

# Development of Enzyme Immunoassay for the Detection of the Herbicide Norflurazon

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An enzyme-linked immunoassay (ELISA) was developed for the herbicide norflurazon [4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]-3(2*H*)-pyridazinone]. Antisera were obtained from two rabbits that had been immunized with a conjugate of butyric acid norflurazon [4-chloro-5-(aminobutyric acid)-2-[3-(trifluoromethyl)phenyl]-3(2*H*)-pyridazinone] and bovine serum albumin. The metabolite, desmethyl norflurazon [4-chloro-5-(amino)-2-[3-(trifluoromethyl)phenyl]-3(2*H*)-pyridazinone] was 22 and 48% cross-reactive for rabbit 1 and rabbit 2 antisera, respectively. The experimental herbicide metflurazon [4-chloro-5-(dimethylamino)-2-[3-(trifluoromethyl)phenyl]-3(2*H*)-pyridazinone] was 37 and 42% cross-reactive for rabbit 1 and rabbit 2 antisera, respectively. Two other phenylpyridazinone-based compounds, pyrazon [5-amino-4-chloro-2-phenyl-3(2*H*)-pyridazinone] and 4,5-dichloro-2-phenyl-3(2*H*)-pyridazinone, were less than 3% cross-reactive for both antisera. The assay was tested with water samples that had been spiked with either norflurazon or one of the above-mentioned compounds. The detection limits for norflurazon and desmethyl norflurazon were 1 and 10 ng/mL, respectively. Seven non-phenylpyridazinone-based pesticides were assayed, and none were cross-reactive with either antisera.

## INTRODUCTION

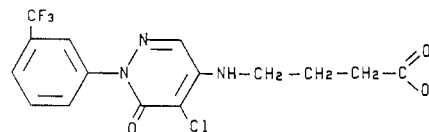
Immunoassays have been developed for a number of pesticides which include endosulfan (Dreher and Podratzki, 1988), metalaxyl (Newsome, 1985), chlorsulfuron (Kelly et al., 1985), 2,4-D (Rinder and Fleeker, 1981), diclofop-methyl (Schwalbe et al., 1984), molinate (Gee et al., 1988), and atrazine (Bushway et al., 1988; Dunbar et al., 1990; Huber, 1985). Currently, no immunoassay has been developed for the herbicide norflurazon or any other phenylpyridazinone-based compound. Norflurazon has been used to control a number of sedges, grasses, and broadleaf weeds in cotton, cranberry, and fruit and nut crop production. Because of increasing demands by federal and state regulatory agencies, there is a growing need to expand all available options for residue analysis. Residue of norflurazon and its associated metabolite, desmethyl norflurazon (Strang and Rogers, 1974; Rahn and Zimdahl, 1973) (part of the tolerance definition for norflurazon [40CFR§180.356]), is typically analyzed by either gas chromatography (GC) using electron capture (Winkler et al., 1981) or high-performance liquid chromatography (HPLC) (Draper and Street, 1981). While GC and HPLC methods can be accurate, they can also be time-consuming and expensive. As a consequence, there is a demand for more cost-effective methods that are rapid, dependable, and efficient. This demand could be partially filled by the use of enzyme-linked immunoassays. The purpose of this study was to develop an enzyme-linked immunoassay for the detection of norflurazon.

## MATERIALS AND METHODS

**Reagents and Equipment.** Norflurazon and desmethyl norflurazon were provided by Sandoz Inc. (Palo Alto, CA). Metflurazon was provided by Prof. Paul Bartels (University of

Arizona). Bovine serum albumin (BSA), rabbit serum albumin (RSA), Tween 20 (TW), 2,2'-azino-bis(3-ethylbenzthiazoline sulfonic acid) diammonium salt (ABTS), dimethylformamide (DMF), and Freund's complete adjuvant were obtained from Sigma Chemical Co. (St. Louis, MO). Biotinylated goat anti-rabbit IgG and horseradish peroxidase-streptavidin conjugate were obtained from Amersham Corp. (Arlington Heights, IL). All other chemicals were of reagent grade or better and were used as obtained. Buffers were prepared as follows: carbonate/bicarbonate buffer solution, Na<sub>2</sub>CO<sub>3</sub> (1.59 g), NaHCO<sub>3</sub> (2.93 g), and distilled water (1 L); phosphate buffer saline solution (PBS), NaCl (8 g), KCl (0.2 g), Na<sub>2</sub>HPO<sub>4</sub> (1.15 g), KH<sub>2</sub>PO<sub>4</sub> (0.2 g), CaCl<sub>2</sub> (0.1 g), MgCl<sub>2</sub> (0.1 g), and distilled water (1 L); citrate/phosphate buffer, 27.8 mL of citrate buffer [citrate (960 mg) and distilled water (50 mL)], 22.2 mL of phosphate buffer [Na<sub>2</sub>HPO<sub>4</sub> (2.68 g) and distilled water (50 mL)], and 50 mL of distilled water. All stocks and standard solutions were stored at 4 °C. ELISA plates (Immulon 2) were obtained from Dynatech (Torrance, CA). Plate absorption readings were made with a Dynatech Model MR600 microplate reader.

**Hapten Preparation.** The hapten was 4-chloro-5-(aminobutyric acid)-2-[3-(trifluoromethyl)phenyl]-3(2*H*)-pyridazinone and is referred to, in portions of the text, as butyric acid norflurazon. The hapten was prepared in the following manner.



Butyric Acid Norflurazon

Mucochloric acid (2,3-dichloro-4-oxo-2-butenic acid) in ethanol was reacted with [(trifluoromethyl)phenyl]hydrazine and then dehydrated and refluxed in a 50% mixture of acetic anhydride and acetic acid to form 4,5-dichloro-2-[3-(trifluoromethyl)phenyl]-3(2*H*)-pyridazinone. This was added at 308 mg (1 mmol) to 30 mL of anhydrous methanol, 103 mg (1 mmol) of 4-aminobutyric acid, and 720 μL (6 mmol) of triethylamine. This

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mixture was refluxed and stirred for 30 h. The solution was rotoevaporated to dryness at reduced pressure. The residue was stirred with 60 mL of ethyl acetate and washed with 15 mL of 5% HCl plus 40 mL of distilled water in a separatory funnel. The acidic material was extracted with 15 mL of 1 N NaOH, and the basic solution was separated and brought to pH 3 and stirred. The resulting cloudy mixture was extracted with 50 mL of ethyl acetate, dried, and separated from the solvent at reduced pressure. The resulting oil was stirred into boiling benzene, which initiated crystallization. The hot solution was then added to hexane and cooled. Crystallization was allowed to continue overnight. The solution was dried, which resulted in 250 mg of white crystals. Thin-layer chromatography was done with a silicon gel plate and developed in chloroform/methanol/water (24:7:1). A single spot with  $R_f$  0.64 was found on the plate by UV absorption.

**Conjugate Preparation.** The hapten was conjugated to BSA and RSA by using a mixed-anhydride method as outlined below. A portion of the hapten (37.5 mg) was dissolved in dry DMF (1.4 mL) and tributylamine (48  $\mu$ L) (0.2 mM). The solution was cooled to 5 °C in a stopped tube. Isobutyl chloroformate (13  $\mu$ L) (0.1 mM) was added to the mixture, and the reaction was allowed to proceed in an ice bath for 30 min. The resultant mixture was stirred into an ice-cooled solution of BSA or RSA (100 mg), distilled water (1 mL), 1 N NaOH (100  $\mu$ L), and DMF (2 mL). The mixture was stirred for 30 min, and then 1 N NaOH (180  $\mu$ L) was added with the pH maintained at 8 throughout the reaction. After sitting for 12 h, the mixture was dialyzed and the pH was adjusted to 4.5. The resulting precipitate was allowed to stand at 4 °C for 4 h and was then separated from the liquid phase by centrifugation at 500g and washed with cold acetone. The resulting product was suspended in water and redissolved by the addition of 0.1 N NaOH for a final pH of 7.8. The solution was dialyzed for 8 h and lyophilized with a final yield of 100 mg.

**Antibody Preparation.** Three New Zealand rabbits were inoculated intradermally with 500  $\mu$ g of the BSA-conjugated hapten and complete Freund's adjuvant, boosted at monthly intervals, and bled on a monthly basis after the second injection. Approximately 30 mL of whole blood per animal was collected and centrifuged to separate blood cells from immune serum. Antisera were tested to find the optimum titer. Cross-reactivity tests were done with antisera from two rabbits collected 6 months after inoculation.

**Enzyme-Linked Immunosorbent Assay.** All reactions were carried out in polystyrene microtiter plates. The assay was performed as follows:

(1) The hapten/RSA conjugate (900 mg) was sonicated in DMF (1 mL) for 1 min and then added to carbonate/bicarbonate buffer solution at pH 9.5 (50 mL). The solution was then added to the wells (200  $\mu$ L).

(2) The microtiter plate was covered with parafilm and incubated overnight at 4 °C.

(3) Wells were washed two times with PBS that contained Tween 20 (TW). Sites not occupied by hapten/RSA conjugate were blocked by 1% gelatin in PBS (900  $\mu$ L) and incubated at 20 °C for 2 h.

(4) The plate was washed two times with PBS/TW.

(5) Diluted rabbit antiserum (dilution established from titer determination) (100  $\mu$ L/well) and sample (100  $\mu$ L/well) were added and incubated together at 37 °C for 45 min.

(6) The plate was washed two times with PBS/TW.

(7) Biotinylated goat anti-rabbit IgG at a 1:1000 dilution in PBS/TW was added (100  $\mu$ L/well) and incubated for 2 h at 37 °C.

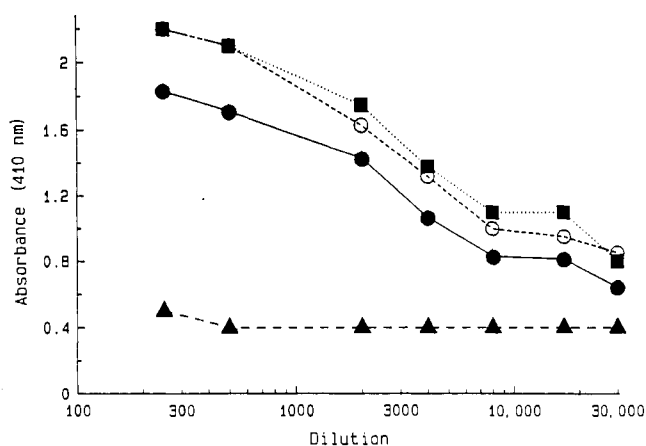
(8) The plate was washed two times with PBS/TW.

(9) Horseradish peroxidase-streptavidin conjugate at a 1:1000 dilution in PBS/TW was added (100  $\mu$ L/well) and incubated for 2 h at 37 °C.

(10) The plate was washed two times with PBS/TW.

(11) Substrate of ABTS (100 mg) in 30 mM citrate/phosphate buffer of pH 4.4 (21 mL) and 30% H<sub>2</sub>O<sub>2</sub> (2  $\mu$ L) was added (100  $\mu$ L/well) and incubated at room temperature in the dark for 20 min.

(12) The absorbance at 410 nm was read.



**Figure 1.** Titer assays for rabbit 1 antiserum (●), rabbit 2 antiserum (○), rabbit 3 antiserum (■), and preimmunization sera (▲).

Titer determinations were done to find the optimum concentration of antisera. A decreasing concentration of antisera from 1:250 to 1:65 000 (100  $\mu$ L) was added to wells (step 5 of the assay, excluding the addition of a test sample). The optimum concentration was that which gave a midpoint absorbance reading. See Figure 1 for absorbance range.

**Preparation of Standard Curves.** Standard curves were prepared for norflurazon, metflurazon, desmethyl norflurazon, pyrazon, and 4,5-dichloro-2-phenyl-3(2H)-pyridazinone (referred to in the text as dichlorophenylpyridazine) by using antisera from rabbits 1 and 2. Compounds were assayed at dilutions of 0.1, 1, 10, 100, 1000, and 10 000 ng/mL of distilled water. Percent inhibition was calculated by subtracting and then dividing the absorbance value of the tested compound by the maximum absorbance value.

**Cross-Reactivity Determinations.** Cross-reactivities were determined for metflurazon, desmethyl norflurazon, pyrazon, and dichlorophenylpyridazine. Cross-reactivity measurements were made by comparing the absorbance values of the tested compounds to those of norflurazon. Cross-reactivity was calculated as follows: [moles of norflurazon at the assay midpoint]/[moles of tested compound at the assay midpoint]  $\times$  100 = % cross-reactivity. The midpoint was the concentration that produced 50% of the observed absorbance reading.

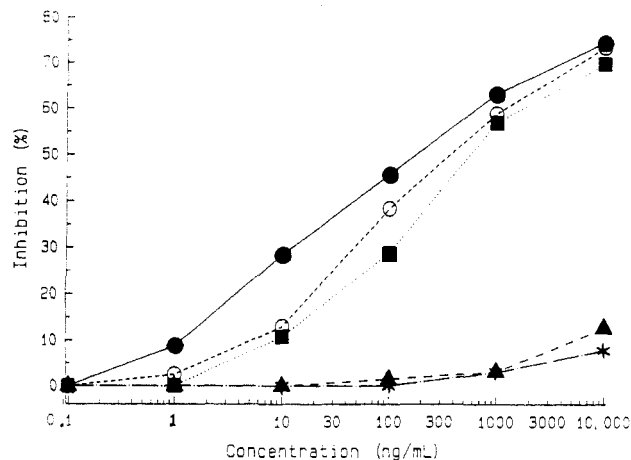
In addition, atrazine (6-chloro-N-ethyl-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine), paraquat (1,1'-dimethyl-4,4'-bipyridinium ion), trifluralin (2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine), glyphosphate [N-(phosphonomethyl)glycine], alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamine], cinmethylin [*exo*( $\pm$ )-1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-7-oxabicyclo[2.2.1]heptane], and fluzafop [( $\pm$ )-2-[4-[[5-trifluoromethyl]-2-pyridinyl]oxy]phenoxy]propanoic acid) were assayed.

**Replications.** Samples were replicated four times per plate with two plates. Absorbance values had coefficients of variation of less than 5%.

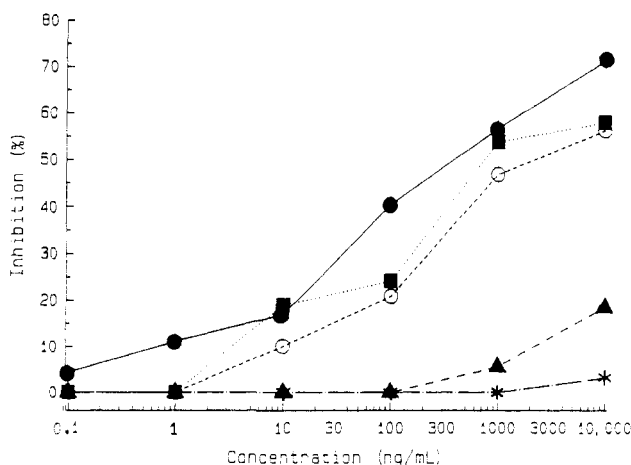
## RESULTS AND DISCUSSION

**Titer Response.** Antibody binding capability to the butyric acid norflurazon-RSA conjugate was demonstrated (Figure 1). Antisera from all three rabbits had similar patterns for the hapten-RSA conjugate with a serial dilution of approximately 1:4000. The curve for preimmune serum (Figure 1), which had been collected prior to inoculation, was identical for all three rabbits and showed no antibody response. Low absorbance values for pre-immune sera demonstrated that no norflurazon-specific antibodies were present at the time of immunization.

**Standard Curve Results.** Standard curves for percent inhibition effects of norflurazon, meflurazon, desmethyl norflurazon, pyrazon, and dichlorophenylpyridazine are presented in Figures 2 and 3. As determined from the



**Figure 2.** Rabbit 1 serum percent inhibition results for norflurazon (●), metflurazon (○), desmethyl norflurazon (■), pyrazon (▲), and dichlorophenylpyridazone (\*).



**Figure 3.** Rabbit 2 serum percent inhibition results for norflurazon (●), metflurazon (○), desmethyl norflurazon (■), pyrazon (▲), and dichlorophenylpyridazone (\*).

curves, the detection limit for norflurazon was 1 ng/mL. This concentration produced approximately 10% inhibition for both antisera. The detection limit for the metabolite, desmethyl norflurazon, was determined to be 10 ng/mL. These results compare favorably to those of a published electron capture GC method and a HPLC method for which detection limits of 10 and 100 ppb (ng/mL), respectively, were reported for combined residues of norflurazon and desmethyl norflurazon (Winkler et al., 1981; Draper and Street, 1981).

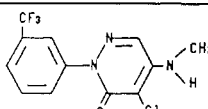
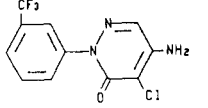
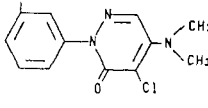
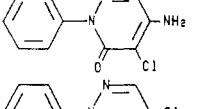
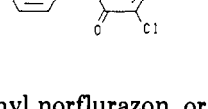
**Cross-Reactivity Results.** RSA and gel were not cross-reactive (data not shown), while BSA was cross-reactive (data not shown) for both antisera. BSA was used to immunize the rabbits, which in turn developed antibodies against BSA.

Only desmethyl norflurazon and metflurazon were 20% or more cross-reactive (Table I). Both pyrazon and dichlorophenylpyridazone were less than 3% cross-reactive. Rabbit 2 antisera had higher cross-reactivity percentages compared to those of rabbit 1. This was especially the case for desmethyl norflurazon.

Atrazine, paraquat, trifluralin, glyphosate, alachlor, cinmethylin, and fluzifop were not cross-reactive at the tested concentrations.

The methylamino portion of norflurazon was a key functional group involved in antibody recognition. The presence of the single methyl group attached to the amino group of norflurazon resulted in a high degree of antibody recognition. The absence of the methyl group, as was the

**Table I. Cross-Reactivity for Phenylpyridazinone Compounds Using Anti-Norflurazon Sera from Two Rabbits**

chemical	structure	% cross-reactivity	
		rabbit 1	rabbit 2
norflurazon		100	100
desmethyl-norflurazon		21.7	48.4
metflurazon		36.6	41.7
pyrazon		1.1	2.6
dichlorophenylpyridazone		0.9	1.7

case with desmethyl norflurazon, or the presence of two methyl groups, as was the case with metflurazon, resulted in reduced antibody recognition. These differences were due to the structure of the hapten. Butyric acid norflurazon had a single butyric acid attached to the amino group. Comparison between the chemical structures shows a closer match-up between butyric acid norflurazon and norflurazon than between butyric acid norflurazon and either metflurazon or desmethyl norflurazon.

The trifluoromethyl group was essential for antibody recognition. This was demonstrated by differences in cross-reactivity for desmethyl norflurazon, pyrazon, and dichlorophenylpyridazone. Desmethyl norflurazon was almost 20 times more cross-reactive than pyrazon, even though the two were identical in structure except for the trifluoromethyl group. Cross-reactivity values for pyrazon were similar to those for dichlorophenylpyridazone. Pyrazon was similar in structure to dichlorophenylpyridazone except for the amine group in place of the chloro group.

In summary, an enzyme-linked immunoassay was developed for the herbicide norflurazon. The assay could detect norflurazon at 1 ng/mL of water. Antisera were cross-reactive with the metabolite, desmethyl norflurazon, and could detect this compound at 10 ng/mL of water.

#### ACKNOWLEDGMENT

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**Registry No.** Norflurazon, 27314-13-2; butyric acid norflurazon, 128526-85-2; mucochloric acid, 87-56-9; [(trifluoromethyl)phenyl]hydrazine, 368-78-5; 4,5-dichloro-2-[3-(trifluoromethyl)phenyl]-3(2H)-pyridazinone, 26806-47-3.