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Effect of Spacer and the Enzyme-linked Immunosorbent Assay

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ABSTRACT

The effect of spacers and the enzyme-linked immunosorbent assay (ELISA) formats on the functional parameters of assays such as lower detection limit, inhibitory concentration at 50 per cent (IC_{50}), and specificity were studied. Enzyme conjugates having hydrophobic and hydrophilic spacers were prepared using *O*-isopropyl methylphosphonic acid (IMPA) and horseradish peroxidase (HRP) as an enzyme label. Comparison was made with reference to enzyme conjugate without any spacer. The present investigation revealed that the presence of a hydrophilic spacer in the enzyme conjugate significantly improves the sensitivity of assays. An enhanced IC_{50} value achieved was 0.01 µg mL^{-1} for free antigen detection by direct immunoassay using hydrophilic spacers and precoating of ELISA plates by secondary antibody. The use of a hydrophilic spacer might have helped in projecting the hapten in the aqueous phase, leading to enhanced antibody binding signal and improved sensitivity of the assay.

Keywords: ELISA, lower detection limit, $IC_{50,}$ hydrphobic, hydrophilic, IMPA

1. INTRODUCTION

Organophosphorous (OP) compounds find out wide range of applications mainly in agriculture settings and chemical warfare[1]. These compounds have distinct advantages as they are relatively easy to make, and biodegradable readily by hydrolysis. Over the years, extensive efforts were carried out to evolve new OP agents as pesticides and chemical weapon. Their residues creates potential hazards for human health due to blocking of neural transmission by permanently binding with acetyl cholinesterase enzyme resulted in the paralysis and mortality[2,3]. These toxic compounds are generally degraded by hydrolysis to first produce alkyl alkyl phosphonic acids (AAPAs) and secondly alkyl phosphonic acids (APAs)[4]. *O*-isopropyl methylphosphonic acid (IMPA) and methyl phosphonic acid (MPA) (Fig. 1) are some hydrolytic degradation product of OP compounds which exerts deleterious effect on the atmosphere and human health. Current analytical methods using gas and liquid chromatography for the detection of small molecular residues (Mw < 1000) like IMPA are sensitive and reliable[5]. However, they are costly and time-consuming due to the complicated sample preparations and derivatisation steps[6-10]. Therefore, there is a growing demand for more rapid and less costly methods for determining small molecular residues in the environmental sample. Enzyme-linked immunosorbent assay (ELISA) is the alternative technique widely used and accepted due to its sensitivity, fastness, and cost-effectivity for the identification of chemicals like pesticides[11–15].

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To achieve better performance of antibody, enzyme tracer and analyte, incubation strategy has been chosen so that analyte can compete strongly even in the low concentration. Similarly, to achieve optimum conditions for the competition the conjugated hapten should have less affinity to antibody than that of analyte. Additionally the catalytic activity of hapten-enzyme conjugate is also of great importance[16]. Considerable variations in the affinity, owing to the hapten structures, have been reported between homologous and heterologous ELISA[17–18]. In case of homologous ELISA less sensitivity has been observed because of strong interaction between antibody and enzyme conjugate.

The Effect of ELISA format and spacer of enzyme conjugate both were found to play an important role in order to improve the sensitivity of immunoassay[19–20]. There are two kinds of assay formats, with either immobilised antigen or antibody. It was observed that in the antigen-immobilised format, varying lengths of spacer arm of the coating antigen had significant effect on the sensitivity of ELISA. A remarkable improvement in the sensitivity of ELISA has been observed by various workers using heterology in spacer structure of coating antigen[21–24]. The use of long flexible chains of highly hydrophilic polyethylene glycol (PEG) for the preparation of

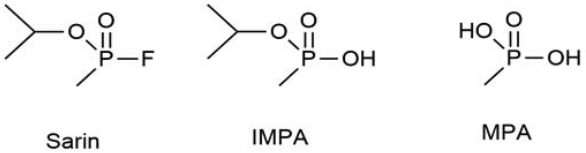


Figure 1. Structure of Sarin, *O*-isopropylmethylphosphonic acid (IMPA), Methyl phosphonic acid (MPA).

coating antigen was also been found to be helpful in order to improve the sensitivity by facilitating the aqueous solubility of hapten, thereby greatly improving protein conjugation. Studies on the effect of spacer length in enzyme conjugate has been studied at length however, not much reports are available discussing the effect of ELISA format and spacers on assay sensitivity.

We are engaged in the development of methods for the retrospective detection of OP toxicant and their markers. In our previous work[25] we reported the production of polyclonal antibodies against the marker of OP toxicant sarin. The obtained antibodies were incorporated into indirect ELISA and tested for assay sensitivity towards sarin and its marker IMPA. The inhibitory concentration 50 per cent (IC_{50}) of free antigen was found to be 0.415 µg mL^{-1} and limit of detection (LOD) was found to be 0.05 µgmL^{-1}. In the present studies antibodies having best binding properties have been used to develop more sensitive ELISA using horseradish peroxidase (HRP) tracers. The aim of the present work was to demonstrate effect of ELISA format and Effect of hydrophobic and hydrophilic spacer-containing OP enzyme conjugates on functional parameters on ELISA using IMPA as a model compound. For present studies polyclonal anti-IMPA antibody (OP-Ab) was prepared in our laboratory from immunogen conjugate 4-(4(isopropoxy (methyl)phosphoryloxy)phenylamino)-4-oxobutanoic acid-BSA[25].

2. MATERIALS AND METHODS

2.1 Reagents

Reagents were obtained from Sigma-Aldrich (Poole UK) unless stated otherwise, including Goat antirabbit IgG peroxidase and Horseradish Peroxidase (HRP). Solvents used were of analytical grade for synthesis. Monitoring of chemical reactions, chromatographic purification and various buffers used in the present studies was similar to the earlier studies described else where[25].

2.2 Instrumentation

The instruments used in the present studies similar to the instruments used in the earlier studies described else where[25].

2.3 Synthesis of Hapten Derivatives

Suitable hapten derivates (2-5) were synthesised by following scheme 1. Four haptens were synthesised by reaction of chlorosarin with different amino acids and peptides. Consequently, incorporation of 2-aminoacetic acid, 4-aminobutyric acid, dipeptides (NH_2-phe-lle-COOH, NH_2-phe-glu-COOH) as a linker to *O*-isopropyl methyl chlorophosphate group was achieved.

2.3.1 Synthesis of OP-1, OP-2, OP-Pep-1, OP-Pep-2

To a stirred solution of 1 (6.4 mmol) in 5 mL of methanol, 15 mL of methanol solution having KOH (16 mmol) and amino acid (8.9 mmol) was added drop wise. Reaction mixture was stirred for 10 min at room temperature followed by filtration and extraction with 1 M HCl-chloroform. The filtrate was dried over anhydrous sodium sulfate and the solvent was evaporated under vacuum. The residue was subjected to column chromatography using silica gel (hexane: ethyl acetate, 8:2) to give 5 as viscous liquid (50 per cent yield).

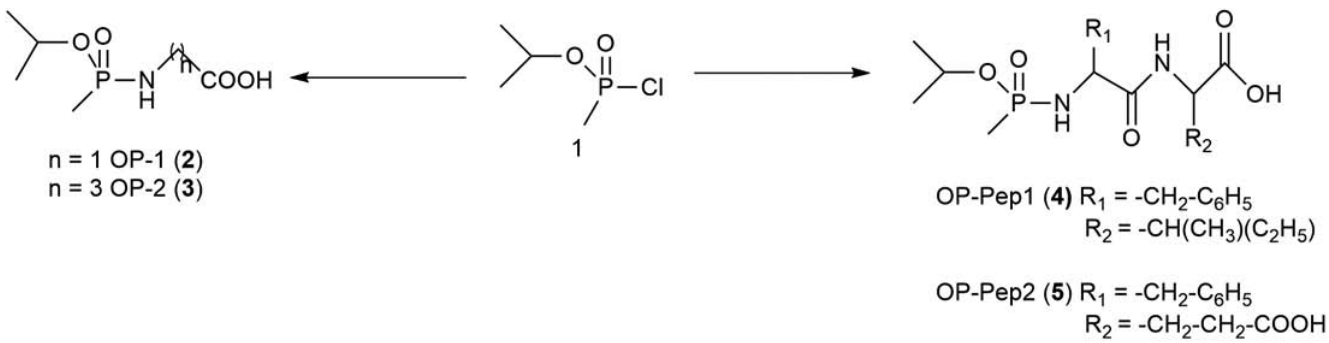
2.3.2 Preparation of OP-1-HRP, OP-2-HRP, OP-Pep1-HRP and OP-Pep2-HRP

The IMPA derivatives with carboxylic end group (OP-1, OP-2, OP-Pep1 and OP-Pep2) were conjugated to HRP enzyme by the *N*-hydroxysuccinimid ester method. To prepare the stock solution *N*-hydroxysuccimide (2.3 mg, 20 µmol) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (20 mg EDAC) were added to a solution of OP-1, OP-2, OP-1-Pep1, and OP-1-Pep2 (10 µmol) in DMF (2000 µL). The mixture was gently stirred at room temperature for overnight. The HRP solution (9.68 mg mL^{-1}; 0.22 µmol mL^{-1}) was prepared in 0.13 mol. L^{-1} $NaHCO_3$ (1000 µL). For preparing the conjugate 0.22 µmol (44 µL) of activated hapten was taken from the stock solution in a vial and added 200 µL (0.022 µmol) of HRP solution. The conjugation mixtures was kept at room temperature for 4 h, and purified by gel filtration chromatography where hapten-protein conjugates were passed through PD10 column in presence of eluent (100 mM phosphate buffer, pH 7.4). Finally the conjugates were stored at -20 °C. The degree of coupling was determined by TNBSA assay calculated at 335 nm, comparing absorbance value of protein before and after conjugation.

2.3.3 Preparation of OP-1-Urea-HRP, OP-1-ADH-HRP, OP-1-ODA-HRP and OP-1-BODA-HRP

It was achieved in two steps:

(a) Coupling of spacers (urea, ADH (adipic acid dihydrazide) and oxy diamine) to HRP: To the 20 mg mL^{-1} aqueous solution of HRP (lot number 28H7848), NHS (40 mg) and EDAC (80 mg) were added and the reaction mixture



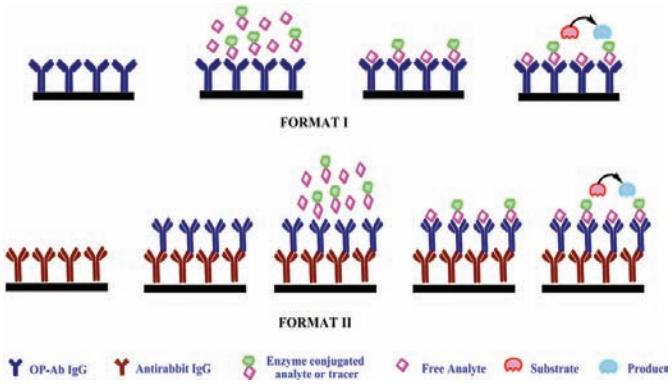
Scheme 1. Reaction scheme for the synthesis of hapten derivates OP-1, OP-2, OP-Pep-1, and OP- Pep-2.

was kept at 4 °C for overnight to activate the carboxylic group of HRP. After activation, an amount of 100 µL was taken and added into three different vials respectively. To this 0.23 µmol of each urea/ADH/oxy diamine in 300 µL of ammonium carbonate (used for urea and ADH)/DMF (for oxy diamine) was added. The solutions were kept at 4 °C for 24 h to allow the HRP-CO-NH-spacer bond formation. All the solutions were then dialysed separately against 3–4 changes of water.

(b) Coupling of OP-1 to HRP-urea, HRP-ADH and HRP-oxy diamine: To prepare the stock solution *N*-hydroxysuccimide (2.3 mg, 20 µmol) and N-(3-dimethylaminopropyl)-N- ethylcarbodiimide (20 mg, EDAC) were added to a solution of OP-1(15 µmol) in DMF (1000 µL). The reaction mixture was kept for 24 h at 4 °C for activation. After activation 100 µL was taken and added to the above prepared HRP-spacer solutions (500 µL). The reaction mixtures were incubated at 4 °C for overnight. The formed tracers were also purified by gel filtration chromatography as described in the previous section.

2.4 Competitive Inhibition Enzyme-linked Immunosorbent Assay

The assay was performed in two formats. In case of format I the microtiter plates were coated directly with the OP-Ab (1:2000) and the HRP tracer was used in the dilution of 1:4000. Whereas, in case of format II plates were coated first with the goat anti-rabbit IgG (0.5 µg mL^{-1}, 250 µL $well^{-1}$) at the concentration of 1:5000 and subsequently with OP-Ab (scheme 2). The assay utilised HRP tracer in the dilution of 1:10000. The inhibition curves were run with dilutions of OP-Ab. The 96-well microtiter plates (Nunc) were coated with OP-Ab/ trapping antibodies (goat anti-rabbit, 100 µL $well^{-1}$) in 0.5 M carbonate buffer (pH 9.6) and incubated overnight at 4 °C. On the following day, plates were washed three times with PBST (PBS buffer containing 0.05 per cent Tween 20, pH 7.6). For running assays in formats I and II the standard in PBS (100 µL) and tracer in PBS (100 µL) were added into the wells. The plate was shaken on a microtiter plate shaker for 1 min, followed by 1 h incubation at 4 °C. Plates were rinsed five times with PBST after each coating and after the competition step to remove the unbound immunoreactives. Then 50 µL per well of substrate solution was added and the reaction was stopped after 15 min by adding 50 µL of 4 M H_2S0_4. The absorbances were measured at 450 nm. Standards were run in triplicate. All inhibition curves were calculated by the four-parameter logistic equation, with the color reaction being inversely proportional to the IMPA concentration. Data analysis was performed by normalising the absorbance using the per cent $B/B_0 = (A\text{-}A_{ex}/A_0\text{-}A_{ex}) \times 100$, where A is absorbance, A_0 is absorbance at zero dose of analyte, and A_{ex} is absorbance at an excess of the analyte.



Scheme 2. CIELISA: A schematic representing both the ELISA formats

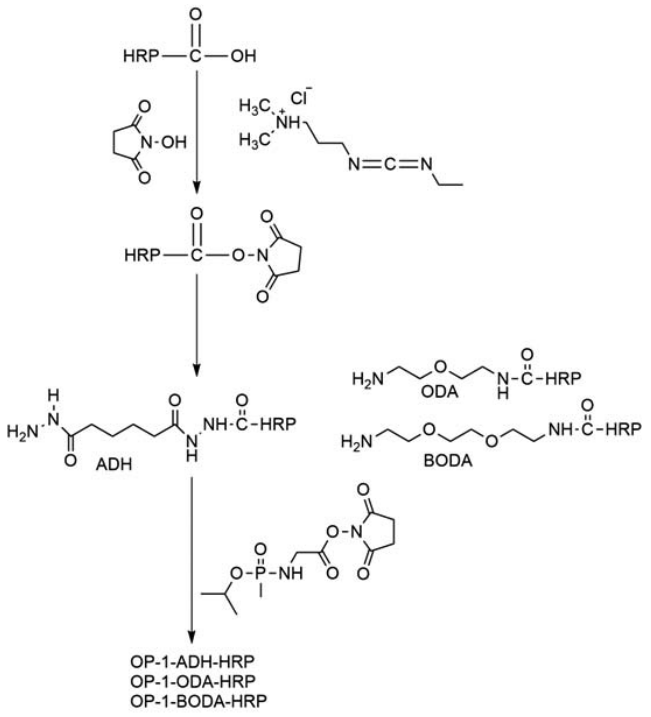
2.5 Analysis of Spiked Sample

To study the spike recovery the same procedure has been used as reported earlier by our group[26] . Stock solution of IMPA (1 mg mL^{-1}) was prepared in methanol. Working standard (50 µg mL^{-1}) of IMPA was prepared by drawing 100 µL aliquot from above prepared stock solution and adjusting to 2.0 mL with 10 per cent methanol in PBS (10 mM). Tap water was collected and spiked with IMPA standards (10 ng mL-1, 50 ng mL-1, and 100 ng mL-1). All the prepared samples and the control were allowed to stand overnight at room temperature before analysed by ELISA and GC-MS. For GC-MS analysis, samples were derivatised by tert-butyldimethylsilylchloride (TBDMS) and analysed. Similarly, an environmental soil sample was also collected from top soil outside the laboratory. A 2 g soil sample was spiked with IMPA in various concentrations (10 ng mL^{-1}, 25 ng mL^{-1}, and 70 ng mL^{-1}). It was extracted with alkaline water (pH 10, adjusted with 0.1 M NaOH) and subjected to solid phase extraction. For GC-MS analysis samples were derivatised by TBDMS.

3. RESULTS AND DISCUSSION

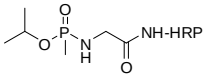
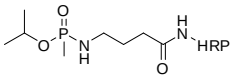
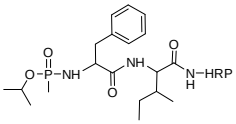
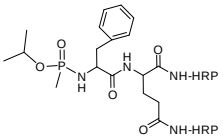
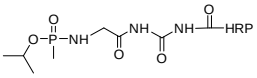
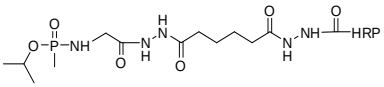
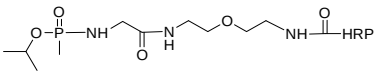
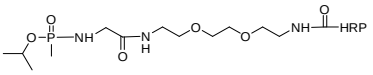
Hapten synthesis is very significant step in the development of immunoassay as it is responsible for getting antibody possessing excellent affinity towards target analyte. Haptens were synthesised by nucleophilic displacement of chlorine with amino group of amino acids or peptides. These hapten were insufficient in eliciting immune response, therefore high molecular weight carrier protein such as HRP was required to attach with synthesised haptens. To prepare the hapten-HRP conjugates, two conjugation procedure was adopted according to the functionalities present in the hapten derivative.The IMPA derivatives with carboxylic end group (OP-1, OP-2, OP-Pep-1, and OP-Pep-2) were conjugated to HRP enzyme by the *N*-hydroxysuccinimide ester method[27]. Coupling of spacers like urea, adipic acid dihydrazide (ADH) and oxy diamine to HRP was achieved in two steps as shown in scheme 3. In the first step carboxylic acid group of HRP was conjugated to spacer through amino functionality utilising the same *N*-hydroxysuccinimide ester method. In the second step HRP-spacer were conjugated to OP-1. All the conjugates (Table 1) were kept at room temperature for 4 h, and purified by gel filtration chromatography (Sephadex PD-10, Amersham Pharmacia, Freiburg, Germany).

The free amino groups in the HRP before and after the conjugation were determined by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBSA). Reaction of primary amines with TNBSA forms a highly chromogenic trinitrophenyl derivative that can easily be quantified by colorimetric read-out at 335 nm. The degree of hapten conjugation to HRP was



Scheme 3. Reaction scheme for the preparation of hapten-HRP enzyme conjugates.

Table 1. Chemical structures of prepared hapten-HRP conjugates

Entry	Hapten-HRP conjugate	Code
1		OP-1-HRP
2		OP-2-HRP
3		OP-Pep1-HRP
4		OP-Pep2-HRP
5		OP-1-Urea-HRP
6		OP-1-ADH-HRP
7		OP-1-ODA[a]-HRP
8		OP-1-BODA[b]-HRP

[a]2,2'-(oxybis(ethylamine) (ODA) [b]2,2'-(ethane-1,2 diylbis(oxy))diethanamine (BODA)

calculated from the absorbance values at 335 nm (which is the characteristic absorption peak of the trinitrophenol group (TNP) using the following equation:

Substitution (%) = [$A_{control} - A_{conjugate}$)/$A_{control}$] × 100.

Control experiments were performed in parallel and consisted of protein mixed with hapten without 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC.HCl). Considering three dimensional structure of HRP, six ε-NH_2 groups of lysine residues are available for coupling to haptens. Hapten density found to be in all the hapten-HRP conjugates was in the range of 2 to 4. Conjugates were additionally characterised by MALDI- MS. The molecular weight of native of the HRP enzyme was determined to be 43000 da. The relative increase in molecular weight of conjugates was manifested as a gradual mass peak shift as a function of hapten to enzyme ratio. This is interpreted as an increase in hapten density of conjugates and therefore provides a method for determining number of haptens per enzyme molecule. The findings of MALDI-MS analysis in terms of hapten density were in close agreement with data obtained from the TNBSA spectrophotometric method of analysis.

All the tracers were used in the same dilution of 1:5000. The dose response study of the seven enzyme conjugates (entry 1 to 8) was carried out with OP-Ab using competition inhibition ELISA (Fig. 2). All enzyme conjugates differing from each other in terms of spacers. OP-1-HRP conjugate was considered as reference to the assay. OP-2-HRP, OP-Pep1-HRP and OP-1-ADH-HRP conjugates were having hydrophobic spacers. Hydrophilic and flexible spacers were used for preparing OP-1-ODA-HRP and OP-1-BODA-HRP conjugates. In contrast to other conjugates OP-Pep2-HRP conjugate included hydrophilic spacers. The assay sensitivity was expressed in terms of IC_{50} (Table 2). It is the effective concentration at which 50 per cent of inhibition in the binding of enzyme conjugates occurs in assays in the presence of analyte.

From the Table 2 it was evident that spacers played a significant part in the free analyte recognition. The insertion of hydrophilic spacers in OP-Pep2-HRP conjugate enhanced the sensitivity significantly. The finding was also supported by study carried out by Nara[19], *et al.* in which insertion of

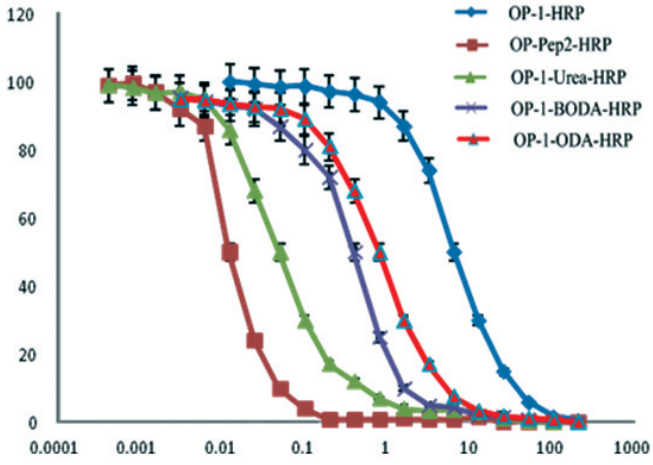


Figure 2. Inhibition curve for IMPA by direct inhibition ELISA.

Table 2. Comparison of different ELISA formats using direct coating of OP-Ab or precoating with goat anti-rabbit IgG

Entry	Enzyme conjugate	ELISA Format I		ELISA Format II	
		IC_{50} ($\mu g\,mL^{-1}$)[a]	$A_{max.}$	IC_{50} ($\mu g\,mL^{-1}$)[a]	A_{max}
1	OP-1-HRP	11.55	0.33	6.25	1.20
2	OP-2-HRP	8.42	0.30	5.27	0.67
3	OP-Pep1-HRP	10.60	0.29	6.18	0.52
4	OP-Pep2-HRP	0.16	0.42	0.01	0.70
5	OP-1-Urea-HRP	0.32	0.48	0.04	0.72
6	OP-1-ADH-HRP	13.34	0.38	7.32	0.64
7	OP-1-ODA-HRP	3.72	0.41	0.78	0.63
8	OP-1-BODA-HRP	3.12	0.38	0.39	0.73

[a]Incubation time required for color development; OP-1-HRP = 20 min, OP-Pep2-HRP = 17 min, OP-1-Urea-HRP = 15 min, OP-1-BODA-HRP = 20 min. Inhibition assay was carried by direct ELISA using format I and II. The assay utilized HRP tracer in various dilution to get the optimum sensitivity. OP-1-HRP 1:2000, OP-Pep2-HRP 1:2000, OP-1-Urea-HRP 1:4000, OP-1-BODA-HRP 1:2000.

hydrophilic spacer resulted in the enhancement of sensitivity and specificity of assay. Possibly the use of a hydrophilic spacer helped in projecting the hapten in the aqueous phase, leading to enhanced antibody binding signal and improved sensitivity of the assay. The present finding suggests that the nature of the spacer (hydrophobic, hydrophilic and amphiphilic) is related to assay sensitivity and not to spacer length.

The absorbance was recorded at 450 nm and data analysis was performed by normalising the absorbance. The concentration of free IMPA was plotted on the X axis and percentage of inhibition (per cent) on the Y axis. Data points are the means of triplicates. Assay formats greatly Effect the sensitivity, absorbance and analysis time of the assay. Two ELISA formats were tested; the first involved the direct coating of OP-Ab (1:2000) to the microtiter plate where as in the other format the plates were precoated with goat anti-rabbit IgG (1:5000) were utilised. The comparison of both the formats using four enzyme conjugates is presented in Table 2. The use of two coating steps (formats II) resulted in higher sensitivity compared to the format using one coating step. Better results were obtained by direct coating of the OP-Ab to the plates however; longer substrate incubation time was required for equal color development. Moreover, plate-to-plate variation was larger than the format II.

Significant lower sensitivity was observed in the competition step when antibodies were used in solution in comparison to the antibodies immobilised on the plate. This is probably due to the higher concentrations of OP-Ab available in solution compared to the immobilised antibodies. Therefore, appropriate concentrations of tracer and IMPA are necessary for an effective competition with the antibodies in solution. As such the Effect of spacer was also observed in case of ELISA format I, however the use of two sequential coating steps (format II) provided the best sensitivity and reproducibility, which can be a consequence of high enzyme tracer dilutions. The precoating step eliminated the blocking steps (*e.g.* with BSA) after coating from the assay. For construction of the calibration curves IMPA stock solution (1 g L^{-1}) was prepared with methanol and then further diluted with pure water to obtain individual standard solutions ranging from 0.003 $\mu g\ ml^{-1}$ to 250 $\mu g\ ml^{-1}$.

An IC_{50} and the lower detection limit determined by standard inhibition curve (Fig. 2) was found to be 0.01 $\mu g\ mL^{-1}$ and 0.004 $\mu g\ mL^{-1}$ respectively while OP-1-Pep2-HRP conjugate was used in ELISA format II. The specificity of the assay in format II was estimated as the percentage of cross-reaction using OP-Ab antibody. The antibodies didn't show significant cross-reactivities for the related compounds (Table 3). Maximum cross reactivity was observed with chlorosarin (100 per cent), *O*-pinacolylphosphonic acid (PMPA, 33 per cent) while other compounds exhibited least reactivity towards OP-Ab. The incorporation of hydrophilic spacer in the enzyme conjugate (OP-1-Pep2-HRP) has decreased the degree of cross-reaction as compared to enzyme conjugates having hydrophobic spacer like OP-2-HRP and OP-1-ADH-HRP and specificity of OP-Ab with other compounds also followed the same trend as obtained in case of enzyme conjugate (OP-1-Pep2-HRP).

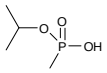
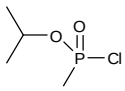
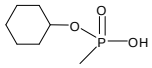
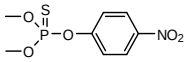
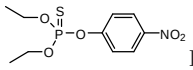
As the assay with OP-Pep2-HRP gave better sensitivity and specificity, this combination was further studied for analytical variables like recovery, precision, and correlation coefficients with GC-MS studies.

To investigate the effect of matrix standard curves were plotted after spiking IMPA in tap water and soil. The obtained lower detection limit with spiked samples was nearly identical to the standard one 0.004 $\mu g\ mL^{-1}$. To study the spike recovery, environmental water from general water supply was used. Tap water and soil samples were spiked with IMPA at various concentrations and each spiked sample was analysed in triplicate by the same method (ELISA format II). Validation of the method was carried out by GC-MS analysis. Table 4 shows the recovery of IMPA from the tap water and soil samples which was carried out by ELISA (format II) utilising OP-1-Pep2-HRP followed by comparison with GC-MS studies. The IMPA recoveries of the developed ELISA ranged from 92 per cent - 118 per cent and were observed in close agreement to those obtained through GC-MS analysis (98 per cent - 112 per cent). This clearly indicates that no significant matrix interferences were observed in both the cases. In water samples just filtration was sufficient for immunochemical analysis. However, extraction followed by sample clean up was required in analysing the soil samples.

4. CONCLUSIONS

The present study described the effect of assay format and the Effect of spacers towards the sensitivity on the detection of IMPA by competitive immunoassay. Once a good polyclonal or monoclonal antibody is developed it can be employed in various formats and sensitivity can be enhanced. The use of OP-Ab in solution for the competition step resulted in lower sensitivity. It was also observed that the nature of spacer is more remarkable factor than the length of the spacer. Hydrophilic spacers appear to be more favourable than the hydrophobic spacers for enhancing the sensitivity. The incorporation of hydrophilic spacer in the enzyme conjugate decreased the degree of cross-reaction as compared to enzyme conjugates having hydrophobic spacer. These finding are in agreement

Table 3. Cross-reactivity studies of IMPA with other related OP compounds

Entry	Toxicant/Pesticide	OP-1-Pep2-HRP conjugate (1:2000)		OP-2-HRP conjugate (1:2000)		OP-1-ADH-HRP conjugate (1:4000)	
		IC^{a}_{50} (µg mL^{-1})	[b]Cross reactivity (%)	IC^{a}_{50} (µg mL^{-1})	[b]Cross reactivity (%)	IC^{a}_{50} (µg mL^{-1})	[b]Cross reactivity (%)
1.	 *O*-Isopropyl methylphosphonic acid	0.01	100	5.2	100	7.3	100
2.	 Chloro sarin	0.01	100	5.2	100	7.2	101
3.	 *O*-Pinacolyl methylphosphonic acid	0.03	33	7.4	70	16	45
4	 *N, N'* Dimethyl *O*-ethyl phosphonic acid	0.08	12.5	14.0	37	22	33
5.	 *O*-Cyclohexyl methylphosphonic acid	12	0.08	17.2	30	28	25
6.	 Methyl phosphonic acid	17	0.05	32	0.16	30	24
7.	 Dimethyl parathion	32	0.08	124	0.3	35	20
8.	 Diethyl parathion	40	0.02	112	0.3	30	24

[a]Determined by direct inhibition ELISA format II using antibody OP-Ab (1:2000). All reactions were performed in triplicate on microtitration plates. [b]Cross-reactivity (%) = (IC_{50} of IMPA/IC_{50} of other compound) X 100.

Table 4. Analysis of IMPA spiked environmental water and soil by ELISA and GC-MS

Spiked Matrix	IMPA spiked (ng mL^{-1})	ELISA[a]				GC-MS			
		IMPA found (ng mL^{-1})	SD[b]	Mean recovery (%)	CV[c] (%)[c]	IMPA found (ng mL^{-1})	Mean recovery (%)	SD[b]	CV (%)[c]
Tap water	10	9.2	0.14	92.0	0.1	10.2	102	0.37	0.3
	50	52.4	0.35	104.8	0.3	54.5	109	0.13	0.1
	100	108.4	0.42	108.4	0.3	104	104	0.21	0.2
Soil	10	11.8	0.24	118.0	0.2	12.2	122	0.16	0.1
	25	27.5	0.30	110.0	0.2	24.5	98	0.26	0.2
	70	75.4	0.16	107.7	0.1	78.4	112	0.15	0.1

[a] Determined by direct inhibition ELISA (format II) using OP-B antibody (1:2000). [b]Standard deviation (SD, n = 4). [c]Coefficient of variation (CV). Data obtained from quadruplicate of the spiked sample.

with the available litreture[19,20]. The developed assay for the detection of IMPA can be used for the direct applications to environmental samples. No matrix effects have been observed, even when the analysis was done without previous dilution.

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