Bull. Environ. Contam. Toxicol. (1997) 59:611-618

Bulletin of Environmental Contamination and Toxicology

Use of ELISA Immunoassay Kits as a Complement to HPLC Analysis of Imazapyr and Triclopyr in Water Samples from Forest Watersheds

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Received: 14 March 1997/Accepted: 11 June 1997

The herbicide triclopyr (3,5,6-trichloro-2-pyridinyl)-oxyacetic acid has been marketed by the Dow Chemical Co. since the mid 1970's as the triethylammonium salt (Garlon 3A) and as the ethylene glycol butyl ether ester (Garlon 4). Shortly after its introduction, McKellar (1977) published a method for the extraction, isolation, and electron capture gas chromatographic analysis of triclopyr and two of its major metabolites in soil and water samples. A number of environmental fate studies of triclopyr have been published since then, each of which has used the McKellar method or a minor modification of it (Oloffs et al. 1986; Deubert et al. 1986; Norris et al. 1987; Whisenant et al. 1989). Glas (1978) developed a method along similar lines for the analysis of triclopyr in plant tissues as did Bovey et al. (1983). While state-of-the-art for their day, these methods are labor-intensive and time-consuming. They use large volumes of dichloromethane which has been designated a hazardous material by the EPA, making its disposal difficult and expensive. These methods also involve derivitization with diazomethane, which is an explosion hazard and requires handling of highly carcinogenic precursors. We have developed a rugged high-performance liquid chromatographic (HPLC) method for analysis of the free-acid form of triclopyr in environmental water samples. This method features solid-phase extraction (SPE) and a lead diacetate cleanup to remove interfering humic substances. We have applied this method to the analysis of over two thousand water samples from a major environmental fate study we conducted on the Ouachita National Forest, Arkansas, but only 10 samples could be processed in one technician-day. Samples from environmental fate studies collected in one day frequently exceed 200-300 so there is a great need for a method which will accelerate the analysis of these samples.

The past few years have seen the development of enzyme-linked immunosorbent assay (ELISA) kits for trace-level analysis of numerous pesticides in environmental water samples (Kaufman and Clower 1991, 1995; Van Emon and Lopez-Avila 1992). Developmental work has thus far focused mostly on pesticides used in crop agriculture, such as the triazine herbicides (Thurman et al. 1990; Goh et al. 1990) and alachlor (Lawruk et al. 1992), and a selection of field-portable and laboratory test kits has been made commercially available (Van Emon and Gerlach 1995). Recently forestry herbicides such as imazapyr, metsulfuron, and hexazinone have received commercial attention as well. The introduction of a magnetic particle-bound polyclonal antibody ELISA kit for triclopyr acid (Ohmicron Diagnostics; Newtown PA) and an antibody-coated 96-well microplate kit for imazapyr (EnSys; Research Triangle Park NC) prompted us to evaluate their applicability to our environmental fate studies. This study presents direct comparisons of these two ELISA kits with our in-house HPLC methods in comparability and

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variability of results when applied to typical forest stream runoff samples. Also included is a rough comparison of analyst time and cost per analysis.

MATERIALS AND METHODS

Analytical standards of triclopyr acid and imazapyr were obtained from Dow Chemical Co. (Midland MI) and American Cyanamid (Princeton NJ), respectively. Methanol, chloroform, and acetonitrile were Burdick and Jackson HPLC grade obtained from Baxter Scientific Products (McGaw Park IL). High purity water was obtained from a Millipore Milli-Q Plus system (Bedford MA) fed by a Barnstead laboratory still (Dubuque IA). Hydrochloric acid (analytical reagent grade) and phosphoric acid (85%) were obtained from Mallinkrodt Chemical Company (Paris KY). Lead diacetate trihydrate (reagent grade) was obtained from J.T. Baker (Phillipsburg NJ). Solid phase extraction was performed using J.T. Baker Light-load Octadecyl 1 g, 6 mL SPE columns (Lot #E08122 and H26550) or Waters Sep-Pak Plus Environmental 1000mg SPE columns (Lot #P1064A1) mounted on Supelco (Bellefonte PA) Visiprep 12-place vacuum manifolds. Water samples for ELISA and for direct injection HPLC were filtered through Millipore Millex-SR 0.5 µm filter units (25 mm diam.). HPLC hardware consisted of LDC CM4000 Multiple Solvent Delivery Systems and SM4000 Programmable Variable Wavelength Absorbance Detectors (Miami FL), Waters WISP 710B autoinjectors (Milford MA), Shimadzu CTO-6A column ovens (Kyoto Japan), and Spectra-Physics SP4400 Chrom-Jet Integrators (San Jose CA).

Samples of surface runoff water were collected during storm events using ISCO 2700 and 3700 water samplers (ISCO, Inc., Lincoln NE) and frozen at or below -10°C until analyzed. Triclopyr was sampled from watersheds on the Fourche and Womble Ranger Districts of the Ouachita National Forest, Arkansas. Imazapyr was sampled from watersheds on the Lower Coastal Plain Substation of the Alabama Agricultural Experiment Station near Camden, Alabama. For HPLC analysis water samples were thawed and shaken. Next, aliquots of 500mL (triclopyr) or 200mL (imazapyr) were treated with 2 mL or 1 mL, respectively, of 1M lead diacetate and allowed to stand at least 40 min to allow precipitates to coagulate and settle. Each treated sample was suction-filtered through a 5.5cm Whatman GF/B glass fiber filter in a Buchner funnel mounted on an Erlenmeyer flask using a vacuum filter grip (VWR Scientific, Atlanta GA #28290-009). The filtrate was adjusted to pH 2.0 with 1N HCl, then subjected to solid phase extraction. Each SPE column was preconditioned with 5 mL methanol followed by 10 mL high purity water acidified to pH 2.0 with HCl. The sample was then passed through the SPE column at no more than 4 drops/sec, not allowing the column to go dry. The column was post-washed with 10 mL acidified water, followed by 10 mL ethanol-free chloroform, then pulled dry under full vacuum for at least 15 min to remove all traces of chloroform. The herbicide extract was eluted with 5 mL methanol, collected in a 5 mL volumetric flask. The eluate volume was adjusted to 5 mL with methanol, shaken thoroughly, and transferred to a 4 mL WISP vial for HPLC analysis. Reversed-phase HPLC was performed on Zorbax Stablebond SB-C8 columns (150 x 4.6 mm + 12.5 x 4.6 mm guard cartridge) maintained at 40°C and eluted isocratically at 1.0 mL/min with 46/54 (v/v) acetonitrile/water (pH 2.0 /w H_3PO_4) for triclopyr or with 15/85 (v/v) acetonitrile/water pH 2.0 /w H_3PO_4) for imazapyr. Detection was by ultraviolet absorbance at 232nm for triclopyr and 240nm for imazapyr. Injection volume was 10 µL for SPE extracts and 100 µL for direct injection water samples. Samples were injected in duplicate and averaged. External calibration standards were injected

before and after every four samples. Detector response was averaged for each set of bracketing standards. One blank and spiked pair was extracted and analyzed with every 10 samples. The minimum matrix detection level (MDL) of our HPLC/SPE method (determined as 10 x the chromatographic baseline noise level) for triclopyr and imazapyr in 500 mL water samples was 0.5 ppb and 0.8 ppb respectively. At the 100:1 SPE concentration factor, a 50 ng/mL spiked water sample produced a peak of height equivalent to 5 ng/µL in the extract. Spike recoveries averaged 99.8±4.0% (n=228) for triclopyr and 97.1 ± 1.6% (n=4) for imazapyr.

Triclopyr RaPID Assay® kits (Lot # 940794, 941774, 950321, and 950616), Magnetic Separation Racks, and RPA-1 RaPID Analyser™ were obtained from Ohmicron Corporation (marketed by Strategic Diagnostics Inc., Newark DE). All analysts using these kits passed certification tests administered by an Ohmicron representative before analyzing samples. All samples were assayed according to kit directions and prior to the expiration dates printed for each lot number. ELISA tube absorbances were read using an Ohmicron RPA-1 Analyser (Newtown PA) with a 450 nm interference filter. Duplicate standards of 0.0, 0.1, 1.0, and 3.0 ng/mL (ppb) supplied with the kit were used for the calibration curve, which was corrected for "non-specific binding" by including an offscale 1000 ppb triclopyr standard as a "reagent blank". The published lower limit of detection (LLD) for the method was 0.03 ppb, but we conservatively chose the 0.1 ppb calibration standard as the LLD. Using all 108 tubes supplied with a "100 tube" kit and the maximum 99 sample memory of the RPA-1 we were able to run 98 samples/kit by this method.

Enviroguard™Imazapyr Plate Kits were obtained from Millipore Corporation, Bedford MA (available from Strategic Diagnostics Inc., Newark DE by special request). All 96 wells were used simultaneously, twelve wells for the calibration standards (0ppb, 0.5ppb, 5ppb, and 50ppb, run in triplicate), and the other 84 wells for individual samples. To minimize pipetting time the standards and samples were first transferred to 1.2mL polypropylene tubes arranged on an 8 X 12 rack (USA Scientific Plastics, Ocala FL) in the exact order of the actual plate. They were then transferred in 100µL aliquots to the antibody-coated wells using a Brinkmann 12-place Transferpette. Similarly, 100µL aliquots of enzyme-hapten conjugate solution were immediately added to each well. The plate assembly was covered with Sealplate adhesive film (Elkay Products, Shrewsbury MA) and agitated on a table shaker at room temperature (22-24°C) for 60 minutes, as specified in the kit instructions. The remaining steps were performed according to kit directions. Absorbances were read at 450 nm using a Ceres 900HDi automatic plate reader (Bio-Tek Instruments, Winooski VT). Response curves and sample analyte concentrations were calculated using Kineticalc II software supplied with the reader data system. The MDL published with the kit was 0.3ppb, later raised to 0.5ppb, identical to the lowest calibration standard.

In order to determine statistical variability we also ran quadruplicate analyses of three composited water samples known to have low, moderate, and high triclopyr and imazapyr concentrations. In this part of the study and in the analyses of all field samples, we made appropriate dilutions of samples to bring them into the range of the ELISA kits used. In addition to the J.T. Baker SPE columns and Ohmicron ELISA kit we also compared Waters Sep-Pak Plus SPE columns against direct aqueous injection. We did not compare SPE/HPLC with Light-load Octadecyl columns for imazapyr because previous work had shown that recoveries were low and highly variable (from 8 to 36%). For a comparison

of the various analyses, we assumed the values obtained from direct injection HPLC to represent the actual analyte concentration and then calculated percent recovery for the other analyses performed on aliquots of the same samples.

RESULTS AND DISCUSSION

Results of the comparison of our HPLC methods and the ELISA methods are presented for triclopyr (Figure 1, Table 1) and imazapyr (Figure 2, Table 2). The correlation between results of the HPLC and ELISA methods on samples collected from field studies are generally good (Figures 1,2). In all cases in which the analyte concentration was greater than the HPLC MDL, the ELISA data are more variable than the HPLC method with coefficients of variation (CVs) for the ELISA method at least 10 times greater than that of the HPLC method (Table 1,2). Percent recovery was similarly variable ranging from 91% to 151% of the actual value. The triclopyr ELISA results, however, show a much closer correlation (r^2=0.92) with HPLC for field samples than those for imazapyr (r^2=0.65). Both kits are subject to false positive results arising from the sample matrix (Lee MS and Richman S, 1991), but the ELISA is also known to be more variable at the ends of the kits' linear dynamic ranges. For triclopyr the range is only about 30 fold (0.1ppb to 3.0ppb) while the range for imazapyr is 100 fold (0.5ppb to 50ppb). Thus, while all field samples had to be diluted so that the triclopyr concentration was within the linear dynamic range, far fewer imazapyr samples had to be diluted. When samples were diluted, we tried to dilute so that the diluted sample would contain an analyte concentration in the middle of the kit's range because this is where the kit is most accurate. As the concentration increases or decreases toward the limits of the linear dynamic range, accuracy is sacrificed and data become more variable. Because fewer imazapyr samples had to be diluted, fewer contained imazapyr concentrations near the middle of the kit's range and so the data are much more variable than those for triclopyr. Dilution also decreased the effects of sample matrix interference. The poorer correlation between HPLC and ELISA for imazapyr is due to a combination of matrix-related false positives and analyses close to the ends of the linear dynamic range for the ELISA kit.

HPLC methods typically exhibit greater variability near the MDL and less variability as the analyte concentration increases. Results of direct injection analysis of triclopyr at concentrations below the MDL are highly variable with a coefficient of variation (CV) of 67% (Table 1). The sample cleanup and concentration which occurs with the use of SPE greatly improves the CV for triclopyr (2.6% and 0.74%, Table 1). Recovery of triclopyr was more quantitative with the J.T. Baker SPE columns than with the Waters SPE columns (Table 1). Imazapyr recovery was essentially quantitative with the Waters SPE column. Previous attempts to analyze imazapyr using J.T. Baker SPE columns resulted in extremely low and highly variable recovery (from 8 to 36%) .

We have been able to integrate ELISA technology into the analysis of samples from our environmental fate studies on two levels. The most useful is based on the fact that ELISA technology may yield false positive but not false negative analyses. Thus ELISA can be used with thousands of samples to quickly eliminate those which do not contain the analyte of interest. In our field study of imazapyr and triclopyr, over 500 samples did not contain the herbicide of interest. A total of 84 or 98 (depending on the kit) samples can be analyzed by ELISA in a day while only 10 can be analyzed per day by our SPE/HPLC analysis. In this case, using ELISA in a screening mode to identify negative samples which did not have to be analyzed by the more time-consuming HPLC

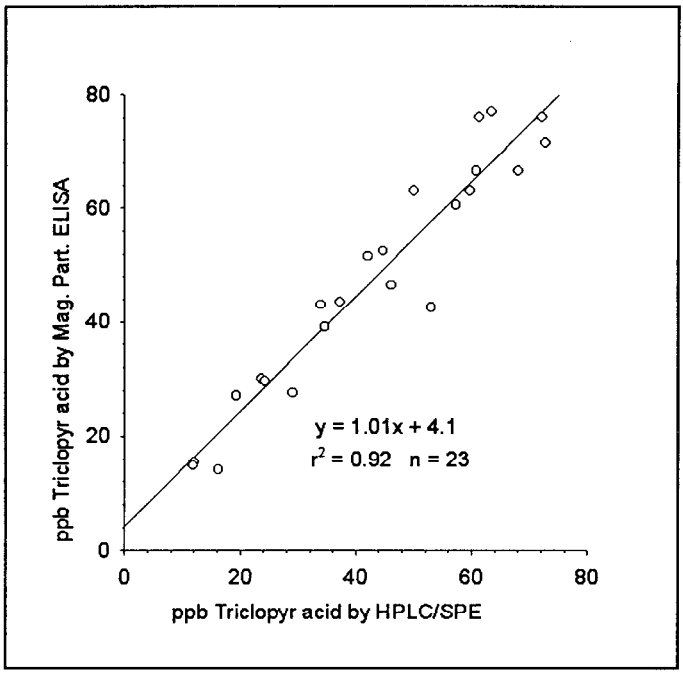

Figure 1. Correlation of HPLC/SPE and magnetic particle ELISA results for triclopyr in stormflow samples from the Ouachita NF, Arkansas.

method saved us 44 technician days and the cost was low, approximately $5 per sample not including technician time.

We have also used ELISA technology to analyze time-sequenced samples, but it is more difficult to effectively use the triclopyr and imazapyr kits in this mode. A major limitation of both immunoassays is their limited dynamic range (< 1:100) which necessitates shrewd guesswork of sample dilution factors to minimize out-of-range results and expensive reruns. In our studies of herbicide dissipation, concentrations of the analyte within a sequence of storm runoff samples decrease rapidly at first and then at a slower rate with later samples. We use HPLC analysis for the first, last, and sometimes middle sample in a sequence of 24 or 28 (the number collected in a series by our automatic samplers) samples to establish analyte concentration. Then, based on this information, intervening samples are diluted to bring them into the linear dynamic range of the ELISA kit to be used. When used in this mode, all outliers and at least 10% of all ELISA analyzed samples are confirmed by HPLC. While less reliable and less cost-effective than when used as a screening device for negative samples, ELISA still provides for savings and increased speed in analyzing large numbers of field samples.

ELISA procedures hold the promise of reducing the labor cost per analysis, relieving time pressures for report deadlines, and permitting "real-time" feedback of early analytical results into decisions affecting later sampling commitments and retention of samples in frozen storage.

Table 1. Comparison of triclopyr concentration[a] determined from direct injection HPLC, solid phase extraction with J.T. Baker (LtLdOctadec) and Waters (Sep-Pak) SPE columns/HPLC, and magnetic particle ELISA

Sample#	Direct Inj. HPLC	SPE/HPLC LtLdOctadecyl	SPE/HPLC Sep-Pak	ELISA Mag.Particle
#1 Avg (n=4)	6.5[c]	7.3	6.7	9.8
STD	4.4	0.19	0.05	1.06
CV	67%	2.6%	0.74%	10.8%
% Recovery	100%	112%	103%	151%
#2 Avg (n=4)	35.8	34.8	33.6	41.3
STD	0.245	0.096	0.171	3.26
CV	0.68%	0.28%	0.51%	7.9%
% Recovery	100%	97.2%	93.9%	115.4%
#3 Avg (n=4)	67.4	67.6	64.1	72.6
STD	0.216	0.387	0.311	2.34
CV	0.32%	0.57%	0.49%	3.2%
% Recovery	100%	100.3%	95.1%	107.7%
MDL[b]	10.0	0.5	0.5	0.1

[a]All concentrattons expressed in µg/L (or ppb).
[b]MDL's determined as 10x the chromatographic baseline noise level or at the level of the lowest ELISA calibration standard.
[c]Doubled injection volumes needed to exceed MDL in this sample.

Table 2. Comparison of imazapyr concentration[a] determined from direct injection HPLC, solid phase extraction with Waters SPE columns/HPLC, and 96-well microplate ELISA

Sample#	Direct Inj. HPLC	SPE/HPLC Sep-Pak	ELISA Microplate
#1 Avg (n=4)	80.5	78.1	109.3
STD	0.81	0.94	18.5
CV	1.01%	1.20%	16.9%
% Recovery	100%	97.0%	135.8%
#2 Avg (n=4)	162.5	159.5	173.6
STD	1.41	1.67	35.1
CV	0.87%	1.05%	20.2%
% Recovery	100%	98.2%	106.8%
#3 Avg (n=4)	485.8	491.4	445.4
STD	1.67	9.44	68.4
CV	0.34%	1.92%	15.3%
% Recovery	100%	101.2%	91.7%
MDL[b]	10.0	0.8	0.5

[a]All concentrations expressed in µg/L (or ppb).
[b]MDL's determined as 10x the chromatographic baseline noise level or at the level of the lowest ELISA calibration standard.

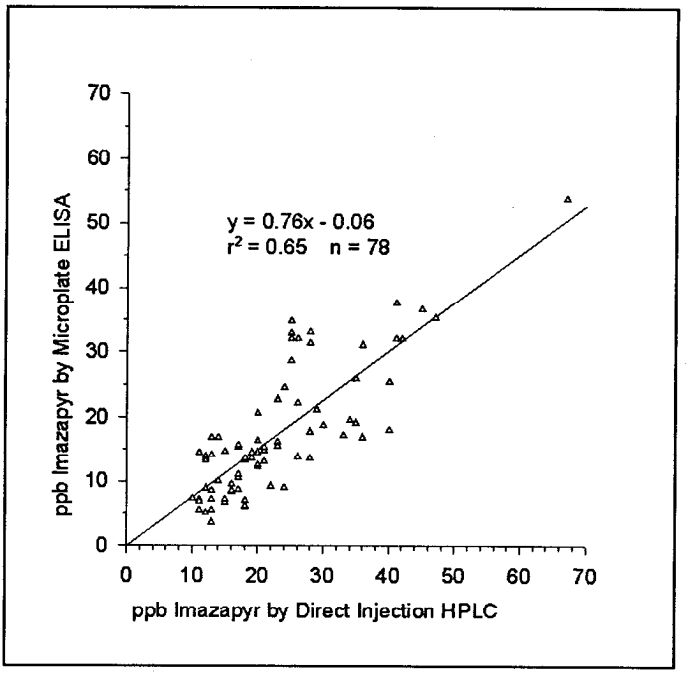

Figure 2. Correlation of direct injection HPLC and microplate ELISA results for imazapyr in storm runoff samples from the Alabama Agricultural Experiment Station Lower Coastal Plain Substation near Camden, Alabama.

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