Published in final edited form as:
Anal Chem. 2010 November 1; 82(21): . doi:10.1021/ac101476f.

Phage Anti-Immunocomplex Assay (PHAIA) for clomazone: Two-site recognition increases assay specificity and facilitates adaptation into a rapid on-site format

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Abstract

The impact of the use of herbicides in agriculture can be minimized by compliance with good management practices that reduce the amount used and their release into the environment. Simple tests that provide real time on-site information about these chemicals are a major aid for these programs. In this work we show that PHAIA, a method that uses phage-borne peptides to detect the formation of antibody-analyte immunocomplexes, is an advantageous technology to produce such field tests. A monoclonal antibody to the herbicide clomazone was raised and used in the development of conventional competitive and noncompetitive PHAIA immunoassays. The sensitivity attained with the PHAIA format was over ten times higher than that of the competitive format. The cross-reactivity of the two methods was also compared by using structurally related compounds, and we observed that the two-site binding of PHAIA “double-checks” the recognition of the analyte, thereby increasing the assay specificity. The positive readout of the noncompetitive PHAIA method allowed adaptation of the assay into a rapid and simple format where as little as 0.4 ng/ml of clomazone (more than 10-fold lower than the proposed standard) in water samples from a rice field could be easily detected by simple visual inspection.

Keywords

PHAIA; anti-immunocomplex assay; phage display; pesticides; Clomazone

INTRODUCTION

Clomazone (2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone), trade names Cerano or Command, is a broad-spectrum herbicide used for the control of weeds in paddy rice, cotton, peas, pumpkins, soybeans, sweet potatoes, tobacco, etc.1 It can be applied early preplant, preemergent or preplant-incorporated, depending on the crop, geographical area, and timing.2 Due to its extended use in rice production there is a concern about the potential contamination of water resources with this chemical. In this regard, Zanella et al.3 reported that significant concentrations of this herbicide were detected in 90% of water samples collected from surface water close to rice fields in Southern Brazil. Proper management can help to reduced its release into the environment because clomazone dissipates in the water

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column, mostly by anaerobic microbial degradation, with an estimated half-life of 8 days.4 Dissipation studies performed in China and Australia showed that by using proper withholding periods, the amount of herbicide that is released into the draining water streams can be minimized.5, 6 We recently performed similar studies in a rice producing area in Northern Uruguay, showing that the irrigation protocol is an important determinant of the final amount of the herbicide release upon field drainage.7

In our previous study, we also presented the development of a clomazone ELISA, which showed excellent correlation with HPLC measurements, and proposed that it can be a simpler and cost-effective alternative to instrumental analysis for regular monitoring of the herbicide. This is certainly relevant because such analysis could be included as an important component of good management practices for rice producers, who in this way could check for possible variations in the herbicide dissipation rate due to changes in temperature, rain regime, ingredients of the herbicide formulation, etc. In order to improve the sensitivity of the assay and to provide an even simpler analytical tool that could be used by untrained personal for on-site quantification of clomazone, we have now developed a second-generation clomazone immunoassay based on phage anti-immunocomplex (PHAIA) technology. In this method the analyte-antibody immunocomplex is specifically recognized by a short peptide displayed on the surface of the M13 bacteriophage, providing a noncompetitive detection of low molecular weight compounds.8 This technology, which provides a positive readout, works with either monoclonal or polyclonal antibodies and performs with higher sensitivity that the competitive format. It has been applied to several targets, including the herbicides molinate and atrazine, the pyrethroid methabolite phenoxybenzoic acid, the drugs cyclosporine and digoxin, and the flame retardant brominated diphenyl ether 47.8, 9 After our initial description of PHAIA8, Inaba et al. also described the application of the method to the detection of the phytohormone gibberellin 4,10 confirming that a significantly improved detection limit could be obtained with this format.

To facilitate the noncompetitive detection of clomazone, we produced an anti-clomazone monoclonal antibody (MAb5.6), which was selected by competitive screening. The MAb5.6 was then used to pan a 7- to 11-mer random peptide library display on the pVIII protein of phage M13. Four different peptides were selected that specifically recognize the immunocomplex, and one of them was used to set up the clomazone PHAIA. The method allowed us to attain sensitivity well beyond the environmental limit recommended for the herbicide and an ELISA that performed with exquisite specificity. Due to its positive readout the method could be easily adapted into a very simple immunotube, variety of dipsticks and biosensor formats.

EXPERIMENTAL SECTION

Materials

Clomazone (Riedel-de Haën, Seelze, Germany) was conjugated to keyhole limpet hemocyanin (KLH) (Pierce, Rockford, IL) or bovine serum albumin (BSA) (Sigma, St. Louis, MO) by nitration and reduction, followed by diazotization as described.11 Mouse anti-M13 monoclonal antibody conjugated to horse radish peroxidase (anti-M13-HRP) was purchased from GE Health Care (Piscataway, NJ) and helper phage M13KO7 was purchased from New England Biolabs (Ipswich, MA). BCA™ Protein Assay Kit for the quantification of purified antibody and protein G affinity columns for IgG purification, were purchased from Pierce (Rockford, IL). BSA, polyethyleneglycol (PEG), Tween 20, 3, 3’, 5, 5’-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO).

Inmunization of mice with clomazone-KLH and monoclonal antibody (MAb) production

Six-week-old female BALB/c mice were initially immunized intraperitoneally with 50 μg of clomazone-KLH in Freund's Complete Adjuvant (Sigma, St. Louis, MO) at a 1:1 protein solution to adjuvant ratio and boosted three weeks later using Freund's incomplete adjuvant. Three days later the animals were sacrificed and hybridomas were prepared as described before.12 Supernatants were initially screened by ELISA for antibodies binding to clomazone-BSA, but not to BSA.

Competitive ELISA

Coating conditions and dilution of antibodies were determined by checkerboard titration to obtain readouts of about 1.0 absorbance unit in the absence of analyte. The competition assay was performed in 96-well microtiter plates coated with 100 ng/well of clomazone-BSA and blocked with 5% skimmed milk in phosphate-buffered saline (PBS). Plates were washed three times with PBS containing 0.05% of Tween 20 (PBST), and 50 μl/well clomazone standard dilutions in PBST plus 50 μl/well of the selected dilution of mouse serum or hybridoma supernatants were added. After 1 h incubation at room temperature, anti-mouse IgG-conjugated to HRP 1/5000 (Pierce, Rockford, IL) was added to the wells and the plate was incubated for 1h. Then the plates were washed and 100 μl of the peroxidase substrate (0.4 ml of a 6 mg/ml DMSO solution of 3,3´,5,5´-tetramethylbenzidine, 0.1 ml of 1% H_2O_2 in water in a total of 25 ml of 0.1 M citrate acetate buffer, pH 5.5) were dispensed into each well. The enzyme reaction was stopped after 20 min by addition of 50 μl of 2 N H_2SO_4, and the absorbance was read at 450/650 nm in a microtiter plate reader (Multiskan MS, Thermo Labsystems, Waltham, MA). Standard curves were normalized by expressing experimental absorbance values (B), as (B/B_0) ×100, where B_0 is the absorbance value at zero analyte concentration. Absolute or normalized values were fitted to a four-parameter logistic equation using Genesis Lite 3.03 (Life Sciences, London) package software.

Competitive screening of monoclonal antibodies

Microtiter plates were coated with 100 μl of 5 μg/ml anti-mouse IgG polyclonal antibodies (Sigma, St. Louis, MO) for 1 h at room temperature, and blocked with 1% BSA in PBS. One hundred μl/per well of hybridoma supernatants were dispensed and incubated for 1 h at room temperature. After washing, 100 ng of the biotinylated clomazone-BSA conjugate in 50 μl PBST was added to each well, followed by 50 μl of clomazone to a final concentration of 10 ng/ml. In a parallel plate the clomazone solution was substituted by PBST. The plates were washed and the amount of biotinylated clomazone-BSA conjugate was revealed by addition of a 1/4000 dilution of streptavidin-HRP conjugate (Pierce, Rockford, IL). The enzymatic activity of HRP was measured as described above. The supernatants that showed the biggest differences between plates were selected.

Phage display peptide libraries and biopanning

A random phage display peptide library with an estimated diversity of 3×10^9 independent clones was constructed on the phagemid vector p8V2.13 This is a constrained library expressing 7 to 11 random amino acid peptides flanked by two cysteine residues and linked to the N-terminus of the major pVIII phage coat protein through a long glycine-rich spacer (GG-C-$(X)_{7–11}$-C-$(GGGGS)_3$-). For the selection procedure, high binding microtiter plates (Greiner, Solingen, Germany) were coated with MAb5.6 at 10 μg/ml in phosphate-buffered saline (PBS) overnight at 4 °C. After coating, the wells were blocked with 5% skimmed milk at 37 °C for 1 h, then washed three times with PBS, 0.05% Tween 20 (PBST). The peptide library (1×10^{11} transducing units) was mixed with clomazone (1 μg/ml final concentration) and BSA (1% final concentration) in a final volume of 600 μl of PBS and

then added to six wells coated with the antibody. After incubation for 2 h at 4 °C with gentle shaking on an orbital plate shaker, the wells were washed five times with PBST, incubated for half an hour in PBST at 4 °C, and washed again five times. Bound phages were eluted by incubation with 100 μl of 0.1 N glycine-HCl, pH 2.2, for 10 min. The phage eluate (600 μl) was immediately neutralized with 30 μl of 2 M trizma base, added to 10 ml of log-phase *E. coli* ARI 292 cells (Affymax Research Institute, Palo Alto, CA) and amplified in Luria-Bertoni (LB) media containing 0.25% K_2HPO_4, 0.1% $MgSO_4$, 0.1% glucose and 100 μg/ml of ampicillin to an OD_{600} = 0.4. M13KO7 helper phage at a multiplicity of infection 10:1 was added. After a period of 30 min at 37 °C without shaking, arabinose and kanamycin were added to a final concentration of 0.02 % and 40 μg/ml, respectively, and the cultures were incubated overnight at 37 °C with vigorous shaking. Phage from liquid cultures were obtained by clearing the supernatants by centrifugation at 12,000 × g for 15 min, precipitated by adding 0.2 volumes of 20% polyethylene glycol 8000, 2.5M NaCl, on ice for 1 hour, and centrifuged at 10,000 × g. Phage pellets were resuspended in 1 ml of sterile PBS and titrated in ARI 292. The 2nd and 3rd round of panning were performed using the sample procedure as described above except that the amount of coating antibody was gradually reduced to 5 and 1 μg/ml, respectively.

Screening of phage eluate for positive clones by phage ELISA

Phage supernatants were prepared as described before.8 Forty eight individual clones were tested by Phage-ELISA by direct addition of 50 μl of phage supernatants into wells coated with 0.5 μg/well of MAb5.6, with or without addition of 50 μl of 40 ng/ml of clomazone in PBST per well. Bound phage was detected by addition of 100 μl of anti-M13-HRP.

Preparation of stabilized phage stocks

Phage clones were individually amplified before8 and after two steps of precipitation with PEG-NaCl, the phage particles were resuspended in 5 ml of PBS, which was supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN) and sodium azide 0.05%. The preparation was filtered (0.22 μm) and stored in aliquots at 4 °C for short term use, or kept at −80 °C until use.

PHAIA method

ELISA plates were coated with 100 μl/well of MAb5.6 (10 μg/ml in PBS) for 1 h at 37 °C, and blocked for 1 hour at 37 °C with 1% BSA in PBS. After washing three times with PBST, the plates were loaded with 50 μl of serial dilutions of clomazone and 100 μl of the appropriate phage dilution in PBST. Plates were incubated for 1 h at room temperature, washed ten times with PBST, and incubated with 100 μl/well of anti-M13-HRP for 1 h. After washing, the peroxidase activity was developed by adding 100 μl of peroxidase substrate (25 ml of 0.1 M citrate acetate buffer pH 5.5, 0.4 ml of 6 mg/ml DMSO solution of 3,3′,5,5′-tetramethylbenzidine and 0.1 ml of 1% H_2O_2). The enzymatic reaction was stopped after 15–20 min by the addition of 50 μl of 2M H_2SO_4, and the absorbance at 450 nm (corrected at 600 nm) was read in a microtiter plate reader (Molecular Devices, Sunnyvale, CA). In order to decrease the matrix interference, the stabilized phage stock was diluted in blocking buffer (1M Tris, 0.3 M NaCl, 0.3 M EDTA, 1% BSA, pH 7.4)

Immunotube assay

The assay was performed using 5 ml Immunotubes (Greiner, Frickenhausen Germany) coated with 1 ml of MAb5.6 (5 μg/ml), blocked overnight at 4 °C with 1% BSA in PBS, and washed 3 times with PBST. Five hundred microliters of serial dilutions of clomazone in PBS, or water samples, were mixed with 500 μl of a 1/50 dilution of the stabilized phage stock prepared in blocking buffer, and the tubes were incubated for 1 h at room temperature.

After washing, the tubes were incubated with a 1/5,000 dilution of anti-M13-HRP for 30 min, washed five times with water, and color was developed with the TMB peroxidase substrate as above.

RESULTS AND DISCUSSION

Generation and characterization of monoclonal antibody MAb5.6

Monoclonal antibodies were initially selected on the basis of their reactivity with clomazone-BSA; final screening was performed by competitive selection as described in Methods. Forty eight positive clones were tested, and two clones showing the biggest difference in signals in the presence or absence of clomazone were selected (results not shown) and subcloned, namely MAb5.6 and MAb21.1. These antibodies and the parent mouse serum were used to set up competitive assays for clomazone in plates coated with clomazone-BSA, as shown in figure 1. The midpoint of the sigmoidal curves, which corresponds to the concentration of analyte causing 50% inhibition of the antibody binding (IC_{50}), were 28.0 ± 1.3 and 109 ± 16 ng/ml for MAb5.6, and MAb21.1, respectively, and 345 ± 93 ng/ml for the parent mouse serum. The competitive screening strategy allowed us to select antibodies with IC_{50} well beyond the average of the polyclonal antibody population. The assay set up with MAb5.6, which showed the lowest IC_{50} exhibited a sensitivity limit, defined as the concentration of analyte causing 20% inhibition of the antibody binding (IC_{20}), which was close to 7.0 ng/ml. This value is not good enough for environmental monitoring, because it has been proposed that the maximum limit for this herbicide in surface water should be lower than 3–4 ng/ml.14

Selection of specific phage-borne peptides to clomazone-MAb5.6 immunocomplex

We have recently shown that the enormous chemical diversity and power of selection inherent to phage display peptide libraries permits the selection of peptides that specifically recognize small molecule-antibody complexes.8 A phage display peptide library expressing random peptides of 7–11 random amino acids flanked by a disulfide bridge and fused to the phage coat protein pVIII was panned with the clomazone-MAb5.6 immunocomplex as described above. In order to favor the isolation of immunocomplex specific peptides, an excess of clomazone was included in the panning experiments to avoid the selection of peptides that could bind to the herbicide binding site of MAb5.6. In addition, the amount of immunocomplex used for the selection was gradually decreased along the panning procedure, under the assumption that increased competition for the combined antibody would remove weak binders. After three rounds of panning, 40 clones were individually grown and tested for binding to the monoclonal antibody- coated wells in the presence or absence of herbicide. Twenty out of 40 clones bound specifically to the immunocomplex in the presence of 20 ng/ml of clomazone, showing minimum or negligible binding to the uncombined antibody (figure 2).

In spite of the fact that we used a phage library containing peptides with 7 to 11 random residues, sequencing of 20 immunocomplex-specific clones (designated ICX) revealed that all of them corresponded to 8-mer peptides, which could be grouped according to four different sequences. The immunocomplex-specific peptides defined a shared consensus motif, CxxAPNxEAC (table 1), where x represents any amino acid. In addition to this, the screening also yielded clones (about 50%) which bound strongly to MAb5.6 regardless of its binding to clomazone. These clones do not bear the consensus sequence characteristic of the immunocomplex-specific peptide, as it is shown on the bottom of table 1 for sequence AbX40.

Phage anti-immunocomplex assay for clomazone

Four clones bearing phagemids that code for four different peptides sequences that specifically recognized the immunocomplex (ICX clones, Table 1) were amplified and prepared as stabilized phage stocks (1×10^{12} transducing units/ml) and were used in checkerboard experiments to determine the antibody coating conditions and the phage dilution that allowed us to maximize the differential signal in the presence or absence of clomazone. The best result for the four ICX phage clones was obtained when 1 μg/ml of MAb5.6 was used for coating and 2×10^9 phage transducing units/well was used for detection. These conditions were selected to set up the noncompetitive PHAIA and to compare the sensitivity of the four different peptide sequences, as shown in figure 3. The binding dose-response curves had a typical sigmoidal shape with signal saturation at high concentration of analyte, but showed differences in assay performance depending on the sequence of the phage- borne peptides. As we have observed before,8 in addition to the role of the consensus residues, which most probably participate in the interactions with the analyte, the non-consensus amino acids appear to work as a ‘fine tuning’ that further determines the peptide binding to the immunocomplex.

The limit of detection (LOD) of the PHAIA assay, which is defined as the concentration of analyte that corresponds to an absorbance value equal to that of the zero analyte concentration plus five standard deviations, was 0.15 ng/ml for the assay set up with the phage-borne peptide ICX11. The midpoint of the curve, which corresponds to the concentration of analyte at which ELISA readings are 50% of the maximal signal (SC_{50}), was 2.6 ± 0.3 ng/ml. This represents a 10-fold improvement in assay sensitivity as compared to the midpoint of the competitive ELISA set up with the same monoclonal antibody (figure 1), which exhibited an IC_{50} of 28.0 ± 1.3 ng/ml. This is consistent with our previous results, 8 which demonstrate that when the same antibody is used the PHAIA format allows us not only to obtain a positive readout but also a significant increase in assay sensitivity.

Double recognition in the two-site format contributes to a highly specific detection of the analyte

The specificity of the clomazone PHAIA was evaluated by comparing the assay response with a panel of common pesticides and herbicides used in rice fields. The compounds analyzed included quinclorac, glyphosate, molinate, bispiribac, propanil, and atrazine. No signal was observed with any of these compounds, even at the highest concentrations tested, 1000 ng/ml (data not shown). The two-site bonding of the analyte provides a larger contact surface that not only contributes to a higher overall binding energy, increasing the assay sensitivity, but also determines a more selective recognition of the analyte. We clearly demonstrated this principle when we compared the cross-reactivity of the conventional competitive assay and that of the PHAIA method with the haptens used for the synthesis of the immunizing conjugates, figure 4. Due to the fact that the clomazone molecule was coupled to KLH through a modification of the aromatic ring, the antibodies raised with these haptens most probably react mainly with the 4,4-dimethyl-3-isoxazolidinone ring of clomazone. This moiety is common to clomazone and the two other haptens, and therefore there is significant cross reactivity in the competitive format. Conversely, the PHAIA peptides were selected to recognize the portion of clomazone that remains exposed to the solvent after binding to the antibody, and therefore their reactivity is highly affected by the modifications of the aromatic ring, being almost completely abolished in the case of the phenol-diazo-clomazone hapten. These results exemplified the exquisite specificity that the two-site format provides over competitive detection of small analytes. In the case of analytes that share a common chemical structure, such as toxin or chemical congeners, an existing group-specific competitive immunoassay could be adapted into compound-specific PHAIA by using the same antibody and selecting compound-specific peptides with the respective

immunocomplex. These data support the hypothesis shown in figure 3 that the phage peptide is interacting directly with the hapten rather than only acting, at least, allosterically through the antibody.

Analytical evaluation of the clomazone PHAIA water samples from rice fields

The usefulness of the assay for the analysis of real samples was evaluated by recovery experiments and comparison with HPLC measurements using water samples collected from rice paddies. Initially a representative water sample (surface water I-1) from rice fields with no record of clomazone application was spiked with various amounts (0.8 to 3.2 ng/ml) of clomazone and the recovery was determined by the PHAIA method, Table 2. Good recoveries were obtained with water samples diluted two-fold or more. Using a two-fold dilution, the actual LOD of the assay was 0.3 ng/ml.

After this initial experiment, the assay performance was compared to that of HPLC. To this end 13 floodwater samples collected at different time points along the rice harvest were analyzed by HPLC as described by Carlomagno et al.7 Four samples were below the LOD of both methods; the rest showed a very good correlation between both techniques ($y = 0.92 x + 1.3$, $R^2 = 0.98$), figure 5. 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 and low cost of the ELISA technique is considered. Indeed, HPLC analysis requires laborious filtering steps, and the application of large volumes of water samples through pre-concentration columns, while in contrast small-volumes (<1 ml) of water samples can be readily analyzed in parallel by ELISA after a 2 min centrifugation step.

Adaptation of the clomazone PHAIA into a rapid and highly sensitive assay for the detection of the herbicide

A major aid to reduce the release of agrochemicals in the environment is the use of simple tests that provide ‘on site’ information about these chemicals, allowing farmers to take programmed or counteractive actions. In that regard, an advantageous characteristic of PHAIA is its positive readout, which can be easily adapted into rapid assay formats. Figure 6 shows the performance of the method using polystyrene tubes coated with antibody MAb5.6, water sample I-1 and the phage clone ICX11. The assay supported detection as low as 0.4 ng/ml of clomazone by simple visual inspection, which is well below the environmental recommended limit. The PHAIA tubes were also used to rank by the naked eye, in a semi-quantitative way the clomazone concentration in the set of fields samples used for the HPLC-ELISA comparison. Four samples ranked as negative (below 0.4 ng/ml) while all other samples were positive, among these, two samples fell in the 0.4–3.2 ng-ml range, and the rest produced signals higher than 3 ng/ml, showing excellent correlation with HPLC and PHAIA in the ELISA format (data not shown). This performance was obtained with inexpensive commercial reagents. It could be improved dramatically in speed of analysis and in sensitivity using simple tube or lateral flow devices with phage-enzyme conjugates and/or portable readers.

CONCLUSIONS

We described the development of an immunoassay for the noncompetitive detection of clomazone, further demonstrating the general applicability of the PHAIA principle. As observed before, using the same antibody for detection, the PHAIA format provides an increased sensitivity (greater than ten-fold) as compared to the conventional competitive assay, with a remarkably enhanced specificity. The method works with real samples, with an actual limit of detection more than ten-fold lower than the environmental limits, and was

adapted into a very simple format that without instrumental aid can be used on-site for regular monitoring and decision making.

Acknowledgments

This work was supported in part by NIH Fogarty International Center Grant TW05718; FPTA 226 INIA, Uruguay; the NIEHS Superfund Basic Research program, P42 ES04699; and the NIEHS Center, P30 ES05707. We thank Dr. R. Zanella, Departamento de Quimica, Universidade Federal de Santa Maria, RS, Brazil, for his kind help with the HPLC technique. BDH is a George and Judy Marcus Senior Fellow of the American Asthma Foundation

REFERENCES

1. Jonsson C, Maia A, Ferreira C, RIbeiro E. Verein Int. Verein Limmnol. 1998; 26:1724–1726.
2. Kidd, H.; James, D. The agrochemicals Handbookhn. Cambridge, UK: 1991.
3. Zanella R, Primel E, Machado S, Goncalves F, Marchezan E. Chromatography. 2002; 55:573–577.
4. Tomco PL, Holstege DM, Zou W, Tjeerdema RS. J Agric Food Chem. 58:3674–3680. [PubMed: 20178392]
5. Quayle WC, Oliver DP, Zrna S. J Agric Food Chem. 2006; 54:7213–7220. [PubMed: 16968085]
6. Li LF, Li GX, Yang RB, Guo ZY, Liao XY. J Environ Sci (China). 2004; 16:678–682. [PubMed: 15495980]
7. Carlomagno M, Matho C, Cantou G, Sanborn JR, Last JA, Hammock BD, Roel A, Gonzalez D, Gonzalez-Sapienza G. J Agric Food Chem. 58:4367–4371. [PubMed: 20302341]
8. Cardozo S, Gonzalez-Techera A, Last JA, Hammock BD, Kramer K, Gonzalez-Sapienza GG. Environ Sci Technol. 2005; 39:4234–4241. [PubMed: 15984805]
9. Kim HJ, Rossotti MA, Ahn KC, Gonzalez-Sapienza GG, Gee SJ, Musker R, Hammock BD. Anal Biochem. 401:38–46. [PubMed: 20152791]
10. Inaba J, Nakamura S, Shimizu K, Asami T, Suzuki Y. Anal Biochem. 2009; 388:63–70. [PubMed: 19454218]
11. Koppatscheck F, Liebl R, Alan L, Kriz A, Melhado L. J Agric Food Chem. 1990; 38:1519–1522.
12. Lorenzo C, Last JA, Gonzalez-Sapienza GG. Parasitology. 2005; 131:669–677. [PubMed: 16255825]
13. Martens CL, Cwirla SE, Lee RY, Whitehorn E, Chen EY, Bakker A, Martin EL, Wagstrom C, Gopalan P, Smith CW, et al. J Biol Chem. 1995; 270:21129–21136. [PubMed: 7545665]
14. 1980; Vol. EEC No. L229/11-29. Brussels: European Economic Community; 1980.

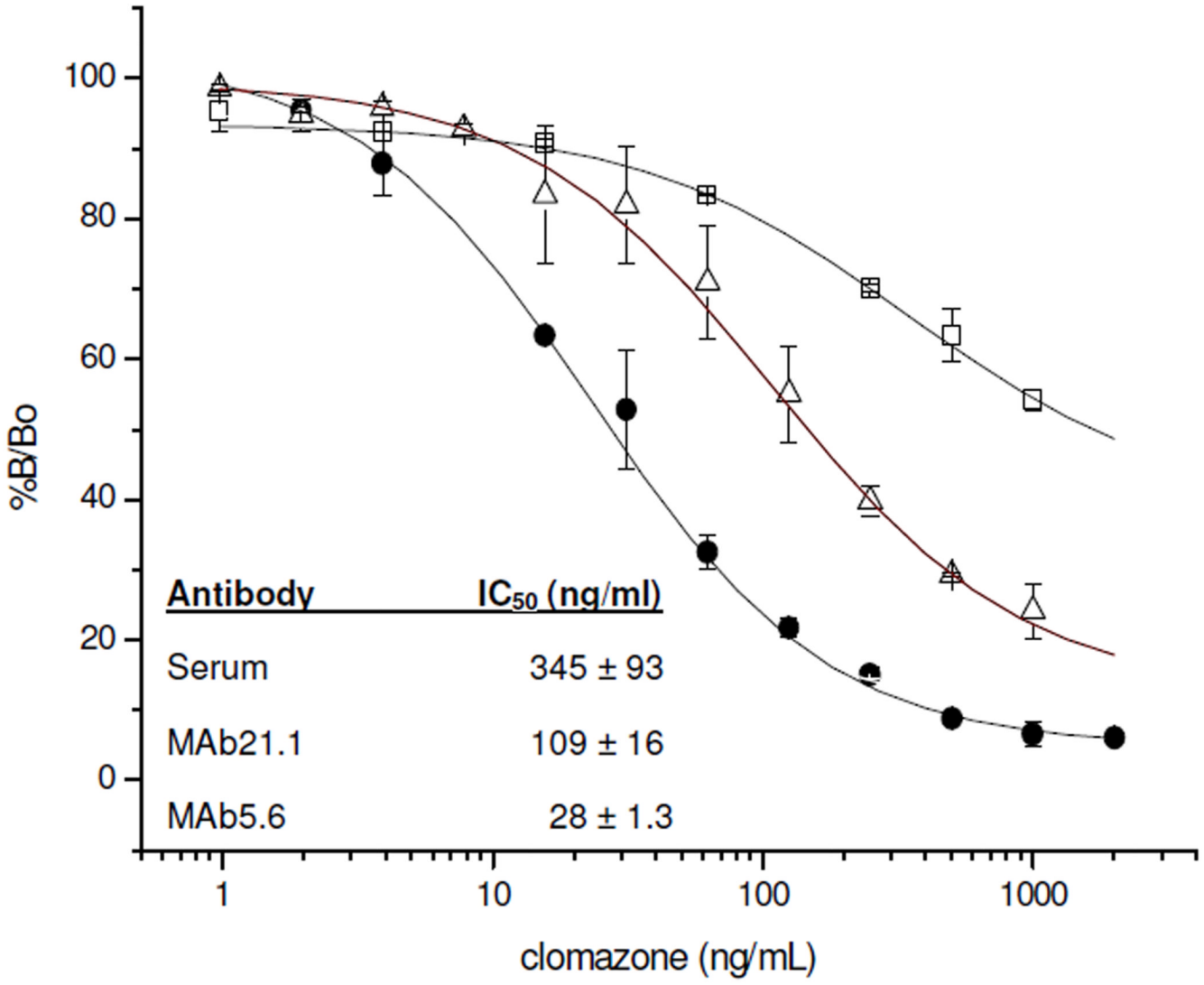

Figure 1. Competitive ELISA set up with monoclonal antibodies and the parent mouse serum against clomazone

The performance of two monoclonal antibodies, MAb5.6 (circles) and MAb21.1 (triangles) and the parent mouse serum (squares) were compared in a competitive format. Coating conditions and antibody dilutions were optimized for each antibody using checkerboard titrations. The IC_{50} for each antibody is shown in the insert.

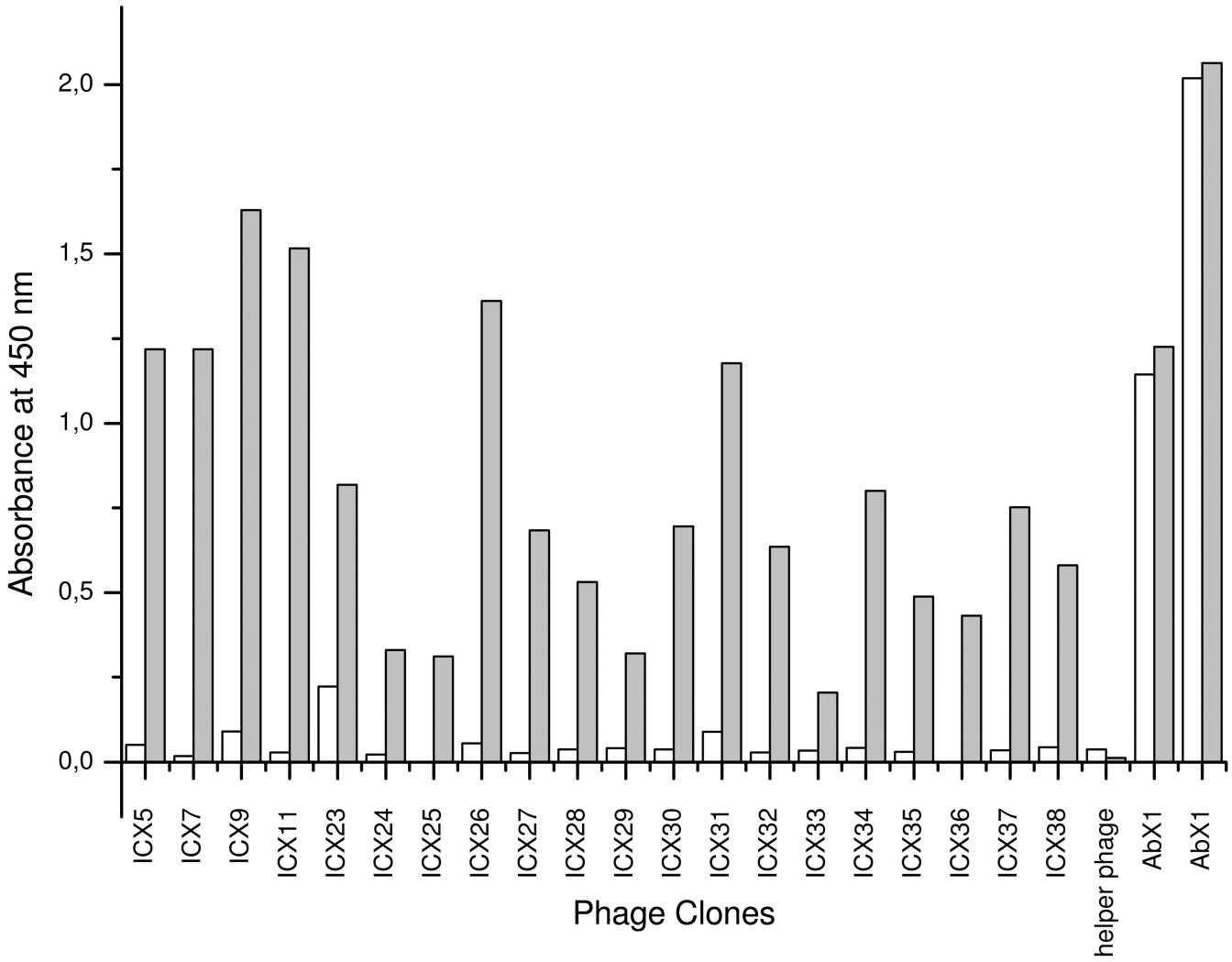

Figure 2. Screening of MAb5.6/clomazone immunocomplex specific phage clones

Twenty out of forty clones reacted specifically with the immunocomplex MAb5.6/clomazone (grey bars, 20 ng/ml clomazone), showing little or negligible signal with the uncombined antibody (white bars, without analyte). The reactivity of the helper phage is shown as negative control, and the reactivity of two representative clones displaying strong binding to immunocomplex and the uncombined antibody are shown on the right (clones AbX1 and AbX40). The 96-well ELISA plate coated with 0.5 μg/ml of MAb5.6 was incubated with 50 μl of phage supernatants plus 50 μl of PBST with 40 or 0 ng/ml of clomazone. Anti M13-phage antibody-HRP was used for detection.

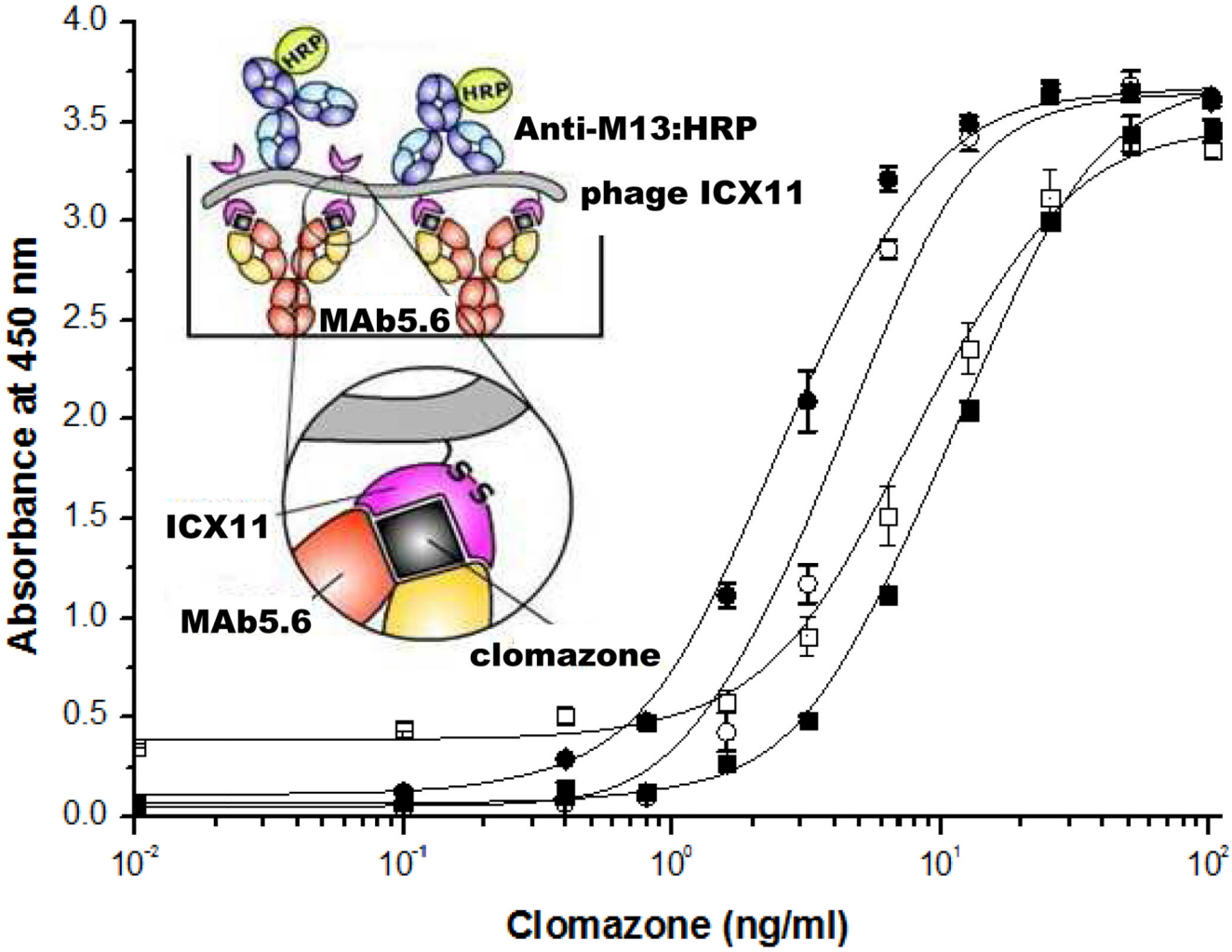

Figure 3. Phage Anti-Immunocomplex Assay (PHAIA) set up with four different phage-borne peptides

PHAIA was performed on ELISA plates coated with MAb5.6; immunocomplex formation in the presence of various concentrations of clomazone was revealed using the anti-immunocomplex phage clones and anti-M13-HRP as depicted in the insert. The curves are: clone ICX11, black circle, Saturation Concentration (SC_{50}) = 2.6 ± 0.3 ng/ml; clone ICX5, white square, SC_{50} = 8.1 ± 1.4 ng/ml; clone ICX9, black square, SC_{50} = 10.8 ± 0.8 ng/ml; and clone ICX7, white circle, SC_{50} = 3.9 ± 0.2 ng/ml).

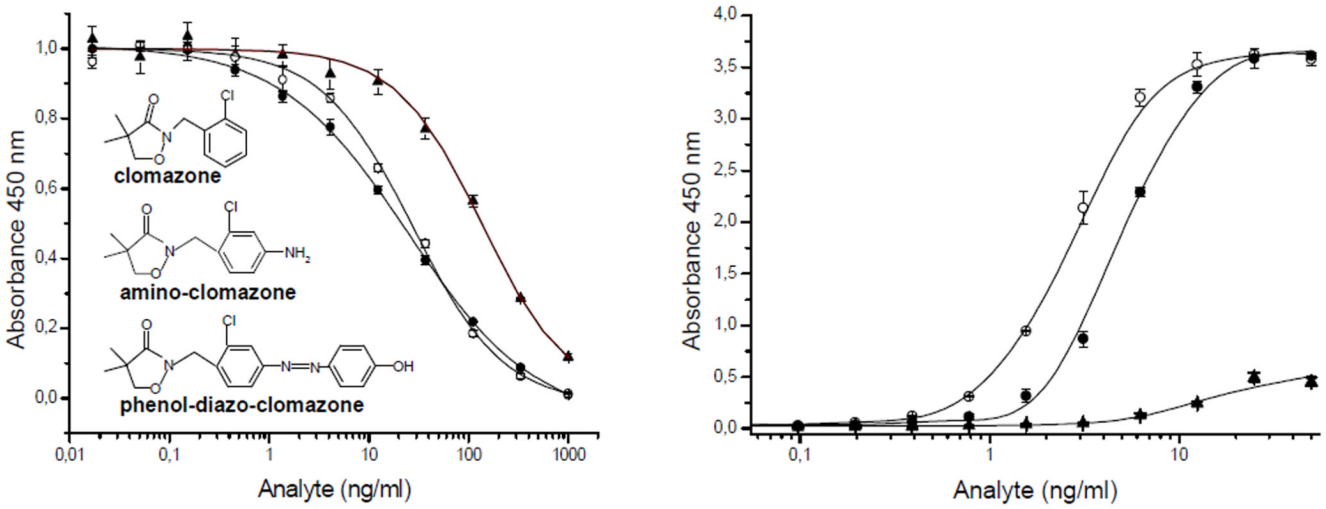

Figure 4. The ternary complex formed in the PHAIA method promotes an increased specificity for the analyte

The conventional competitive assay (left) and PHAIA (right) were tested with serial dilutions of the herbicide (white circles), aminoclomazone (black circles) or phenol-diazo-clomazone (triangles). Percentage of cross-reactivity (100 × clomazone SC_{50} divided by cross-reactive compound SC_{50}) for aminoclomazone and phenol-diazo-clomazone were 110 and 20% for the competitive assay, and 54% and < 0.2% for PHAIA, respectively.

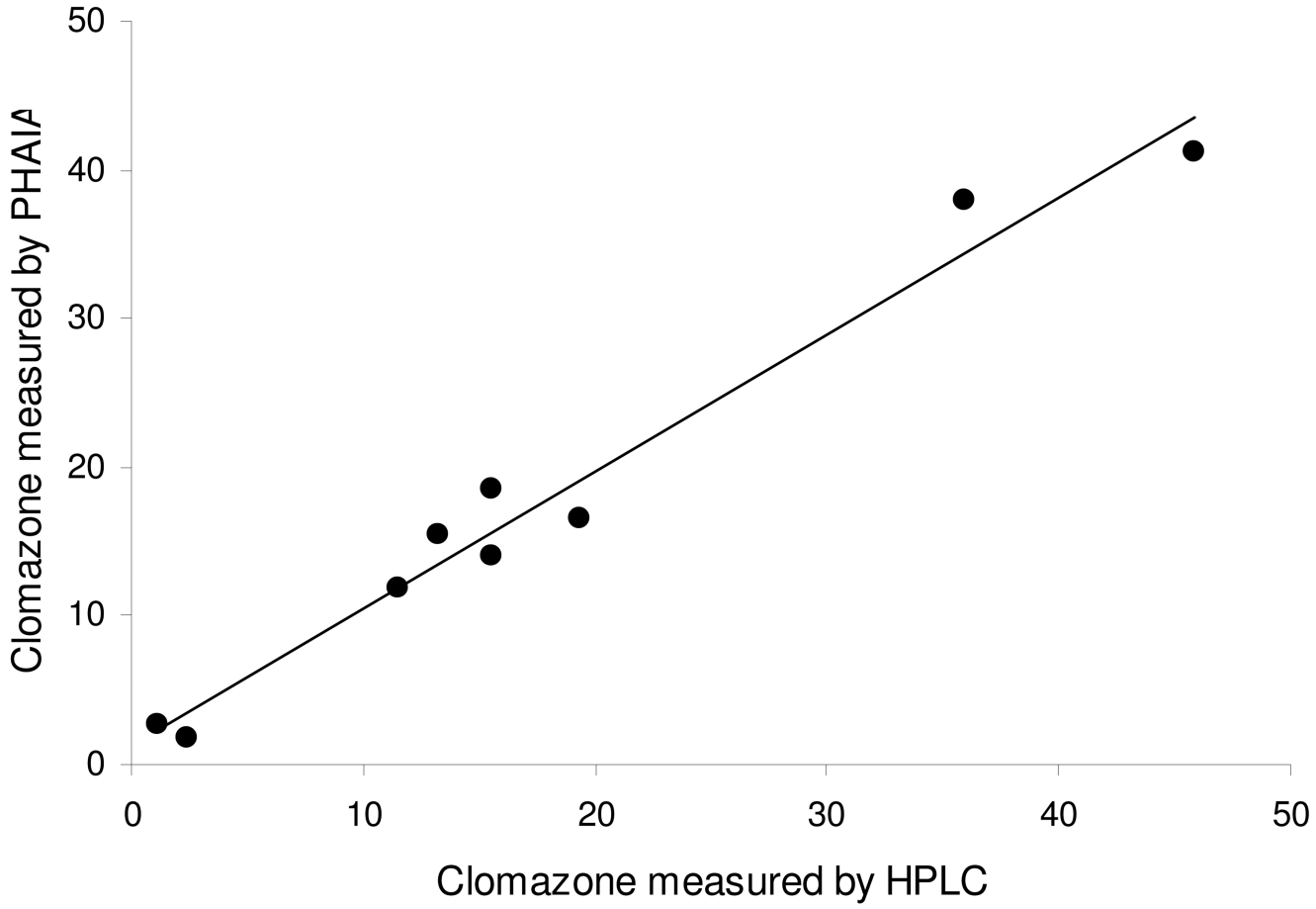

Figure 5. Comparison of clomazone measurement by PHAIA and HPLC

Eleven water samples were used to evaluate the correlation between the reference technique, HPLC (LOD = 0.5 ng/ml) with the PHAIA assay (LOD = 0.3 ng/ml). Samples were analyzed by clomazone PHAIA ELISA in only two hours.

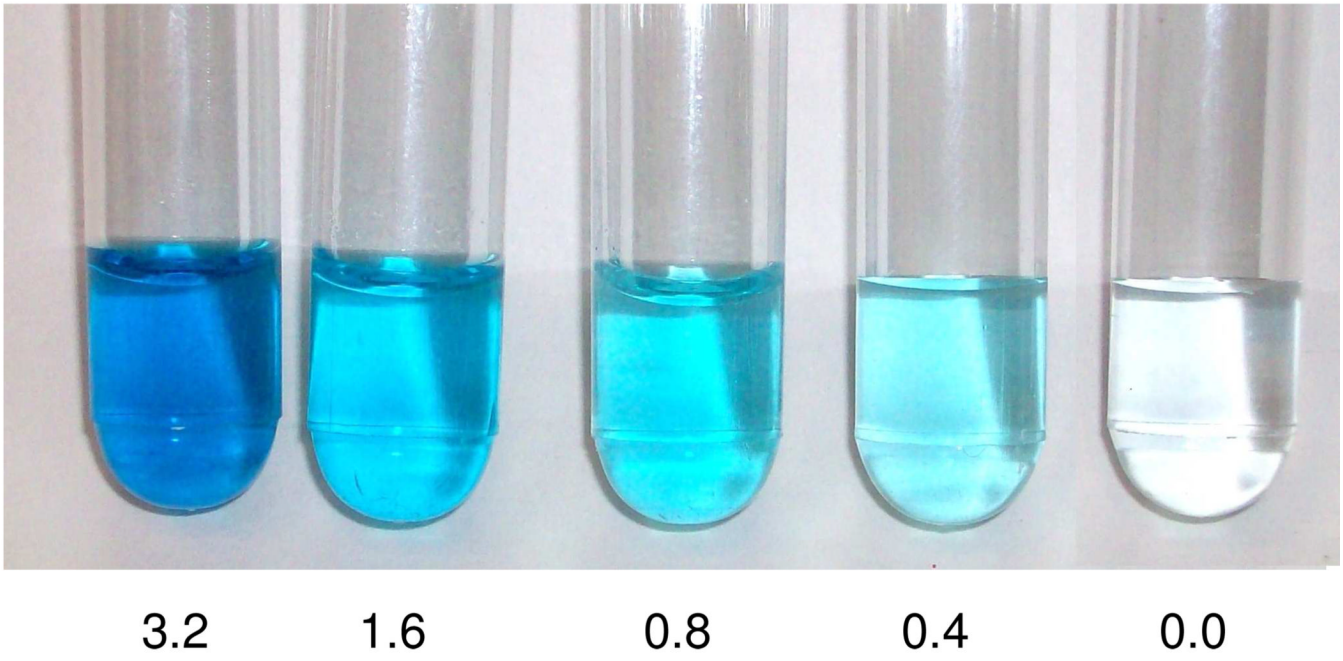

Figure 6. PHAIA immunotube assay

Tubes coated with MAb5.6 were incubated with clomazone standards (ng/ml) in PBST and phage ICX11, and the bound phage was detected with anti-M13-HRP.

Table 1

Phage borne peptides isolated with the MAb5.6/clomazone Immunocomplex.

Name	Sequence	Frequency
ICX5	C I S **A P N** M **E A** C	(5)
ICX9	C A L **A P N** Q **E A** C	(4)
ICX7	C T Q F **P N** P **E A** C	(9)
ICX11	C L E **A P N** I **E** G C	(2)
AbX40	C G F D G S Y Y F C	

The number of isolates bearing the same sequence is indicated between parentheses.

Table 2

Recovery of clomazone in surface water I-1 measured by PHAIA

Percentage of water sample (%)	Clomazone (spiked) (ng/ml)	Clomazone (measured) (ng/ml, *n* = *3*)	mean recovery (%, *n* = 3)
100	3.2	4.46 ± 0.37	139
	1.6	2.4 ± 0.17	150
	0.8	1.73 ± 0.08	216
50	3.2	3.36 ± 0.02	105
	1.6	1.97 ± 0.15	123
	0.8	1.05 ± 0.04	131
25	3.2	3.1 ± 0.21	97
	1.6	1.63 ± 0.04	102
	0.8	0.92 ± 0.01	115
12.5	3.2	2.68 ± 0.41	84
	1.6	1.40 ± 0.10	88
	0.8	0.84 ± 0.05	105