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Comparative study of immunoassay and Gas chromatography for chlorpyrifos detection in laboratory spiked water samples

Zoology

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ABSTRACT

Indirect and competitive enzyme-linked immunosorbent assay (I-ELISA and C-ELISA) were developed optimized and compared with gas chromatography (GC) for chlorpyrifos (CPF), detection. Chlorpyrifos (haptens) was conjugated to BSA proteins to obtain immunogens. Cross-reactivity (CR) for CPF was found less than 20 % for I-ELISA while showed no CR for BSA. Characterization for limit of detection was done against chlorpyrifos residue which reveals GC was the fastest and most sensitive mehod with the LOD of 0.1 ng ml–1 while C-ELISA and I-ELISA shows LOD of 100 ng ml–1 and 10 ng ml–1 respectively. All methods were further applied to analyzed % recoveries for chlorpyrifos spiked samples and mean recovery found to be between 85% to110% and comparable LOD'S for all three techniques thereby immunoassay can be used for screening of food and environmental samples for CPF residues without complicated clean-up as in case of GC.

INTRODUCTION

Due to widespread use of chlorpyrifos in agriculture, its residue occurrence in environment has been reported by Neidert et al (1994), Cochran et al (1995), Kamrin et al (1997) which poses potential health hazards analyzed by Rao et al (2010), Parmar and Kocher (2013). Most of the officially accepted methods of pesticide residue analysis deal with the instrumental methods, such as gas-liquid chromatography and liquid chromatography coupled with mass spectrometry. In spite of high sensitivity, these methods rely on highly skilled technical expertise capable of isolating the pesticide components from complex matrixes and subsequently their detection at very low levels. Alternative to these conventional methods i.e. immunochemical techniques (ELISA) began recently to gain acceptance as a fast and cost-effective tools for separating and/or detecting trace amounts of pesticides as proved by Tomlin et al (1997) . This technique was recognized as a valuable tool in residue analysis and complements the conventional analytical methods. Because these techniques provides accurate and rapid sample testing and the generation of the results is more cost-effective when compared with conventional chromatographic analysis. Among the organophosphate group, chlorpyrifos pesticides is widely used in agricultural and domestic settings Hennion et al (1998), Karima et al (2007). Therefore, this growing demand for more rapid and economical methods for determining pesticide residues immunoassays can meet such demands. So present study addresses the sensitive and selective ELISA (indirect and competitive) development for chlorpyrifos using the sera against BSA hapten –conjugate. Further the study deals with comparison of these immunoassays and gas chromatographic to find out their limit of detection (LOD) and % recoveries of chloprpyrifos in laboratory-spiked samples.

MATERIAL AND METHOD

Screening of antisera

The titer of the serum from each animal was determined by measuring the binding of serial dilutions to microtiter plates coated with different concentrations of the homologous coating antigens. Microtiter plates were coated with Hapten-BSA (10 µg/ml, 100 µl/ well) in PBS (10 mM, pH 7.2) and incubated overnight at 4°C. On following day, the plates were washed five times with PBST (pH 7.4) and were incubated for 1 h at 37°C. The dilution of antiserum ratios were 16000, 32000 and 64000. After another washing step, 100 µl /well of goat anti-rabbit IgG, conjugated with distilled water, diluted (1: 10000) and incubated for 1 h. Then the plates were washed again, and (0.04% OPD in PBS containing 0.01% H2O2) was added (100 µl/well). After incubation at 37°C for 30 min, the reaction was stopped by adding 3 N NaOH (50 µl/well) and the absorbance was read at 492 nm. The 1st bleed corresponds to preimmune sera collected on 0th day of the experiment (collected before injecting the rabbits with the antigen). The antibody titres were calculated from preimmune sera collection at 0 day up to 75 DPI.

Determination of cross-reactivities

Anti-CPF antibodies developed in the experiment above were tested for cross reactivity with the carrier protein i.e. BSA which was calculated according to the formula given below:

$$\% \text{ Inhibition} = \frac{A_{max} - A_{test}}{A_{max}} \times 100$$

Cross reactivity (CR) of the compounds structurally related to chlorpyrifos i.e its analogue (artificially synthesized hapten) was investigated by preparing standard curves using the indirect assays and determining their I50 values (concentration at which binding of the antibody to the enzyme tracer is inhibited by 50%). The cross-reactivity values were calculated as follows: (I50 of chlorpyrifos/I50 of compound) × 100.

ELISA development

Firstly, 100 µl of antigen was coated to the surface of an high binding ELISA plate (Nunc, Denmark) using coating buffer (carbonate –bicarbonate buffer) to immobolise the antigen. After coating it was kept for 4 hours at 37°C and plate was washed with PBST thrice after that Blocking was done with 1% BSA solution by pouring 300 µl in each well and the plate was kept overnight at 4 °C. The plate was washed again with PBST thrice. The wells of the plates were then coated with 100µl of serum samples (100 times diluted with 0.1% BSA in PBS) and incubated for 2 hours at 37°C. The plate was washed thrice with PBST. Then 100 µl of conjugate (1:4000) goat anti rabbit HRPO conjugate (Genie, Bangalore) was added in all the wells.The plate was washed with PBST thrice. After that 100 µl of OPD-H2O2 was poured and incubated for 20 minutes in dark. After 20 minutes, the reaction was stopped using in 1 M H2SO4. The absorbance was measured using an ELISA reader (Tecan) at 492 nm. Antibody titre was calculated in terms of % inhibition by the formula:

$$\% \text{ Inhibition} = \frac{\text{Mean absorbance of treated serum - Mean absorbance of control serum*}}{\text{Mean absorbance of treated serum*}} \times 100$$

* Serum of treated group before treatment i.e. at 0 DPI was considered as control.

In case of competitive inhibition ELISA although all the procedure was similar, but additionally one step was introduced i.e. after blocking with 1% Lactogen in PBS (37 °C, 1 h) the plate was incubated with rabbit antiserum dilutions of 100 and 500 times and

antigen with 0.01 ng-100ng at 37 ºC for 1 hour.After that plate was kept to allow for competition between sample containing free antigen and bound antigen for limited antibody sites.

Standard curve immunoassay for chlorpyrifos

Chlorpyrifos free water samples were used for spiking of known concentration of standard protein with different concentrations (known doses) of pure CPF to prepare the standard curve for chlorpyrifos between the linear working range (0.000001-100 µg/ml).

Collection of chlorpyrifos free water samples

chlorpyrifos free water samples were collected from the Department of Entomology, PAU, Ludhiana. Gas chromatography (GC) was used for the confirmation of free chlorpyrifos samples. These water samples were further detected with indirect plate ELISA.For positive control (1-1000µg/ml) pure chlorpyrifos was used with antibody dilution of 1:5000. In case of negative control no antigen was used, while in test chlorpyrifos free water sample were used as antigen with similar antibody dilution of 1:5000. Confirmation of CPF free water sample was done by taking absorbance at 492 nm on ELISA reader.

Prepartion of stock solution for spiking of pure chlorpyrifos into chlorpyrifos free water samples

For spiked samples a known concentration of standard (pure chlorpyrifos) was added (spiked) into chlorpyrifos free water sample as detected by GC. Different concentrations of pure CPF ranging from 0.000001-100 µg/ml were added to detect the presence of chlorpyrifos. Percent inhibition in terms of binding inhibition (%) was calculated by indirect plate ELISA at 492 nm. Same samples were used for chlorpyrifos detection limit by gas chromatography (GC). For ELISA a chlorpyrifos stock solution was prepared in methanol at the concentration of 1000 µg/ml and for GC a stock solution was prepared in acetonitrile at the concentration of 10µg/ml. The ELISA stock standard was diluted to yield working standards of 1000, 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/ml. While the stock solution in case of GC was diluted to make working standards of 10, 1, 0.1, 0.01, 0.001 and 0.0001ng/ml.

RESULT AND DISCUSSION

Cross reactivity determination

Detection of cross-reactivity (CR) of pure chlorpyrifos with Bovine serum albumin (BSA)

The inhibition ratio was calculated by the difference in absorbance between the buffer containing chlorpyrifos and without chlorpyrifos i.e. negative control. As % inhibition is inversely proportional to its cross reactivity value, therefore results revealed that pure chlorpyrifos showed cross reactivity with its own compound ranging from 97.3-97.4%, while for BSA it showed almost negligible cross reactivity (1.48-3.43%). Table.2.However no report is available on testing of the cross-reactivity of the anti-CPF antibodies with KLH carrier protein also (Cho et al 1997).

Detection of cross-reactivity of pure chlorpyrifos with artificially synthesized hapten

Cross reactivity (CR) of the compounds structurally related to CPF i.e artificially synthesized hapten. CR of pure CPF obtained was 0.22%. In the same concern literature shows that assay specificity evaluated by using compound that are structurally related to target analyte i.e. pure CPF, the cross reactivity of two analogues of chlorpyrifos i.e. methyl and TCP (trichlorophenol pyridynl) was found to be 0.039 and 2.27 % , respectively (Cho et al 2002). The results were further supported by Schneider et al (1995) and Chen et al (2010) with CR of 0.16 and 0.14 %, respectively for carbfuran and parathion pesticide.

Table 1. Detection of cross-reactivity (CR) of pure chlorpyrifos with Bovine serum albumin (BSA) by indirect plate ELISA

% Inhibition of antichlorpyrifos antibodies (wells 1-9)									
%Inhibition	Negative control (wells 1-3)			Pure CPF (wells 4-6)			BSA (wells 7-9)		
	-	-	-	2.69±0.44	2.71±0.46	2.54±0.46	98.52±0.51	96.57±0.57	97.63±0.56
%Cross reactivity	-	-	-	97.31	97.29	97.46	1.48	3.43	2.37

All the values are in mean ±S.E

(-) refers to as blank control with no colour development

Analysis of chlorpyrifos residue detection level in laboratory spiked samples through immunoassay

Through indirect plate ELISA (I ELISA)

Limit of detection for commercially available chlorpyrifos as antigen with raised antibodies was determined by indirect plate ELISA. Results in table 3 showed that the dilution of antigen from 1-12 wells lied between the ranges of 50µg-0.0001ng, while antibodies concentrations were taken to be 1:100. This plate showed trend of increased % binding inhibition of antigen for antibody that was indicated by minimum colour development

and vice-versa. Interpretation of results from the table 3 indicated that 100ng/ml of antigen with 1:100 antibody dilutions showed perfect combination for attaining the maximum % inhibition (96.52±0.52) to attain the cut of dose for finding out the LOD for pure CPF.

Table 2. Percent inhibition of antichlorpyrifos antibodies for determination of limit of detection (LOD) for pure chlorpyrifos by indirect plate ELISA

Type of antigen in rows	% Inhibition of antichlorpyrifos antibodies (wells, 1-12)											
	1	2	3	4	5	6	7	8	9	10	11	12
A-B (-ve) control	-	-	-	-	-	-	-	-	-	-	-	-
C-F (pure Chl)	-	-	-	2.69±0.44	12.49±0.42	31.54±0.46	50.89±0.65	84.83±0.56	96.52±0.51	91.57±0.56	94.43±0.54	92.43±0.54
Antibody A,B (NS)1:100, C-H (IS) 1:100 Conjugate A-H:1:10000 NS- referes to normal seum IS- refers to immune serum												

All the values are in mean ±S.E

(-) refers to as blank control with no colour development.

Through competitive ELISA © ELISA)

Antibodies produced against prepared hapten of CPF were used for quantitative detection through competitive inhibition between synthesized and commercially avialiable CPF by using indirect plate ELISA. Results indicated from table 4, A-D set that with increase in concentration of pure CPF added for competition, lesser amount of antibodies was available for binding with already coated synthesized antigen and hence there was a decrease in absorbance, which was seen as decrease in intensity of sunset-orange colour in the wells. The results showed that at the concentration of 10 ng/ml of antigen or above, no colour was developed thereby indicating maximum % binding inhibition (97.43±0.54) and the sensitivity remained 10 ng/ml Table 4. While in case of gas chromatography, LOD was found to 0.1ng/ml.

Table 3. Competitive inhibition ELISA for detection of pure chlorpyrifos

Type of antigen in rows	% Inhibition of antichlorpyrifos antibodies (wells 1-12)											
	1	2	3	4	5	6	7	8	9	10	11	12
A (4-12) Pure CPF	-	-	-	2.87±0.54	-	-	97.43±0.54	76.57±0.57	57.43±0.54	36.57±0.57	26.57±0.56	18.57±0.52
B (4-12) Pure CPF	-	-	-	2.67±0.54	-	-	96.43±0.54	72.57±0.57	56.43±0.54	33.57±0.57	24.57±0.56	19.57±0.52
C (5-10) Pure CPF	-	-	-	-	2.67±0.54	2.69±0.53	2.34±0.41	1.78±0.43	1.45±0.47	0.98±0.67	-	
D (5-10) Pure CPF	-	-	-		2.45±0.57	2.75±0.54	2.63±0.47	1.69±0.44	1.48±0.48	0.93±0.65		
Antibody AB (NS)1:100, C-H (IS) 1:100												

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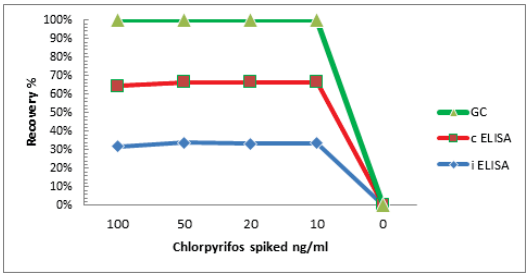
Conjugate A-H:1:10000
NS- referes to normal serum
IS- refers to immune serum

Comparisons of gas chromatography (GC), indirect and competitive ELISA (iELISA, cELISA) techniques for recovery of spiked concentration of pure chlorpyrifos

Recovery through three methods i.e. GC, indirect plate and competitive inhibition ELISA was calculated. Results revealed that similar concentration range of 0.1-100ng /ml of CPF in all the methods with its range of 80-120% (Table 5). It indicated that all the methods were found to be almost are comparable for estimation of pesticide amount present in the samples, but varied with the LOD for each method. In case of present study LOD was found to be 0.1 ng /ml 10 ng/ml and 100 ng/ml for gas chromatography indirect plate and competitive inhibition ELISA respectively. All the recoveries value fall into the acceptable range of 80 -120% which were also supported by Krotzky and Zeeh (1995).

Figure1 . % Recovery comparisons of gas chromatography (GC), indirect and competitive ELISA (iELISA, cELISA)



SUMMARY:

Conclusion drawn from present study revealed that both of the techniques (GC and ELISA) were highly correlated as the amount of chlorpyrifos recovered in both cases was almost equal thereby proving the fact that immunoassay techniques is equally sufficient for pesticide residue detection and could be used as effective tool for daily routine test with various limit of detection.

References

1. Tomlin, C. (1994). The Pesticide Manual: A World Compendium. 10th ed. British Crop Protection Council. Cambridge: Royal Society of Chemistry. 3351–52.
2. Hennion, C., & Barcelo, D. (1998). Strengths and limitations of immunoassays for effective and efficient use for pesticide analysis in water samples: a review. Anal. Chim Acta 362, 3.
3. Karima, H. E., Haggag, Sayeda, & Farghaly, F. (2007). Effect of Metalaxyl and CPF-Methyl Against Early Blight (Alternaria solani, Sor.) and Whitefly (Bemisia tabaci, Genn.) in Tomato and Eggplant. J Appl Sci Res 3, 723-32.
4. Cho ,Y., Matsuoka, N., & Kamiya, A. (1997). Determination of organophosphorous pesticides in biological samples of acute poisoning by HPLC with diode-array detector. Chem Pharm Bull 45, 37–40.
5. Cho, Y. A., Lee V., Park, E .Y., Lee, Y. T., Bruce, D. H., & Chang, A. K., et al. (2002) Development of an ELISA for the Organophosphorus Insecticide CPF. Bull Korean Chem Soc 23, 481-87.
6. Chen,Y. P., Ning, B., Liu, N., Feng, Y., Liu, Z., & Liu, X., et al. (2010) Rapid and sensitive fluoroimmunoassay based on quantum dot for detection of chlorpyrifos residue in drinking water. J Environ Sci Health 45, 508-15.
7. Schneider, P., Gee, S. J., Kreissig, S. B., Harris, A. S., Kramer, .P M., & Marco M. P., et al. (1995) troubleshooting during the development and use of immunoassays for environmental monitoring. New Frontiers in Agrochemical Immunoassay 103-105.
8. Krotzky, A. J., & Zeeh, B. (1995) Immunoassays for residue analysis of agrochemicals: proposed guidelines for precision, standardization and quality control (Technical Report). Pure Appl Chem 67, 2065-88.
9. Parmar, P.,& kocher, D. K. (2013) Study of Immunological Parameters from the Serum of Rabbits Exposed to Chlorpyrifos Pesticide Research indian journal of applied research 15-16.
10. Kamrin, M. A., (1997). (In press). Profile, Toxicity, environmental impact and fate. CRC Press, Boca Raton, FL.