Polyclonal and Monoclonal Enzyme Immunoassays for Picloram Detection in Water, Soil, Plants, and Urine

Raymond J. A. Deschamps,*,‡ J. Christopher Hall,‡ and Mark R. McDermott§

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1, and Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Two indirect enzyme immunoassays for picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) detection were compared in terms of sensitivity, accuracy, and precision. The assay using a rabbit anti-picloram serum had a linear working range from 5 to 5000 ng/mL with a mean I_{50} value of 140 ng/mL and a lower detection limit of 5 ng/mL. The assay using a monoclonal antibody obtained from a mouse hybridoma cell line yielded a linear working range from 1 to 200 ng/mL with a mean I_{50} value of 10 ng/mL and a lower detection limit of 1 ng/mL. Neither assay showed appreciable cross-reactivity with the structurally related pyridine herbicides clopyralid, fluroxypyr, and triclopyr or with the phenoxyacetic acid herbicide 2,4-D. From the analysis of fortified river water, soil extracts, plant extracts, and urine, the monoclonal antibody based assay was shown to be more sensitive, more accurate, and more precise than the polyclonal antiserum based assay. Only the monoclonal assay was suitable for quantitative determinations of picloram.

INTRODUCTION

Immunoassays are considered replacements for, or complements to, conventional methods of pesticide residue detection since they can potentially provide quantitative data more quickly and at lower cost than conventional techniques (U.S. Congress, 1988). Such immunoassays may be based on polyclonal or monoclonal antibodies. The former is a heterogeneous mixture of proteins isolated from serum that represents a variety of antibody molecules of differing specificities and affinities. In contrast, the latter is a homogeneous reagent possessing a single antibody specificity and affinity. In a variety of assay systems, either monoclonal or polyclonal antibodies may have certain advantages over the other. For a detailed description and comparison of polyclonal and monoclonal antibodies, the reader is referred to the text by Zola (1987).

The majority of published immunoassay techniques for pesticide detection employ polyclonal antibodies. In a review of immunoassays for agrochemicals, Mumma and Brady (1987) cite 49 assays employing polyclonal antibodies and only 12 employing monoclonal antibodies. The reason for this discrepancy in popularity may be that polyclonal antibody based assays, at first examination, are easier to develop. Obtaining polyclonal antibodies involves synthesizing an immunogen, immunizing an animal species of choice, and collecting the serum containing the antibodies, which may be used without further processing or may be purified before use. Monoclonal antibodies are obtained by hybridizing the nonimmortal antibody-producing cells from an immunized mouse or rat with immortal tumor cells, selecting those hybrids producing the antibody of interest, and culturing them for mass production of the antibody.

When used as reagents for the quantitation of pesticide residues, monoclonal antibodies have certain advantages over polyclonal antibodies: (i) hybrid cells can be cultured indefinitely, either in vivo or in vitro, to yield a potentially unlimited supply of homogeneous, standardized reagent; (ii) during the hybridoma selection process, the investigator can select a hybrid cell producing the desired antibodies in terms of specificity and affinity; (iii) the monoclonal antibody will be free of antibodies that are specific for irrelevant antigens that may interfere with the assay's performance; and (iv) cross-reactivity with structurally similar molecules (e.g., other members of a pesticide class) can be selected for or against depending upon whether the investigator desires an assay to detect a single pesticide or a class of pesticides (Vanderlaan et al., 1987; Vinas, 1985). Despite these issues favoring monoclonal antibody based assays, it is possible to develop excellent immunoassays based on polyclonal antibodies.

Earlier, we reported a radioimmunoassay (RIA) procedure for picloram determinations in water and urine employing polyclonal antibodies produced in rabbits (Hall et al., 1989). In the present study, we describe the development of two indirect enzyme immunoassays (EIA), the first using the polyclonal antisera described previously (Hall et al., 1989) and the second using a monoclonal anti-picloram antibody developed in our laboratories. The polyclonal antiserum and the monoclonal antibody were developed from a common immunogen. The immunoassays were compared in terms of the characteristics of the standard curves and performance based on the determination of picloram in fortified water, soil extracts, plant extracts, and human urine samples.

MATERIALS AND METHODS

Materials. Analytical standards of picloram, clopyralid (3,6-dichloro-2-pyridinecarboxylic acid), fluroxypyr [[(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid], and triclopyr [[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid] along with radiolabeled picloram ([2,6-^{14}C]picloram, specific activity 264 MBq/mmol) were provided by the Dow Chemical Co., Midland, MI. Female Balb/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME, or from Charles River Inc., Montreal, PQ. Cell culture media (RPMI and NCTC-109) as well as the HAT selective medium components (hypoxanthine, aminopterin, and thymidine) were obtained from Gibco Inc., Burlington, ON.

Preparation of Immunogen. The immunogen used for both polyclonal and monoclonal antibody production was the picloram–bovine serum albumin (BSA) conjugate described previously (Hall et al., 1989).

‡ University of Guelph.
§ McMaster University.

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Preparation of Coating Conjugates. Picloram was conjugated to rabbit serum albumin (RSA) according to two different procedures. One procedure yielded a conjugate with peptide linkages between picloram and the protein (RC4), while the other yielded a conjugate with primarily ester linkages (RC5).

RC4. Following a procedure described by Fleeker (1987), equimolar amounts of picloram (46 mg), *N*-hydroxysuccinimide (NHS, 22 mg), and *N,N'*-dicyclohexylcarbodiimide (DCC, 39 mg) were dissolved in 2.5 mL of dioxane. After incubation at room temperature for 18 h, the solution was filtered to remove any precipitate. The filtrate was dried at 35 °C under vacuum. The residue was resuspended in 3 mL of 0.10 M borate buffer (pH 9.0) containing 500 mg of RSA and was agitated gently for 1 h. The resulting solution was dialyzed against cold flowing tap water for 24 h and lyophilized.

RC5. Picloram (50 mg) was dissolved in 5 mL of thionyl chloride ($SOCl_2$) in a small boiling flask. The solution was refluxed for 2.5 h at 85 °C to form the acid chloride of picloram. Excess thionyl chloride was removed under vacuum at 60 °C on a rotary evaporator. The residue was dissolved in 2 mL of tetrahydrofuran (THF). The picloram acid chloride solution was added slowly with stirring to 200 mg of RSA in 10 mL of 0.02 N NaOH. Before the addition of the acid chloride solution was completed, precipitate formed which did not resolubilize after stirring for 18 h at room temperature. Dilution of the reaction mixture to 200 mL with 0.02 N NaOH succeeded in dissolving most of the precipitate. The resulting suspension was centrifuged to remove any precipitate. The supernatant was dialyzed against cold flowing tap water for 24 h and lyophilized.

Production of Polyclonal Anti-Picloram Antibody. Anti-picloram antisera were obtained from New Zealand White rabbits following the protocol described by Hall et al. (1989).

Production of Monoclonal Anti-Picloram Antibody. *Immunization.* Ten 11-week-old mice were injected intraperitoneally with a total volume of 250 μL of a 1:1 (v/v) mixture of 70 μg of immunogen dissolved in phosphate-buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.4) and Freund's complete adjuvant (Difco Laboratories Inc., Detroit, MI). Secondary inoculations were given 3 and 11 weeks after the initial immunization. One week following each secondary inoculation, the mice were bled from the retro-orbital plexus and the anti-picloram serum antibody titer was determined by using the RIA procedure described by Hall et al. (1989). A serum sample was considered positive for anti-picloram antibody activity if binding of the picloram radiolabel was more than twice the level of nonspecific binding. After the final secondary inoculation, binding values ranged from 1900- to 4400-fold greater than the level of nonspecific binding. Approximately 4 months after the third injection (6 months from the initial immunization), the two mice possessing the highest antisera titers were given a final injection of 200 μg of immunogen in 100 μL of PBS delivered via a lateral tail vein. Three days later, the mice were sacrificed by cervical dislocation.

Hybridization. The spleen of each mouse was freed of connective tissue and was placed into a Petri dish containing serum-free RPMI medium. The spleen was cut into several small pieces and was gently forced through a 400-mesh stainless steel screen into a second Petri dish that also contained RPMI medium. The cell suspension was transferred to a sterile centrifuge tube, and any large tissue aggregates were removed by the sedimentation procedure described by Shortman et al. (1972). The suspension was centrifuged (200*g*) for 10 min, and the cell pellet was resuspended in fresh medium. Cells in trypan blue viability stain were enumerated microscopically. The spleen cells were mixed with an equal number of SP/2.0 myeloma cells in the semilog growth phase in RPMI medium. The cell mixture was centrifuged (200*g*) for 10 min, and the cell pellet was suspended in 1 mL of poly(ethylene glycol) (3000–4000 molecular weight range) at 37 °C. The suspension was mixed continuously for 1 min, followed by the addition of 1 mL of RPMI medium and another 1 min of continuous mixing. An additional 9 mL of RPMI medium was added slowly with mixing. The fusion products were centrifuged at 200*g* for 10 min, the supernatant was discarded, and the cell pellet was resuspended in RPMI medium supplemented with 10% fetal bovine serum, 10% NCTC-109 medium, and 1% HAT to obtain selective growth of the hybrid cells (Zola, 1987). The cell suspension was dispensed (100 μL/well) into six sterile 96-well microtitration plates. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 in air. The fusion procedure was repeated with the second mouse. Four days following the fusion, the cell cultures in the microtitration plates were resupplied with medium by removing 100 μL of supernatant from each well and replacing it with 100 μL of fresh medium. This was repeated daily for 3 days.

Fusion Product Screening. Ten days after the fusion, the cell culture supernatants were screened for the presence of mouse IgG antibodies. Microtitration plates were coated with goat anti-mouse IgG (5 μg/mL, 100 μL/well) diluted in PBS and incubated for 2 h at 37 °C. The plates were washed three times with Tris/Tween (0.02 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.4). The harvested culture supernatant (100 μL) was transferred to the coated plates followed by a further 1-h incubation at 37 °C. After the plates were washed with Tris/Tween, goat anti-mouse IgG–alkaline phosphatase conjugate (Zymed Laboratories Inc., South San Fransisco, CA) diluted 1:750 in PBS was added and the plates were incubated for 1 h at 37 °C. The plates were washed as before with Tris/Tween, and substrate was added (Sigma 104 phosphatase substrate, 1 mg of disodium *p*-nitrophenyl phosphate per milliliter of 1% diethanolamine buffer, pH 9.8). The color reaction was allowed to proceed for 30 min, after which time it was stopped with 50 μL/well of 2 N NaOH. Absorbance at 405 nm was then determined with a microplate reader. Of 12 plates, most wells were positive for mouse IgG.

The aforementioned process for detection of IgG in the supernatants was repeated for specific anti-picloram activity by substituting a picloram–RSA coating conjugate (RC4) for goat anti-mouse IgG in the coating step. RC4 had peptide linkage between picloram and the protein molecule. From this assessment, 385 wells from the 12 plates showed a strongly positive anti-picloram activity ($A_{405} > 1.00$). A double screen was conducted on those cultures showing a strongly positive anti-picloram response whereby the culture supernatants were tested for activity against RSA and RC4 coating conjugate in separate sets of plates. Only one culture showed cross-reactivity for RSA, and 171 cultures retained a strong anti-RC4 activity. Therefore, of the 171 cultures, 37 were selected to be transferred to 24-well culture plates for further proliferation. The remaining cultures that tested strongly positive against RC4 coating conjugate in both screens (134 cultures) were transferred to sterile 96-well plates to be held in reserve.

After time was allowed for the cell cultures in the 24-well culture plates to grow, the EIA assessment was repeated on the culture supernatants. Three cultures were found to be no longer producing antibodies, and these were discarded. The remaining cultures were transferred to 25-cm^2 flasks. Subsequent assessment assays in which attempts were made to competitively inhibit binding of the antibodies in the culture supernatant to RC4 coating conjugate with free picloram were unsuccessful. It was postulated that the cultures selected up to this time contained antibodies specific for the peptide link between picloram and RSA in the coating conjugate, as no activity was shown against either RSA or picloram alone, although the activity against the coating conjugate was strong. Assessments of the same cultures using a new picloram–RSA coating conjugate (RC5) with primarily ester linkages revealed only one culture with specific anti-picloram activity. Four more cultures containing antibodies specific for picloram were found among the 134 cultures that were held in reserve in 96-well plates. These cultures were screened for cross-reactivity with three other pyridine herbicides, clopyralid, fluroxypyr, and trichlopyr. None of the cultures showed appreciable cross-reactivity with the other pyridine herbicides. Throughout the assessment process, the cultures were gradually depleted of HAT in the medium by resupplying the cultures with medium containing successively lower amounts of hypoxanthine, aminopterin, and thymidine.

Limiting Dilution. The culture showing the best results from the EIA assessment was selected for the limiting dilution procedure to achieve the clonality of the hybridoma cells. The cells were counted in trypan blue viability stain. On the basis of this figure, dilution factors were calculated to yield 10, 5, and 1 cell/

100 μL of solution. By use of a multichannel pipet, 100 μL/well of dilution calculated to yield 1 cell/well was added to 8 columns of a 96-well microtitration plate. Three columns received the dilution calculated to yield 5 cells/well and the last column was given the dilution calculated to yield 10 cells/well. The wells of the plate were checked daily for the presence of a single colony. Once a colony was visible, it was fed with 125 μL of RPMI medium. Supernatant (125 μL) was removed from the well for screening by EIA when the cells of the colony were one-fourth to half confluent. Cells from colonies testing positive for anti-picloram antibody activity were transferred to 24-well plates, rescreened by EIA, and transferred again into 25-cm^2 flasks if they remained positive for picloram antibodies. The limiting dilution procedure was repeated to ensure monoclonality. After a final assessment by EIA, the cells producing the monoclonal antibodies specific for picloram were collected for the production of ascitic fluid in mice.

Ascites Fluid Production. Mice were given an injection of 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane), a hybridoma growth promoting compound. Seven days later, the mice were injected with 3×10^6 hybridoma cells in 200 μL of PBS supplemented with 5% fetal bovine serum. Approximately 2 weeks following the injection of cells, ascites fluid was withdrawn, centrifuged to remove red blood cells, and frozen at −20 °C until used.

Sample Preparation. Water was collected from the Speed River, Guelph, ON, and stored at 4 °C. The water was fortified with an acetone solution of picloram. Soil (40 g) was shaken for 15 min with 200 mL of a 1:1 methanol/water solution. The mixture was filtered through a glass fiber filter, and the methanol was removed under vacuum at 50 °C. The volume of the resulting aqueous solution was returned to 100 mL with P_i buffer (0.1 M phosphate, 1 mM $MgCl_2$, pH 7.5) and filtered through a 0.45-μm nylon filter. The filtered extract solution was fortified with an acetone solution of picloram. Grass clippings (20 g) were homogenized in 100 mL of 0.1 N KOH with 10% KCl. The homogenate was shaken for 30 min and filtered through a glass fiber filter. The filtrate was acidified to pH 2 with 3 N H_2SO_4, refrigerated at 4 °C for 30 min, and centrifuged at 3000*g* for 10 min. The volume of the supernatant was made up to 100 mL with P_i buffer, and aliquots were fortified with an acetone solution of picloram. Prior to analysis, 10.00 mL of the fortified solution was forced through a C_{18} reversed-phase liquid chromatography column. The column was washed with 5 mL of water and dried with a gentle stream of forced air for 1 min. The column was eluted with 9 mL of methanol. The eluate was evaporated to dryness, and the residue was redissolved in 10.00 mL of P_i buffer. Human urine was fortified with picloram, and 10-mL aliquots were acidified to pH 2 with 3 N H_2SO_4. The picloram was extracted three times with 3-mL portions of diethyl ether. The ether fractions were pooled and evaporated to dryness. The residue was redissolved in 10 mL of P_i buffer and centrifuged at 12000*g* for 10 min.

Recoveries for the extractions described above were determined by using [^{14}C]picloram added to soil, grass clippings, and urine. Recoveries were 95% for the soil extraction, 90% for the plant extraction, and 90% for the urine extraction.

Indirect Enzyme Immunoassay. The following procedure is a modified version of that described by Hall et al. (1989).

Microtitration plates were coated by adding to each well 200 μL of RC5 coating conjugate dissolved in P_i buffer (0.1 μg of coating antigen/mL). The plates were incubated overnight at 4 °C. The plates were emptied and washed three times with washing solution (P_i buffer supplemented with 0.1% Tween 20). If the plates were not to be used immediately, they were wrapped with plastic and stored at 4 °C for up to 24 h.

Sites on the polystyrene well surface unoccupied by coating conjugate were blocked by adding 200 μL of 0.1% (w/v) gelatin solution in P_i buffer and incubated for 20 min at 4 °C. The plates were emptied and washed as described above.

Antisera diluted 1:20 000 or ascites fluid diluted 1:10 000 in P_i buffer supplemented with 0.05% Tween 20 surfactant (Hunter and Lenz, 1982) were preincubated 1:1 (v/v) with picloram standard or sample solutions. Aliquots of the preincubated mixture were transferred to the wells of the microtitration plate (200 μL/well). One column in each plate received P_i buffer (200 μL/well) to determine nonspecific binding of the antibody-labeled horseradish peroxidase enzyme in the following step. The plates were incubated for 1 h at 4 °C.

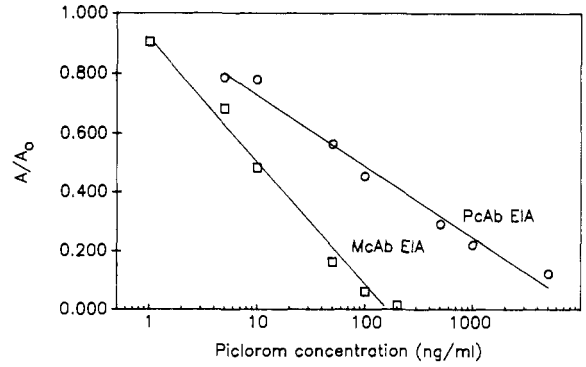

Figure 1. Standard curves for monoclonal antibody based enzyme immunoassay (McAb EIA) and polyclonal antiserum based enzyme immunoassay (PcAb EIA) for picloram determination.

After the plates were washed as before, 200 μL of goat anti-rabbit or goat anti-mouse IgG–horseradish peroxidase conjugate diluted 1:5000 in P_i buffer was added to each well, and the plates were incubated for 1 h at 4 °C, emptied, and washed.

Substrate [1 mg/mL 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium; 1 mg/mL urea hydrogen peroxide in citrate buffer, 0.024 M citrate, 0.047 M phosphate, pH 5.0] was added and color was allowed to develop for 30 min. The color reaction was stopped by the addition of 100 μL of 0.5 M citric acid. Absorbance of each well was measured at 405 nm with a microtiter plate reader. All absorbance values were corrected for nonspecific binding of the antibody-labeled enzyme. Absorbance values of the standards and the samples (A) were divided by the maximum absorbance value (A_0) representing those wells in which binding of antibody to the coating conjugate was not challenged with free picloram in solution. The A/A_0 values for standards were plotted against the log of picloram concentration to construct a standard curve. Concentrations of samples were calculated on the basis of the standard curve.

RESULTS

Enzyme Immunoassay Standard Curves. Picloram standards in P_i buffer were used to generate standard curves for both immunoassays. A linear relation between the log of picloram concentration and relative absorbance (A/A_0) was found in the range 5–5000 ng/mL for the polyclonal assay and 1–200 ng/mL for the monoclonal assay (Figure 1). The monoclonal assay, therefore, had a standard curve with a much steeper slope compared to the polyclonal assay. Typical coefficient of determination values (r^2) were 0.97 for the monoclonal assay and 0.95 for the polyclonal assay.

Assay Sensitivity. The polyclonal assay had a mean I_{50} value of 140 ng/mL with a lower detection limit of 5 ng/mL. The monoclonal assay was more sensitive with a mean I_{50} value of 10 ng/mL and a lower detection limit of 1 ng/mL. Both assays were more sensitive than the RIA for picloram reported by Hall et al. (1989), which had an I_{50} value of 760 ng/mL and a lower detection limit of 50 ng/mL.

Assay Precision. By use of the absorbance values in 12 separate wells for each of the picloram standards, the interwell variability was determined for the two EIA procedures (Table I). The polyclonal assay showed a mean interwell coefficient of variation (CV) of 6.4% over the standard curve. The mean interwell CV over the standard curve for the monoclonal assay was slightly lower at 5.3%. Interassay CV of the picloram standard A/A_0 values determined on four separate runs for the polyclonal assay

Table I. Interwell Variability of Indirect Enzyme Immunoassay Standard Curve Using Polyclonal or Monoclonal Antibodies

antibody	picloram std, ng/mL	absorbance ± SE	CV, %
polyclonal	5	0.715 ± 0.007	3.2
	10	0.676 ± 0.010	4.9
	50	0.606 ± 0.006	3.3
	100	0.580 ± 0.011	6.6
	500	0.505 ± 0.014	9.4
	1000	0.472 ± 0.011	7.8
	5000	0.465 ± 0.013	10
monoclonal	1	0.797 ± 0.010	4.2
	5	0.637 ± 0.006	3.4
	10	0.484 ± 0.007	5.4
	50	0.261 ± 0.006	7.5
	100	0.185 ± 0.003	5.2
	200	0.154 ± 0.003	5.9

Table II. Interassay Variability of Indirect Enzyme Immunoassay Standard Curve Using Polyclonal or Monoclonal Antibodies

antibody	picloram std, ng/mL	A/A_0[a] mean	SE	CV, %
polyclonal	5	0.797	0.010	2.1
	10	0.768	0.024	6.1
	50	0.565	0.029	10
	100	0.482	0.023	9.5
	500	0.342	0.027	16
	1000	0.250	0.029	23
	5000	0.167	0.022	23
monoclonal	1	0.916	0.018	5.1
	5	0.685	0.016	6.0
	10	0.512	0.016	8.5
	50	0.178	0.012	17
	100	0.095	0.009	23
	200	0.034	0.010	26

[a] A/A_0, absorbance of standard/maximum absorbance (i.e., concentration of picloram equals 0).

Table III. Intraassay Variability of Picloram in Four Fortified Plant Extract Samples from Enzyme Immunoassay Standard Curve Using Polyclonal or Monoclonal Antibodies

picloram added, ng/mL	picloram recovered: PcAb EIA[a] mean, ng/mL	CV, %	McAb EIA[b] mean, ng/mL	CV, %
4	9.9	87	3.9	29
20	39	89	24	15
40	99	84	52	21
400	780	59	450	10

[a] Polyclonal antibody enzyme immunoassay. [b] Monoclonal antibody enzyme immunoassay.

ranged from 2.1% to 23% with a mean of 12.8% (Table II). For the monoclonal assay, the interassay CV of A/A_0 values determined on seven separate occasions ranged from 5.1% to 26% with a mean of 16%. In both cases, CV values increased with an increase in picloram standard concentration due to decreasing A/A_0 values. Singh et al. (1989) showed similar results for their enzyme immunoassay for the antibiotic sulfamethazine. Absorbance values representing nonspecific binding showed well to well coefficient of variation of 8% and 5% for the monoclonal and polyclonal assays, respectively. Plate to plate CV values for nonspecific binding were 9% for the monoclonal assay and 8% for the polyclonal assay. Intraassay CV values were obtained on picloram determinations in four fortified plant extract samples (Table III). The polyclonal assay showed a much higher variability with a mean CV value of 80% over the four plant extract samples compared to only 19% for the monoclonal assay over the same samples.

Table IV. Recovery of Picloram from Fortified Water Samples Determined by Enzyme Immunoassay Using Polyclonal or Monoclonal Antibodies

picloram added, ng/mL	picloram recovered,[a] ng/mL: PcAb EIA[b]	McAb EIA[c]
20	27 ± 5.1 (18)	11 ± 0.98 (12)
200	569 ± 79 (18)	165 ± 7.4 (12)
2000	3590 ± 550 (18)	1920 ± 80 (12)

[a] Mean ± SE (number of determinations). [b] Polyclonal antibody enzyme immunoassay. [c] Monoclonal antibody enzyme immunoassay.

Table V. Recovery of Picloram from Fortified Soil Extract Samples Determined by Enzyme Immunoassay Using Polyclonal or Monoclonal Antibodies

picloram added, ng/mL	picloram recovered,[a] ng/mL: PcAb EIA[b]	McAb EIA[c]
4	23 ± 4.4 (24)	2.1 ± 0.15 (36)
20	90 ± 23 (24)	13 ± 0.53 (36)
40	110 ± 23 (24)	33 ± 1.1 (36)
400	1010 ± 280 (24)	480 ± 12 (12)

[a] Mean ± SE (number of determinations). [b] Polyclonal antibody enzyme immunoassay. [c] Monoclonal antibody enzyme immunoassay.

Table VI. Recovery of Picloram from Fortified Plant Extract Samples Determined by Enzyme Immunoassay Using Polyclonal or Monoclonal Antibodies

picloram added, ng/mL	picloram recovered,[a] ng/mL: PcAb EIA[b]	McAb EIA[c]
4	9.9 ± 2.5 (12)	3.5 ± 0.23 (24)
20	39 ± 10 (12)	24 ± 1.0 (24)
40	99 ± 24 (12)	51 ± 2.4 (24)
400	780 ± 130 (12)	450 ± 13 (24)

[a] Mean ± SE (number of determinations). [b] Polyclonal antibody enzyme immunoassay. [c] Monoclonal antibody enzyme immunoassay.

Specificity of the Antibodies. Three structurally related pyridine herbicides, clopyralid (3,6-dichloro-2-pyridinecarboxylic acid), fluroxypyr [[(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid], and triclopyr [[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid], were tested for cross-reactivity with the polyclonal and monoclonal anti-picloram antibodies. Neither antibody cross-reacted appreciably with the other pyridine herbicides as the I_{50} values in all cases were greater than the highest concentration of herbicide tested (50 000 ng/mL for the polyclonal antibody, 10 000 ng/mL for the monoclonal antibody). In addition, the monoclonal anti-picloram antibody showed no cross-reactivity to 2,4-dichlorophenoxyacetic acid (2,4-D) up to a concentration of 20 000 ng/mL. We have previously shown that the polyclonal anti-picloram antiserum also has no specificity for 2,4-D (Hall et al., 1989).

Determination of Picloram in Water, Soil Extract, Plant Extract, and Urine Samples. Recovery of picloram from water (Table IV), soil extracts (Table V), plant extracts (Table VI), and urine (Table VII) indicated that only the monoclonal assay was suitable for quantitative determinations. Overall recoveries for the monoclonal assay were 78%, 73%, 112%, and 167% for water, soil extract, plant extract, and urine, respectively. For the polyclonal assay, overall recoveries were 200%, 388% and 221% for water, soil extract, and plant extract, respectively. The polyclonal assay for determination of picloram in urine was not successful because of extreme interference from an

Table VII. Recovery of Picloram from Fortified Human Urine Samples Determined by Enzyme Immunoassay Using a Monoclonal Antibody[a]

picloram added, ng/mL	picloram recovered,[a] ng/mL, by McAb EIA[c]
4	11 ± 0.58 (12)
20	30 ± 2.2 (12)
40	50 ± 2.1 (12)
400	450 ± 12 (12)

[a] Polyclonal assay was not successful due to unknown contaminant. [b] Mean ± SE (number of determinations). [c] Monoclonal antibody enzyme immunoassay.

unknown contaminant. Picloram concentration estimates were taken from a standard curve made in P_i buffer. Interference from components of the sample matrix account for the systematic error in the concentration estimates. Such interferences from sample components have been reported by Wie and Hammock (1982).

DISCUSSION

For accuracy of quantitation, a standard curve with a steep slope is desirable. The monoclonal assay had a standard curve with a much steeper slope than the polyclonal assay (Figure 1), and the advantage gained is illustrated by the mean overall recovery of picloram from various fortified samples: 108% for the monoclonal assay compared to 274% for the polyclonal assay (Tables IV–VII). The high variability of the picloram determinations from the polyclonal assay (Table III) can also be attributed to the relatively flat standard curve.

A certain amount of the error in the picloram concentration determinations from both assays is systematic in nature and is due to the fact that the matrix of the fortified samples and the picloram standard matrix (P_i buffer) differed in chemical composition. In preliminary studies, we have found that the ionic strength of the matrix solution and possibly the presence of organic coextractives (e.g., phenols) influence the amount of sample matrix interference. These effects were minimized if the antibodies were diluted in P_i buffer supplemented with 0.05% (v/v) Tween 20 as described by Hunter and Lenz (1982). In our previous study (Hall et al., 1989), the RIA procedure for picloram showed a high degree of accuracy (82–110% recovery) when the standards were prepared in picloram-free river water or urine obtained from the same source as the fortified samples. Singh et al. (1989) used swine plasma as the reference matrix in their EIA to determine the antibiotic sulfamethazine in a swine plasma sample matrix with excellent accuracy. For the present study, we chose to prepare standards in phosphate buffer rather than in picloram-free extracts for the following reason. Soil or water samples from different geographical regions or urine samples from different subjects will vary widely in composition. The analyst has two options: (i) to attempt to categorize the samples (e.g., by soil type) and prepare a separate standard curve for each category or (ii) to pool various blank samples and prepare a composite reference matrix. We believed that our choice to simply prepare the standards in buffer was more feasible. Upon examination of the recoveries of picloram from the various samples matrices (Tables IV–VII), it is evident that this choice had more severe consequences with respect to the polyclonal antibody based assay than with the monoclonal antibody based assay. The advantage of a standard curve with a steep slope is that small errors in absorbance values will not translate to large errors in concentration estimates.

One disadvantage of a standard curve with a steep slope is the narrow linear working range (Figure 1). Rather than making several dilutions of a sample in the hope of obtaining one dilution in the proper range, it may be more efficient to conduct a separate assay with a wide working range to rank samples so that appropriate dilutions can be made with certainty for accurate quantitation by a second assay. The polyclonal system described here would be adequate for the role of ranking samples. Alternatively, one could modify the parameters of the monoclonal assay (e.g., increase the antibody concentration) to achieve a standard curve with a flatter slope and a wider working range.

It is commonly stated that for polyclonal antibody production the design and the preparation of the immunogen are most critical (Hammock et al., 1987; Hammock and Mumma, 1980; Jung et al., 1989). Several studies have illustrated the influence of hapten structure, bridging groups, immunogen structure, and coating conjugate structure on immunoassay performance (Vallejo et al., 1982; Wie and Hammock, 1984; Wie et al., 1982). The goal of immunogen design and preparation is to maximize the quantity of specific antibodies in the antisera having high affinity for the antigen (analyte). In general, one advantage of the monoclonal technique over polyclonal antibody production is that the antigen does not have to be pure for the purposes of immunization as long as, during the screening process, high-affinity antibodies specific for the antigen of interest can be distinguished from low-affinity antibodies and those that are specific for irrelevant antigens. Likewise, the design of the immunogen used to produce monoclonal antibodies may not be as critical as that required for polyclonal antibody production. An effective screening program will enable the investigator to select and expand the hybridoma cell clone(s) producing the desired antibody, even if such clones are rare. In the present study, the same immunogen that yielded a polyclonal antisera with a low average affinity also yielded a monoclonal antibody of high affinity.

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