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Determination of Imazamethabenz in Cereal Grain by Enzyme-Linked Immunosorbent Assay

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Imazamethabenz-methyl is a post-emergent herbicide that is registered for use in Canada and the United States on barley and wheat at a maximum residue limit of 0.1 ppm. It consists of a 2:3 mixture of methyl 6-(4-isopropyl-4-methyl-oxo-2-imidazol-2-yl)-m-toluate (1, Fig.1) and p-toluate, and is moderately water soluble (The Pesticide Manual, 1987). It is readily hydrolysed in plants to the free acids. Some aspects of the chemistry of this class of imidazolinone herbicides have been reviewed recently (Wepplo 1990).

Immunochemical methods are frequently found to be effective in providing a rapid and cost-effective alternative to conventional residue analyses (Harrison et al. 1988) and often may be applied directly to crop extracts without prior purification steps. Circumvention of the cleanup is possible when the analyte is relatively polar in nature and the levels to be determined are in the ppm range as, for example in the case of the determination of maleic hydrazide (Harrison and Nelson 1990), triadimefon (Newsome 1986) or metalaxyl (Newsome 1985).

The present enzyme-linked immunosorbent assay (ELISA) was developed to facilitate the determination of the free acids of imazamethabenz-methyl, using as few purification steps as possible. A hydrolysis step was included to convert any methyl ester present to the acid and permit the determination of both compounds as the acid.

MATERIALS AND METHODS

Imazamethabenz, imazamethabenz methyl ester, and the m- (2, Fig 1) and p- hydroxymethyl metabolites (MTA and PTA, respectively) were generously supplied by American Cyanamid, Princeton, NJ. The m- and p- toluic acids, tri-n-butylamine, iso-butyl chloroformate, and dioxane were purchased from the Aldrich Chemical Co., Milwaukee, WI.

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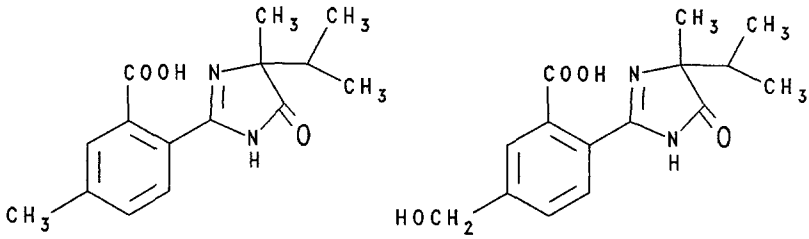

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Figure 1. Structures of the m- isomers of imazamethabenz (1) and its hydroxymethyl metabolite (2).

Bovine serum albumin, ovalbumin, human serum albumin, goat anti-rabbit IgG peroxidase conjugate (second antibody), Tween 20, o-phenylenediamine dihydrochloride, and 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from the Sigma Chemical Co., St Louis, MO. Freund's complete and incomplete adjuvant were purchased from Gibco, Grand Island, NY. C-18 solid phase extraction cartridges (Chromsep) containing 500 mg of adsorbent were obtained from Chromatographic Specialties, Inc. Brockville Ont. and were eluted before use with 10 mL of methanol followed by 10 mL of water and then 5 mL of 0.5 M phosphate buffer, pH 3.5.

Phosphate-buffered saline (PBS) contained 20 mM NaH_2PO_4 and 140 mM NaCl, adjusted to pH 7.2 with NaOH. Antiserum was diluted 1:8000 with 0.1% bovine serum albumin in PBS.

The immunogen was prepared by coupling imazamethabenz to human serum albumin with EDC in 0.05 M phosphate buffer, pH 7.0 as described previously (Newsome and Collins 1989). Coating protein used to sensitize the wells of the microtiter plate was prepared by coupling imazamethabenz to ovalbumin using the mixed anhydride formed by treatment of imazamethabenz with iso-butyl chloroformate in the presence of tri-n-butylamine as described by Newsome and Collins (1989).

Flat-bottomed polystyrene microtiter plates of 300-μL capacity were obtained from Dynatech Laboratories, Inc., Alexandria VA, and were sensitized by adding to each well 180 μL of a solution consisting of 0.24 μg/mL of coating protein and 10 μg/mL ovalbumin in 0.05 M sodium carbonate

buffer pH 9.6. The plates were incubated overnight at 4°C, washed with Tween in PBS, then water, and stored in plastic bags at -20°C. The plates were washed between reagent additions with a Titertek Microplate washer model 120, using 0.1% Tween 20 in (PBS).

Antiserum was obtained from New Zealand White rabbits immunized by subcutaneous injection with a 1:1 emulsion of immunogen in Freund's complete adjuvant, followed by injections at monthly intervals with emulsions in incomplete adjuvant. Each animal received 0.5 mL of a 1 mg/mL-dose administered at five injection sites and was tested for antibody titer and imazamethabenz binding at monthly intervals. After eight months, the animals were exsanguinated under anesthesia, and the serum was prepared and stored at -20°C in 200 μL aliquots.

The samples were extracted by mixing 10 g of pulverized grain with 50 mL of methanol in a 125-mL Erlenmeyer flask using a Kinematika homogenizer. After filtering through Whatman No.1 paper using gentle vacuum on a Buchner funnel, 2 mL of 2 N NaOH were added to the filtrate. The filtrate was transferred to a 250-mL round-bottomed flask. The methanol was removed on a rotary evaporator. The residue was allowed to stand for 90 min to effect hydrolysis of any methyl ester present. The residue was then transferred with 50 mL of distilled water to a 125-mL separatory funnel and extracted with 25 mL of hexane which was discarded. The aqueous layer was transferred to a 250-mL round-bottomed flask, and the flask was evacuated on a rotary evaporator to remove traces of hexane. The pH of the sample was then adjusted to 3.5 with 3 N HCl; the volume was measured, and one-half was added to a C-18 SPE cartridge.

After passage of the sample through the cartridge using gentle vacuum, the stationary phase was eluted with 2 mL of 0.5 N phosphate buffer pH 3.5, which was discarded, then 3 mL of 50% aqueous methanol. Two hundred-microliter aliquots of the latter fraction were placed in 13x100 mm tubes and evaporated to dryness by evacuating them in a desiccator overnight.

For determination by ELISA, the dried samples or 25 μL aliquots of imazamethabenz standard containing 0-32 ng were taken up in 1.0 mL of diluted antiserum and incubated 30 min at 4°C. Aliquots of the equilibrated serum (200 μL) were added to triplicate wells of a sensitized microtiter plate immediately after removal from -20°C storage. The plate was incubated at -4°C for 30 min, the wells were emptied and washed, and 200 μL of a 1:1000 dilution of second antibody was added. After reaction for 30 min at room temperature, the wells were again emptied and washed and 200 μL of substrate

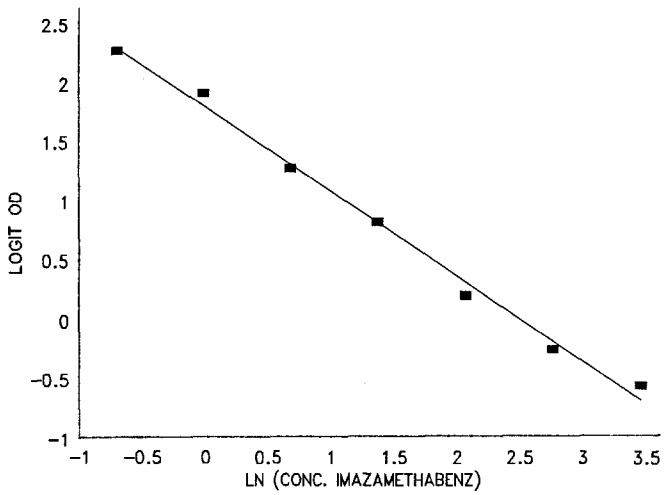

Figure 2. Standard curve for the determination of imazamethabenz. Points are the means of triplicate wells.

consisting of 35 mg of o-phenylenediamine dihydrochloride and 20 μL H_2O_2 in 50 mL citrate buffer pH 5.0 (Voller et al. 1979) added. The enzymic reaction was terminated after 1 h by the addition of 50 μL of 4 M H_2SO_4. The OD of each well was read on a Titertek Multiscan MCC (Flow Laboratories, Inc. Mississauga, Ont.) plate reader and the data transmitted to a spreadsheet for analysis. The amount of imazamethabenz in the samples was calculated from a least squares plot of the logit of the OD against the ln of the concentration of standard.

RESULTS AND DISCUSSION

A typical standard curve for the determination of imazamethabenz at concentrations from 0.5-32 ng/mL is shown in Figure 2.

The mean CV of the data points was 3.0% and the mean IC_{50} (concentration producing 50% inhibition of binding to the plate) for standard curves run on five different days was 12.6 ng/mL (CV 15.2%).

The antiserum was relatively specific for imazamethabenz and the methyl ester as indicated by the data in Table 1.

The methyl ester was bound by the antiserum more readily than imazamethabenz free acid, a phenomenon which was also observed with methyl ester and free acid of 2,4-D (Newsome and Collins 1989) and is probably due to the absence of an ionizable carboxyl group in both the immunogen and ester. The MTA and PTA metabolites were

recognized to a much lesser extent while m- and p- toluic acids did not bind to a significant extent.

Table 1. Comparative binding of imazamethabenz and related compounds in the imazamethabenz ELISA

Compound	IC_{50} (ng/mL)	%[1]
imazamethabenz	13.8	100
imazamethabenz-methyl	2.2	627
MTA metabolite	141	10
PTA metabolite	167	8
m-toluic acid	> 250	0
p-toluic acid	> 250	0

[1] $100(IC_{50}\text{imazamethabenz}/IC_{50}\text{test compound})$

Initial attempts to determine imazamethabenz directly in methanol extracts of wheat or barley (10 g/50 mL) were without success due to interfering coextractives which produced false positive results. The standard curve was not sufficiently sensitive to determine residues at the desired levels, so concentration and purification of the of the extract was effected using C-18 solid phase extraction cartridges.

Satisfactory recoveries of imazamethabenz were obtained from barley or wheat fortified at 12.5-200 ppb, as shown by the data in Table 2. The repeatability of the method was examined by conducting replicate determinations on four samples of barley spiked at 12.5 ppb. Mean recoveries were 89.8% ±12. Four blank samples analyzed concurrently gave a mean value of 2.9 ppb ±1.1.

Table 2. Recoveries of imazamethabenz added to barley and wheat

Imazamethabenz Added (ppb)	Imazamethabenz Found[1] (ppb)	
	Barley	Wheat
0	2.0	0.7
12.5	12.3	8.1
25	21	17
50	41	46
100	78	111
200	158	236

[1] Values are the means of duplicate determinations and have been corrected for the commodity blank.

The addition of alkali to the extract and a hydrolysis period of 90 min was necessary to ensure conversion of any imazamethabenz methyl ester in the sample to the free acid. Samples spiked in duplicate with 50 and 100 ppb of the methyl ester yielded mean recoveries of 84 and 90% respectively of imazamethabenz when carried through the procedure.

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