

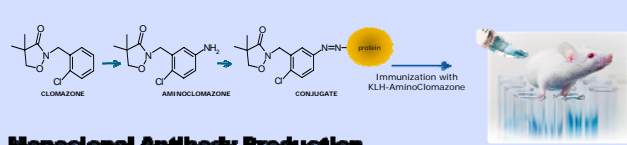
NONCOMPETITIVE IMMUNOASSAY FOR THE DETECTION OF CLOMAZONE USING CICLIC PEPTIDES ISOLATED FROM PHAGE DISPLAY LIBRARIES

Martin A. Rossotti, Mariana Carlomagno, Cecilia Matho and Gualberto González-Sapienza.
Cátedra de Inmunología, Facultad de Química, UDELAR, Montevideo, Uruguay.

INTRODUCTION

Immunoassays are sensitive, quantitative, rapid and economic tools, widely used in environmental analytical measurements. To date, there are few technologies for the development of noncompetitive immunoassays for small molecules (haptens), the most common of which relies on the use of anti-immunocomplex antibodies. This approach is laborious, case specific, relies upon monoclonal antibody technology for its implementation and often exhibits high cross-reactivity with the unligated primary antibody. We recently have developed a new technology, PHAIA (*phage anti-immunocomplex assay*), where short peptide loops isolated from phage display libraries can be used as substitutes of anti-immunocomplex antibodies for noncompetitive immunodetection of small molecules. The aim of this work is to use of this kind of peptides derived from phage display libraries as haptens substitutes to allow the development of potentially more sensitive assays and contribute to diminish the amount of chemical synthesis needed.

Chemical Synthesis of the Conjugate and Immunization



Monoclonal Antibody Production

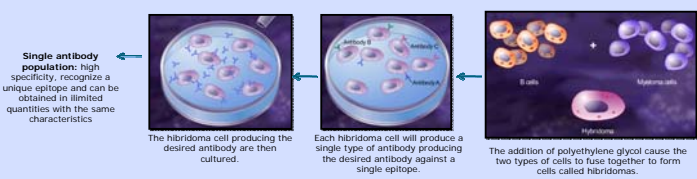


Figure 1. Production of the monoclonal antibodies for the development of the Immunoassay for Clomazone.

Phage Display and PHAIA Technology

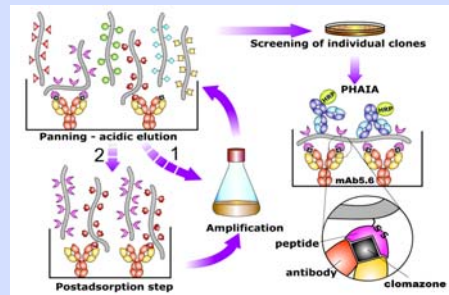


Figure 2. Scheme of the procedure for the selection of anti-immune complex phage and setup of phage anti-immune complex assay (PHAIA). A possible scheme of the antibody-hapten-peptide trivalent interaction that allows development of PHAIA is shown in the lower right part of the figure. Note that the overall affinity of the peptide for the immune complex is postulated to be the result of their interaction with the analyte and the antibody.

METHODS:

Phage display peptide libraries provides billions of different peptides expressed as fusion partners with the bacteriophage coat proteins pIII or pVIII. Phage that display a suitable ligand are retained by the target, (immunocomplex formed by a monoclonal antibody against the herbicide Clomazone (mAb5.6) and the Clomazone molecule) while non adherent particles are washed away. Bound phages are detached from the surface by acid elution, used to reinfect bacteria and reproduced for a new round of selection (Figure 2). After three rounds of selection, phages obtained as supernatants of bacterial cultures were tested for binding to the immunocomplex by ELISA. DNA from positives phages clones was extracted and sequenced to deduce the amino acid composition of the displayed peptide. Four different sequences with a share consensus motif were titrated and employed in a noncompetition assay with clomazone and aminoclozomazone to test the specificity of that union (see fig. 5).

RESULTS:

Four different sequences were isolated from phage library against the mAb5.6/Clomazone Immunocomplex.

Table 1. Amino acid sequences of the positives clones

Clone	Sequence
ICX5	C I S A P N M E A C
ICX7	C T Q P N P E A C
ICX9	C A L A P N Q E A C
ICX11	C L E A P N I E G C

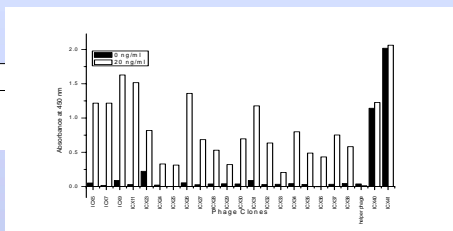
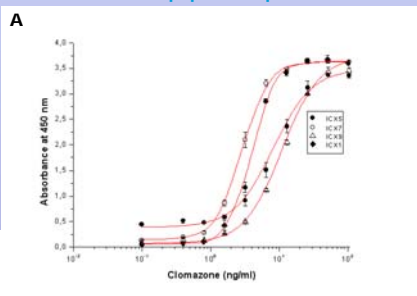


Figure 3. Screening of phage eluate. Forty eight phage clones were selected for screening by phage ELISA in the presence of clomazone or absence of analyte. Most of them bound specifically to the immunocomplex, showing negligible signal with the uncombined antibody.

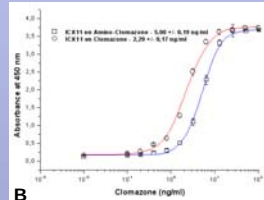
Immunodetection of Clomazone and effect of the peptide sequence in the assay performance

Figure 4. Noncompetitive ELISA developed with each sequence shows no substantial difference between the sensitivity of them but using the ICX11 peptide displayed on the phage we developed an ELISA which allowed us to detect as low as 0.33 ng/ml of clomazone. This sensitivity was 20-folds better than that attained with the conventional competitive assay setup with the same antibody.



The peptide ICX11 showed a higher affinity for the immunocomplex of clomazone/Ab5.6 ($SC_{50} = 2.3$ vs. 2.5 ng/ml) than for the immunocomplex between the aminoclozomazone with the same antibody

The detection limit was estimated from the reading of zero analite concentration plus five standard deviation. $LD = 0.33$ ng/ml



CONCLUSIONS :

Using the immunocomplex Ab5.6/Clomazone it was possible to isolate peptides bearing phages that can be used to setup a noncompetitive ELISAs for this pesticide. This strategy allowed to attain 20-fold better sensitivities than that of the conventional competitive assay setup with the same antibody.

As phage particles are very robust and can be easily purified, we have found a simple and rapid method for the development of immunoassays. Also phage particles can be produced in large amounts and constitute highly standardize reagents that substitute the conventional competing antigens, diminishing the extent of chemical synthesis and lowering the amount of organic solvents waste.

The clomazone PHAIA was also easily adapted into two rapid and highly sensitive assay, that in minutes and by simple visual inspection allowed the detection of up to 0.4 ng/ml of the herbicide, which corresponds to 7-fold lower the recommended maximum concentration of the herbicide in surface waters.

Clomazone-PHAIA has a good correlation with HPLC and show almost null matrix effect.

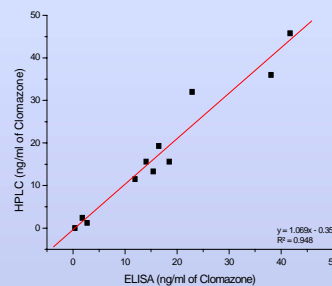


Figure 5. Ten samples of water was used to evaluate the correlation between the reference technique, HPLC ($LD = 0.5$ ng/ml) with our assay ($LD = 0.33$ ng/ml). Different waters were analyzed by Clomazone-PHAIA ELISA in only two hours.

Table 3. Recovery of Clomazone in Surface Waters Measured by Noncompetitive ELISA

Final concentration of water (%)	AR2 - Surface Water - 0 ng/ml measured using HPLC			mean recovery (%; n = 3)
	Spiked Clomazone (ng/ml)	Measured Clomazone (ng/ml)		
100	3.2	4.46	139	
	1.6	2.44	152	
	0.8	1.73	216	
50	3.2	3.36	105	
	1.6	1.97	125	
	0.8	0.9	112	
25	3.2	3.1	97	
	1.6	1.6	100	
	0.8	0.9	112	
12	3.2	2.7	84	
	1.6	1.4	88	
	0.8	0.8	100	
6	3.2	2.7	84	
	1.6	1.5	94	
	0.8	0.8	100	

The effect of the matrix effect was evaluated by monitoring the recovery of spiked clomazone in surface waters measured by noncompetitive ELISA. To obtain a good correlation is necessary a two-fold dilution of the sample.

Detection by simple visual inspection of 0.4 ng/ml of Clomazone

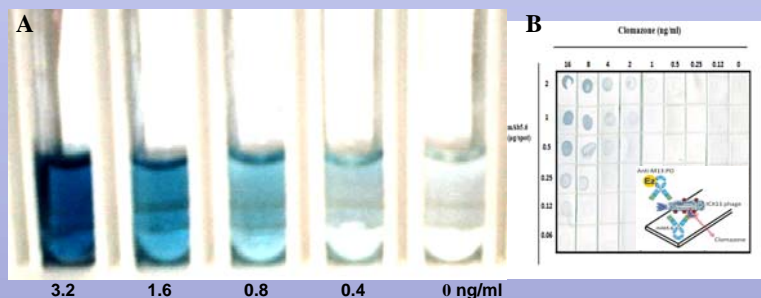


Figure 6. Clomazone PHAIA adapted into a Dipstick Assay (A) and ImmunoTubes assay. We can detect by simple inspection as low as 0.4 ng/ml, 7-fold lower to the recommended maximum concentration of the herbicide in surface waters.