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A Clomazone Immunoassay to Study the Environmental Fate of the Herbicide in Rice (*Oryza sativa*) Agriculture

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Abstract

The environmental impact of rice agriculture is poorly studied in developing countries, mainly, due to limitations of the analytical capacity. Here we report the development of a clomazone ELISA as a fast and cost-effective tool to monitor the dissipation of this herbicide along the harvest. Antibodies were prepared using different strategies of hapten conjugation, and the best hapten/antibody pair was selected. It proved to be a reliable tool to measure the herbicide in the 2.0-20 ng/mL range in field samples, with excellent correlation with HPLC results. The assay was used to study the dissipation of the herbicide in floodwater of experimental rice paddies in Uruguay. Large differences in the residual amount of herbicide were observed depending on the flooding practices. Due to its robustness and simplicity, the assay may be useful to delineate and monitor management practices that can contribute to minimizing the release of the herbicide in the environment.

Keywords

ELISA; clomazone; rice; HPLC; *Oryza sativa*

Introduction

Agricultural production is one of the key components of the domestic and export economy of developing countries. Such is the case of Uruguay, which worldwide is among the top-ten exporters of rice (*Oryza sativa*), and where a vast land area is dedicated to this crop. Unlike other regions of the world, the cereal is commonly grown in rotation with pastures. Typical rotation consists of one or two years of rice, where only one crop per year is grown, followed by 3 to 4 years of pastures. This alternate use of the land minimizes the build up of pests and diseases, and reduces the use of agrochemicals (1). This agricultural practice is expected to be more benign to the environment than alternative, more intensive production systems, but the actual impact of this culture methodology is largely unknown. One of the main herbicides used on this crop is clomazone (2-(2-Chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone), which

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is used in 78% of the planted area. Before flooding the fields, clomazone alone or in combination with other herbicides is applied for the control of weeds (2). Dissipation studies performed in China and Australia indicated that after flooding, the herbicide starts to be degraded or volatilizes into the atmosphere, and that following safe withholding periods its release into water streams can be prevented (3;4). This is important, because there are numerous studies revealing that in high doses, clomazone can cause endocrine disruption in fishes or represent a threat to the biota (5). In addition, contamination of drainages and rivers holds the potential for unacceptable levels of clomazone in drinking water. The European Economic Community (Directive N° 0/778/EEC) sets a maximum permissible concentration of pesticides in water for human consumption of 0.1 ng/mL (6), and the environmental required limit for surface water is less than 3 ng/mL (7).

Instrumental analytical methods such as gas chromatography (GC) or high performance liquid chromatography (HPLC) are the reference techniques for the analysis of pesticides. However, the equipment is expensive to buy and operate and require highly trained personnel and large amounts of organic solvents. In addition, in order to attain the sensitivity required to determine the rigorous limits of herbicides in waters, it is necessary to use methods for extraction and pre-concentration of the sample, adding costs to the analysis and making sample preparation labor-intensive and tedious (8). An alternative to this situation, particularly suitable for low income countries, is the introduction of methodologies that provide reliable data and are yet cost-effective (9). Immunoassays have consolidated as a mature technology and many immunoassays have been incorporated as official methods by the Environmental Protection Agency of the USA. Immunoassays rely on the high affinity and specificity with which antibodies recognize their target analytes. They attain low detection limits, allow the parallel processing of many samples, clean up steps are less stringent or unnecessary, and are simple, fast and portable. The most common format is the enzyme-linked immunosorbent assay (ELISA). While immunoassays do not completely substitute for the conventional instrumental methods, they have enormous potential as screening tools for large scale and sustainable monitoring (10).

The aim of this work was the development of an ELISA for the detection of clomazone, to provide a fast and cost-effective tool to monitor the fate of this herbicide in rice cultivation. Immunoassays for clomazone have been reported before (11), but they did not possess enough sensitivity nor have they been used to study the dissipation of the herbicide in regard to this crop. In the current studies polyclonal antibodies were prepared using different strategies of hapten conjugation. The best hapten/antibody pair was selected and buffer conditions were adjusted to minimize matrix effect, enhance recovery and improve long-term reagent stability. The assay was further compared against HPLC using water samples from rice fields. The optimized method was then used in time-course studies in pilot culture fields under controlled conditions of herbicide applications, flooding and drainage, providing valuable information about the fate of the herbicide. These results and the introduction of this technique will allow delineation of management practices that permit rice farmers to achieve good levels of production with minimum environmental impact. Also the environmental protection officers would take advantage of this approach.

Materials and Methods

Materials

Methyl alcohol HPLC grade (Baker, Phillipsburg, USA), phosphoric acid (BDH Chemicals Ltd., Poole, England), clomazone HPLC grade (Riedel-de Haën, Seelze, Germany) was used as primary standard for ELISA and HPLC. The herbicides quinclorac, atrazine, simazine, propanil, bispyribac, pirazosulfuronethyl, metsulfuronmethyl, imazethapyr, glyphosate were

from Cyperex (Uruguay). All other reagents were from Sigma, St. Louis, MO, USA, unless specified otherwise.

Synthesis of aminoclomazone (A-Clo) conjugates

Clomazone (technical grade, Cibeles Co., Uruguay) was conjugated to keyhole limpet hemocyanin (KLH, Pierce, Rockford, IL, USA) or bovine serum albumin (BSA) electrophoresis grade as described by Koppatschek et al.(11). Briefly, 0.3 mL of concentrated nitric acid and 1.2 mL of concentrated sulfuric acid were mixed and added drop wise to a stirred solution of 1.19 g of clomazone in 4 mL of sulfuric acid at -10°C, over 30 min. The reaction was followed by TLC employing ethylacetate:hexane (1:3) as solvent. The nitration mixture was warmed to 0°C and poured carefully into 24 mL of an ice/water mixture. Concentrated ammonium hydroxide was added to the mixture, resulting in the precipitation of the nitrated product. The precipitate was removed by filtration, washed with cold 1 M HNO₃, and air dried. Incorporation of the nitro group to clomazone was confirmed by NMR. Then, 0.3 mg of platinum oxide catalyst were added to 0.27 g of nitro-clomazone in 78 mL of ethanol. The mixture was shaken in a 250 mL thick-walled bottle on a Parr hydrogenator under hydrogen at 45 psi. After 60 min, the pressure dropped to 2 psi. The resulting clear, yellow solution was filtered through filter paper to remove the catalyst, and the solvent was removed below 30°C under reduced pressure. The reduction of nitro-clomazone to aminoclomazone (A-Clo) was evaluated by NMR. The conjugation of A-Clo to the carrier proteins was done on ice. Ten milligrams of A-Clo were added to 1.5 mL of 0.1 N HCl, followed by drop wise addition of 0.052 M NaNO₂ until a positive starch-iodine test was obtained. After 30 min of additional stirring, 0.5 mL of the reaction mixture was added to 0.02 g of carrier protein dissolved in 2.5 mL of borate buffer 0.1 M, pH 9.0. The mixture was stirred for 2 h, with regular addition of 0.1 N NaOH to maintain the pH close to 9.0. The bright orange reaction mixture was dialyzed for 24 h against phosphate-buffered saline, pH 7.5 (PBS).

Synthesis of 2-{2-chloro-4-[(4-hydroxyphenyl)diazenyl]benzyl}-4,4-dimethyl-1,2-oxazolidin-3-one (Phe-Clo) conjugates

Ten milligrams of A-Clo were added to 1.5 mL of 0.1 N HCl, followed by drop wise addition of 0.052 M NaNO₂ until a positive starch-iodine test was obtained. After 30 min of additional stirring, 0.5 mL of the reaction mixture was combined with 2.5 mL of 0.039 mmol of phenol dissolved in 0.1 M borate buffer, pH 9.0. The mixture was stirred for 2 h, with regular addition of 0.1 N NaOH to maintain the pH close to 9.0, and then 0.1 N HCl was added drop wise to pH 2.0, to induce precipitation of the product. The resulting adduct was filtered and purified by preparative TLC, and its structure confirmed by NMR. The hydroxyl group of the phenol spacer was used to conjugate this hapten to BSA and ovalbumin (OVA) (12). Briefly, 5 mg of Phe-Clo were dissolved in 1 mL of DMSO, and 200 µL of this solution were activated with 8.8 mg of CDI (1,1'-carbonyl-diimidazole, Pierce, Rockford, IL, USA) 2 h in the dark. Then 200 µL of the carrier protein (5 mg in 100 mM NaHCO₃, pH 8.5) was added slowly under continuous stirring and allowed to react overnight at 4°C. Conjugation was evaluated by UV spectrometry (not shown).

Antibody production—One hundred micrograms of KLH-A-Clo or OVA-Phe-Clo were dissolved in 250 µL of PBS and vigorously mixed with 250 µL of Freund's Complete Adjuvant (Pierce, Rockford, IL, USA) to form a thick emulsion. This emulsion was then injected subcutaneously into several points on the back of New Zealand white rabbits. After 4 and 8 weeks the animals were immunized intramuscularly with additional doses of 100 µg of hapten conjugates emulsified in Freund's Incomplete Adjuvant. Ten days after the final booster the animals were bled, and after clotting and centrifugation the sera were aliquoted and kept at -20°C until used.

Checkerboard titration—Polystyrene high-binding microtiter plates (Greiner bio one, Germany) were coated with 100 µl of serial dilutions of coating antigen in PBS, overnight at 4°C, starting with 1 µg/mL in the first row, 0.5 µg/mL in the second and so on. The plates were washed three times with PBS-0.05% Tween 20 (PBS-T), and blocked by incubation with PBS-0.1% Tween 20 for 1 h. Then 100 µl of serial dilutions of rabbit sera in PBS-T were dispensed, starting with a 1/100 dilution in the first column, 1/200 in the second, and so on. After 1 h at room temperature, the plates were washed three times with PBS-T, and 100 µl of anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (Pierce, Rockford, IL, USA) diluted 1/5000 in PBS-T. After 1 h of incubation at room temperature, the plates were washed 5 times with PBS-T and the wells were loaded with 100 µl of peroxidase substrate (0.4 mL of 6 mg 3,3',5,5'-tetramethylbenzidine in 1 mL of DMSO, 0.1 mL of 1% H₂O₂ in water in a total of 25 mL of 0.1 M citrate acetate buffer, pH 5.0) and incubated for 20 min at room temperature. The enzyme reaction was stopped by the addition of 50 µl of 2 N H₂SO₄, and the absorbance was read at 450 nm in a microtiter plate reader (FLUOstar OPTIMA, BMG Labtech, Germany)

Clomazone ELISA—Based on checkerboard titration, the appropriate dilutions of coating antigen and antibody were selected to obtain a maximum absorbance reading close to 1 absorbance unit (AU). The competitive ELISA was then set up essentially as described above. Briefly, 96-well microtiter plates were coated and washed as described, and then 50 µl/well of clomazone standard or sample were combined with 50 µl of the appropriate dilution of the antibodies. After 1 h of incubation at room temperature the plates were washed and developed as above. All dilutions were performed with PBS-T except when interference buffer (1M Tris, 0.3 M NaCl, 0.3 M EDTA, 1% BSA, pH 7.4) was used. The software employed for the analysis of data was Origin 7.5

HPLC analysis—The equipment used consisted of a Waters HPLC (Milford, MA, USA) model 1525 Binary HPLC Pump with a 20 µL sample loop, associated with a Waters 2487 Dual λ Absorbance Detector. The analytical column was a Phenomenex (Torrance, CA, USA) 5µm C18, of 250 × 4.60 mm. The samples were analyzed as described by Zanella et al. (13) with modifications. The analytical column was operated isocratically at room temperature, using methyl alcohol:Milli-Q water (65:35) acidified to pH 3.0 with phosphoric acid. The flow-rate was set at 0.8 mL/min and runs were monitored by UV detection at 220 nm and 254 nm. A standard curve with 0.1, 1.0 and 8.0 µg/mL of clomazone was build and used for quantization. Water samples were pre-concentrated by adsorption on SPE columns (Strata™ C18-E from Phenomenex, Torrance, CA, USA). Briefly, water samples were filtered with Whatman paper 1 and then with 0.4 µm cellulose membranes (Millipore, Bedford, MA, USA), and the pH was adjusted to 3.0 by addition of phosphoric acid. Then, the SPE column was activated by passing consecutively 3 mL of methanol, 3 mL of Milli-Q water, and 3 mL of Milli-Q water pH 3, and 250 mL of water were applied to the column at 5 mL/min. After that, the SPE column was eluted with 1 mL of methanol, which was used for analysis.

Pilot studies of clomazone dissipation—The persistence of the herbicide was studied in experimental rice cultures, implemented by the National Institute of Agricultural Research (INIA) during the harvest 2008/2009 in Treinta y Tres, Uruguay. The pilot experiments were designed to evaluate the effect of two different irrigation treatments on the fate of the herbicide. During the study, climatic variables were monitored in the Meteorological Station of the experimental unit. Two adjacent plots of 112 m² were used. Clomazone, 384 g/ha as an emulsifiable commercial formulation, 120 l/ha of broth, was applied to both plots at the same moment 13 days post-emergence of the culture. After 2 days, plot #1 was flooded with a water layer of 10 cm (early flooding), which was maintained during the flooding cycle. Plot #2 was flooded under the same conditions but 16 days after application, which is the standard

procedure used by local rice farmers. Samples were regularly collected from the water layer. The samples were maintained at 4°C, and analyzed within the following week.

Results and Discussion

Antibody production and checkerboard titration

The IgG titer of New Zealand rabbits immunized with A-Clo-KLH was determined by indirect ELISA, on plates coated with A-Clo-BSA, Phe-Clo-BSA or BSA as a negative control. Very high titers were obtained against the A-Clo conjugate (but not to Phe-Clo), and no reactivity with BSA was observed. The animal showing the highest response to the hapten was chosen. The serum titer (midpoint of the titration curve) was 500 folds higher for A-Clo than Phe-Clo, showing that the bulky spacer somehow interfered with the binding of the clomazone moiety to the antigen binding site of the antibody.

Standard curve of the clomazone ELISA

The amount of coating antigen and proper dilution of the antibody were established by checkerboard titration as described above. The serum dilution producing an ELISA reading close to 1 AU with the smallest amount of coating antigen was chosen. This was obtained with 8.0 ng/mL of coating antigen and a serum dilution of 1/40,000 for aminoclomazone-BSA, and 1.0 µg/mL of coating antigen and a serum dilution of 1/8,000 for Phe-Clo-BSA. The standard curves of the homologous assay (immunizing and coating hapten are the same) and heterologous assay (immunizing and coating hapten differ) are shown in figure 1. The homologous ELISA had a midpoint (Inhibitory Concentration causing 50% inhibition, IC₅₀) of 3.5 ± 0.3 ng/mL with a limit of detection (LOD = IC₂₀) of 1.1 ng/mL. Similarly, the heterologous ELISA presented an IC₅₀ = 4.2 ± 1.1 ng/mL and LOD = 0.9 ng/mL. This result was somewhat unexpected because, in general, heterologous assays set up with polyclonal antibodies tend to produce assays with improved sensitivity (14;15).

Both ELISAs were then used to validate the method with real water samples. Five samples were collected from the source river or rice fields where clomazone had not been used, and were spiked with various concentrations of the herbicide. The results (not shown) were unsatisfactory with unacceptable recoveries for both methods. Based on our previous experience, the inclusion of a bulk protein that may interact with humic acids, chelating agents that sequester metal ions, and an increase ionic strength, may help to decrease the matrix effect. To test this hypothesis, different proportions of 'interference buffer', 1M Tris, 0.3 M NaCl, 0.3 M EDTA, 1% BSA, pH 7.4, were included during the competition step of the assay. Figure 2 shows the standard curves of the homologous method performed under these conditions, the inclusion of interference buffer causes a moderate decrease of the maximum signal, but the parameters of the curve are basically not modified. Initial experiments demonstrated that the best recoveries were obtained when 10% of interference buffer was used, and therefore this buffer composition was selected to validate the assays. Figure 3 displays the results obtained with the homologous assay. In the 2 to 20 ng/mL range, there was a good correlation ($y = 0.94x + 0.23$, $R^2 = 0.85$) between the amount of clomazone used for spiking and its measured concentration in four different water samples. Similar recoveries were obtained with the heterologous method; however the latter appeared to be less robust and was not further studied.

Assay specificity

The specificity of the assay was tested using a panel of herbicides that are representative of the compounds currently used in rice agriculture in Uruguay. The cross reactivity of the assay for each compound was calculated as % cross-reactivity = $100 \times [\text{IC}_{50}(\text{clomazone})/\text{IC}_{50}(\text{cross-reacting compound})]$ Table 1. Except for the clomazone-related hapten synthesized in this

study, none of the other herbicides exhibited significant cross reactivity at the highest concentration tested.

Inter-method comparison

The assay was also validated by comparing ELISA and HPLC clomazone measurements in representative water samples. Clomazone was assayed in fifteen water samples taken from various local rice fields at different time points along the harvest. Four samples were below the LOD of both methods, and for the rest, there was a very good correlation between both techniques ($y = 0.98x - 0.15$, $R^2 = 0.98$), figure 4. The fact that both methods produced equivalent results in the 2-18 ng/mL range is of great relevance for this study, particularly when the simplicity of the ELISA technique is considered. Indeed, HPLC analysis requires tedious filtering steps, and passage of large water volumes through preconcentration columns, while on the other hand a large number of a small-volume (1 mL) water samples can be readily analyzed in parallel by ELISA after a 2 min centrifugation step.

Dissipation of clomazone in water samples from rice culture

The dissipation of the herbicide was analyzed in a water management study implemented at El Paso de la Laguna Experimental Unit (33° 16'S 54° 10'W) of the National Institute of Agricultural Research during the 2008-2009 harvest in Treinta y Tres, Uruguay. An important aim of this project is to define the best conditions of flooding and drainage that optimize productivity while minimizing the release of agrochemicals into the environment. The assay developed in this work was used in an initial model experiment in which two culture plots were flooded at different times after application of the herbicide as described. As expected, early irrigation of the plot was accompanied by high concentrations of clomazone in the water layer figure 5. After an initial concentration of 77 ng/mL found the day of flooding, there was a temporary reduction, in the first three days to a minimum value of about 45 ng/mL, at which time the clomazone concentrations increased rapidly to reach a maximum of 129 ng/mL at day 6 after flooding. If we consider an irrigation water layer thickness of 10 cm, this concentration corresponds to approximately one third of the total amount applied to the plot. The amount of clomazone decreased afterwards, until day 41 when the concentration of the herbicide in the water layer was below the LOD. Late irrigation was associated with lower concentrations of clomazone, peaking at day 9 after flooding (26 ng/mL) and waning until day 30 when the compound became undetectable. There was a remarkable difference between the two conditions of irrigation. Apparently, late irrigation, which is the procedure commonly used by local farmers, favors stronger herbicide adsorption onto the soil, its microbial or photoinduced degradation, or its volatilization (16), which should be further studied. The high levels of clomazone attained with early irrigation may be a matter of concern, and care should be taken to avoid that climatic conditions, such as heavy rain, may cause uncontrolled release of the herbicide during peak days. Based on studies performed by the Californian Department of Pesticide Regulation (17), it has been recommended that growers withhold treated water in their farms for a minimum of 21-28 days, to minimize environmental impacts associated with herbicides. Under the conditions investigated in our study, the residues of herbicide reached environmentally safe levels 40 and 30 days after flooding, for the early and late irrigation treatments, respectively, which reveals the importance of local monitoring of these parameters.

These pilot experiments showed the potential of the method, producing the first set of data about the fate of the herbicide in the production system used in Uruguay. Based on its robustness and simplicity the method could become a useful tool to facilitate further optimization of management practices that will allow farmers to achieve good production standards while preserving the environment. The method could be particularly suitable for monitoring water quality in rice growing regions of developing countries that currently are not able to do such water testing due to cost or lack of capability to perform instrumental analysis. In previous

studies we have demonstrated the potential of other ELISA formats to allow broader uses of the method than the type of assays used in this study. For example (18), dipstick formats might be developed that would allow the farmer who is not trained as an analytical chemist to do semi-quantitative assays at the point of use of the herbicide. The ability to actually monitor the clomazone content of the irrigation water under local conditions prior to release of the water to the environment can ensure the use of “best practices” under a wide range of field conditions. We are currently exploring the adaptation of the clomazone immunoassay into Phage anti-immunocomplex assay (PHAIA) which can increase the sensitivity by 5-20 folds and it is easily adapted into rapid formats (18;19).

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Figure 1. Clomazone competitive ELISAs using PBS as reaction buffer

The homologous (black) and heterologous (white) assays were set up using A-Clo-BSA and Phe-Clo-BSA, respectively. The concentration causing 50% inhibition (IC_{50}) was 3.5 ± 0.3 ng/mL for the homologous assay and 4.2 ± 1.1 ng/mL for the heterologous, with detection limits of 0.9 and 1.1 ng/mL, respectively.

Figure 2. Effect of interference buffer on assay performance

Four conditions were tested using 0% (squares), 5% (circle), 10% (triangle) or 20% (diamonds) interference buffer in the competition step of the ELISA.

Figure 3. Recovery of spiked water samples

Four water samples collected from the source river (circle), a rice field where clomazone had not been used (diamonds), and two flooding channels (triangles and squares) were spiked with various concentrations of clomazone and then analyzed by ELISA.

Figure 4. Comparison of ELISA and HPLC determination of clomazone in field samples collected at different time points along the harvest

Samples were analyzed by triplicates (ELISA) and duplicates (HPLC).

Figure 5. Concentration of clomazone in pond water of two rice plots cultivated under different conditions of irrigation

Plot#1 (circles) and plot#2 (squares) were flooded 2 and 16 days after application of the herbicide, respectively, and maintained with a 10-cm water layer throughout the study.

Table1
Cross reactivity of agrochemicals currently used in rice production in Uruguay

Herbicide name	Herbicide structure
Clomazone
Aminoclomazone

Herbicide name	Herbicide structure
Atrazine

Herbicide name	Herbicide structure
Simazine	<chem>CCN=C1N(C(=N1)Cl)NCC</chem>
Quinclorac	<chem>Clc1cc(Cl)nc2c1cc(Cl)c(C(=O)O)c2</chem>
Propanil	<chem>CCC(=O)Nc1cc(Cl)cc(Cl)c1</chem>

Herbicide name	Herbicide structure
Bispyribac
Pirazosulfuronethyl
Metsulfuron methyl

Herbicide name	Herbicide structure
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Imazethapyr	
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Herbicide name	Herbicide structure
Glyphosate	<p>The chemical structure of Glyphosate is shown. It consists of a central phosphorus atom (P) bonded to four groups: a carboxymethylamino group (-CH₂NHCOOH), a hydroxyl group (-OH), another hydroxyl group (-OH), and an oxygen atom (=O). The carboxymethylamino group is further detailed with a carbonyl group (C=O) and a hydroxyl group (OH) attached to the methylene group (-CH₂-).</p>