−response curve for imazethapyr in TBS. Each point represents the mean of three measurements; error bars represent ±1 SD.

they found that some antibody−antigen reactions at solid−liquid interfaces show a high degree of irreversibility. It is thus thought that in this sequential injection mode, displacement of bound imazethapyr by the liposomes is negligible. In comparison, competitive assays were conducted (data not shown) in which imazethapyr and liposomes were injected simultaneously onto the immunocolumn. The detection limit and sensitivity of this assay protocol were approximately 1000-fold poorer than those found with the sequential injection method. It is believed that the liposomes, which have multiple imazethapyr tags on their surface, have greater opportunity to bind to the antibodies. Thus, a higher concentration of the univalent analyte imazethapyr is required to effectively compete with the multivalent liposomes for antibody binding sites as compared to the sequential assay format.

Dose−Response Curve. The dose−response relationship of imazethapyr using the capillary column in the sequential injection format is shown in Figure 4. The dose−response curve for imazethapyr in TBS shows a working range from 0.1 to 100 ng/mL. A typical coefficient of variation (CV) for triplicate measurements was <3%. However, it was observed that reproducibility was reduced during the course of many analytical runs because introduction of the methanol solution created a large temperature differential and caused bubbles to form in the system. As a result, all solutions must be degassed under vacuum before use each day and after use for several hours. In this research, the antibody was covalently attached to protein A by DMP to give high stability and reproducibility. The capillary column could be reused for up to 150 analysis runs. After that, the response decreased slowly. The decrease in response may be due to the physical detachment of the bound antibody from the capillary and/or the inactivation (denaturation) of some of the antibody binding sites over extended periods of time.

Detection of Imazethapyr in Water Samples. The recoveries of the added imazethapyr from the water samples were determined by carrying out the FILIA procedure on each sample and ranged from 93 to 114% (Table 1). Generally, the recoveries exhibit a slight positive bias, which is a typical immunochemical response (Brady et al., 1995). The presence of contami-

Table 1. Recoveries of Imazethapyr Added to Water Samples

water	imazethapyr spike, ppb	imazethapyr recovered				
		mean, ppb	*N*	%	SD, ppb	% CV
tap	1.0	1.0	9	103	0.1	11
	10.0	11.0	9	110	2.4	22
	100.0	113.9	9	114	7.3	6
pond	1.0	1.0	9	97	0.1	8
	10.0	10.1	9	101	2.8	28
	100.0	93.2	9	93	12.2	13

nants in the water filtrates might affect the antibody−antigen interaction, which could result in an increase in the binding of imazethapyr in the sample or block some nonspecific binding sites for liposomes which make the area of peak B smaller. However, from analytical runs without the capillary column it was thought that this positive bias resulted mostly from nonspecific binding of the contaminants to the sample loop and tubing. In the flow injection system, which was not coupled to the column, liposomes were introduced after an injection of TBS and the fluorescence generated from the lysis of the bound liposomes was considered as the control (100% nonspecific binding). In comparison, after injections of tap water and pond water, the fluorescence was measured and was slightly less than the control. Therefore, the contaminants in the water samples masked some nonspecific binding sites for liposomes and, as a result, a smaller area of peak B produced the false positive results. These contaminants also were somewhat detrimental to the stability of the capillary column. The column lost antibody activity faster than with the samples in TBS. As a result, the column could be reused only for half as many analytical runs as with TBS. This may be the reason for the moderately high percent CV in the recovery test. After the column was reused for approximately 50 samples, reproducibility of the data was reduced.

Conclusion. This work demonstrates the feasibility of using capillary flow injection liposome immunoanalysis for the determination of imazethapyr herbicide. The detection limit achieved by this method is significantly lower than the 5 ng/mL obtained by chromatographic methods using no preconcentration (Devine, 1991). A single assay can be performed in <10 min with recoveries of added imazethapyr from tap and pond water ranging from 93 to 114%. This suggests that it can be readily adapted to the analysis of real water samples. Future studies will investigate the application of this technique to the determination of imazethapyr extracted from soil samples.

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