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PAPER

Direct hapten-linked competitive inhibition enzyme-linked immunosorbent assay (CIELISA) for the detection of *O*-pinacolyl methylphosphonic acid

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Immunoassay detection of *O*-pinacolyl methylphosphonic acid (PMPA) employing direct coating of *N*-2-aminoethyl-*O*-pinacolyl methylphosphonate (hapten B) on microtiter plates is reported. Coating was achieved by covalently linking hapten B to a glutaraldehyde (GA) polymer network directly bound to the polystyrene (PS) surface of a standard 96-well microtiter plate. 4-(2-(*O*-Pinacolylmethylphosphoryl amino)ethyl amino)-4-oxobutanoic acid (hapten A)–ovalbumin (OVA) conjugate served as the coating antigen for comparison with direct hapten B-coated plates in the CIELISA format. The developed assay employing direct hapten B coated plates demonstrated enhanced sensitivity with the IC_{50} value for PMPA being 0.027 μg mL^{-1}. The assay could detect PMPA even at the concentration of 0.006 μg mL^{-1}. The mean recovery of standard PMPA (spiked in water) was found to be 83.7%.

Introduction

Organophosphorus nerve agents such as sarin, soman, VX and their analogues constitute a most lethal class of chemical warfare agents (CWAs) hence included in schedule 1 category of chemical weapon convention (CWC).[1] In general, nerve agents block neural transmission by permanently binding with acetylcholinesterase enzyme which is responsible for neural transmission thereby causing the paralysis and mortality.[2] These toxic nerve agents are generally degraded by hydrolysis to produce alkyl alkylphosphonic acids (AAPAs) followed by alkylphosphonic acids (APAs)[3] (Fig. 1).

These hydrolytic degradation products of nerve agents are also included in the schedule 2B4 category of CWC, hence are considered important markers of nerve agents for the verification of CWC related chemicals. Amongst various environmental samples, water and soil is an important matrix which can be contaminated during deliberate or inadvertent spread of CWAs.[3] Therefore, analysis of CWAs and their degradation products from environmental matrices is of paramount importance from a verification point of view of CWC.[4] The target selected in the present studies was soman which gives *O*-pinacolyl methylphosphonic acid (PMPA) and methylphosphonic acid (MPA) on hydrolysis. Several analytical methods have been developed for their identification, involving capillary electrophoresis (CE),[5] an electrochemical sensor,[6] liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS),[7] CE microchip,[8] matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[9] and gas chromatography-mass spectrometry (GC-MS)[10] which are characterized by low limits of detection and high precision and sensitivity. However, these methods have few shortcomings: the need for time-consuming sample cleanup prior to detection, sophisticated equipment, lack of high sample throughput screening and inadequacy of onsite analysis. Thus, there is a need of a method that can detect a specific CWA or its marker rapidly and in a quantitative manner. In addition, the results should have minimal interference from other structurally similar but unrelated compounds. Immunological methods such as enzyme-linked immunosorbent assay (ELISA)[11] are increasingly becoming important for pesticides and other environmental residual analysis due to the high inherent selectivity of antibodies.[12] To the best of our knowledge not too many haptens and ELISA formats

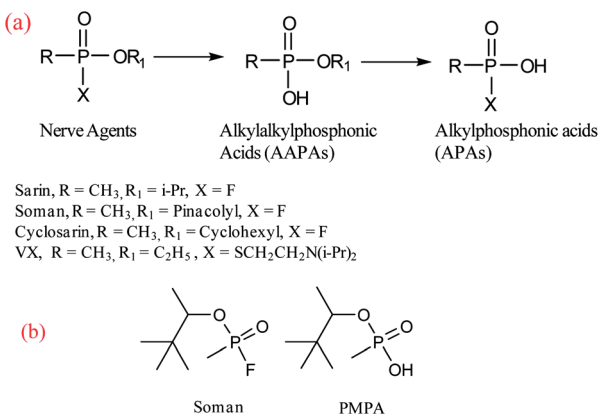

Fig. 1 (a) Hydrolytic pathways of nerve agents; (b) structure of soman and PMPA.

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have been studied so far for the detection of toxicants either for the generation of antibodies or to obtain the specificity with enhanced sensitivity.[13] Development of sensitive immunoassay for toxicant detection depends primarily upon the quality of the antibody used.[14] In most hapten based ELISAs, hapten molecules are usually bound to polystyrene (PS) microtiter wells indirectly by coating the wells with protein–hapten conjugates. However, the formation of such a conjugate is not always reproducible, which makes it difficult to evaluate the hapten–protein stoichiometry.[15] In order to avoid this problem the direct and covalent hapten linkage to the PS support has been reported as an interesting alternative.[16] Subsequently, quite a few approaches[17] have been developed on modified PS microtiter wells of ELISA plates, resulting in improved analytical performances of the assays. A PS covered with a polymerized glutaraldehyde (GA) network has been successfully used to immobilize macromolecules on PS *via* the NH_2 group, and also for the direct linkage of the hapten glutamate to solid surfaces.[18] Herein, we report the direct coupling of a newly synthesized PMPA derivative (hapten B) with PS microtiter plates *via* a GA network. PMPA was used as a hapten proxy for the target soman while haptens A and B were the derivatives of PMPA. Hapten A was employed for the preparation of immunogen and coating antigen whereas hapten B linked directly to the PS ELISA support through covalent linkage. The polyclonal antibody was generated and used for the development of a sensitive competitive immunoassay for the detection of PMPA in water.

Experimental

General

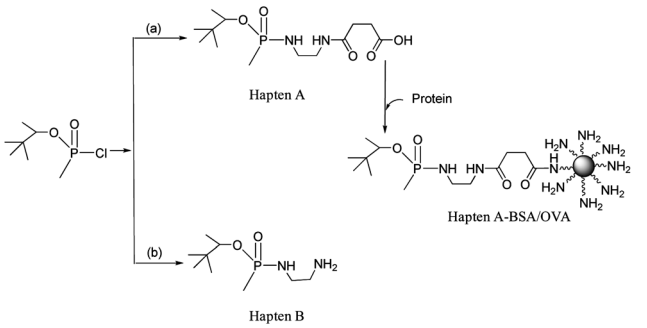
Reagents were obtained from Sigma-Aldrich (India) (unless stated otherwise), including bovine serum albumin (crystalline, fraction V) (BSA) and ovalbumin (OVA). GA (25% in water) was purchased from Baker (Griesheim, Germany). Solvents were of analytical grade for synthesis. The reaction progress was monitored by thin-layer chromatography (TLC) using GF254 silica gel on glass plates with a fluorescent indicator. Chromatography was performed using silica gel (60–120 mesh). PD-10 gel filtration columns (Pharmacia, Sweden) were obtained from GE Healthcare. 96-well, polystyrene Maxisorb plates were from Nunc (UK). The goat anti-rabbit IgG-HRP (immunoglobulin-horseradish peroxidase) conjugate secondary antibody was obtained from Dako (Denmark). The single beam scanning UV (Ultraviolet)-Visible Spectrophotometer (Camspec M501) was from Camspec Analytical Instruments Ltd., Leeds, UK. The ELISA plate reader used in this study was from Molecular Devices (USA). Mass spectra were acquired on a Micromass Q-TOF (Quadrupole Time-of-Flight) high-resolution mass spectrometer equipped with electrospray ionization (ESI) on a MassLynx 4.0 data acquisition system, MALDI-MS (matrix-assisted laser desorption/ionization-mass spectrometry) data were recorded on a MALDI 4700 TOF from Applied Biosystems.

The GC-MS analysis was performed in electron ionization (EI) mode with an Agilent 6890 gas chromatograph equipped with a model 5973N mass selective detector (Agilent technologies). An SGE BPX5 capillary column with 30 m length × 0.32 mm i.d. × 0.25 μm film thickness was used. The column oven temperature was programmed from 80 °C (hold for 2 min) to 170 °C at 15 °C min^{-1} thence at 60 °C min^{-1} to a final temperature of 280 °C (hold for 5 min). Helium at a flow rate of 1.2 mL min^{-1} was used as a carrier gas under constant flow mode. The samples were analyzed in the splitless mode at an injection temperature of 250 °C. The injected volume was 2 μL, and injection was done with a 5 μL microsyringe. The EI source was kept at 230 °C and 70 eV ionization energy, and the quadrupole temperature at 150 °C. Quantitation studies were performed in selected ion monitoring (SIM) mode. ^{1}H-NMR and ^{31}P-NMR spectra were recorded in $CDCl_3$ solutions on a JEOL ECX NMR spectrometer operating at 400 MHz (for ^{1}H) and 100 MHz (for ^{31}P), using tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in parts per million (ppm) down field from tetramethylsilane. Spin multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Coupling constants are reported in Hertz (Hz).

Hapten synthesis

The synthetic routes for haptens A and B are presented in Scheme 1. Both the compounds were prepared from the common precursor *O*-pinacolyl methylphosphonochloridate.[19] 4-(2-Aminoethylamino)-4-oxobutanoic acid required for the preparation of hapten A was synthesized by the reported method.[20]

Synthesis of 4-(2-(*O*-pinacolylmethylphosphoryl amino)ethyl amino)-4-oxobutanoic acid (hapten A). To a stirred solution of *O*-pinacolyl methylphosphonochloridate (1.26 g, 6.4 mmol) in 5 mL of cooled MeOH, a solution of KOH (896 mg, 16 mmol) and 4-(2-aminoethylamino)-4-oxobutanoic acid (1.42 g, 8.9 mmol) in 15 mL of methanol was added drop wise. After stirring for 10 min, the reaction mixture was filtered and extracted with 1 M HCl–chloroform. The extract was dried over anhydrous sodium sulfate and the solvent was evaporated. The residue was subjected to column chromatography on silica gel (hexane : ethyl acetate, 8 : 2) to afford 4-(2-(*O*-pinacolyl methylphosphono) ethylamino)-4-oxobutanoic acid (hapten A) as viscous liquid. Yield 50%. ^{1}H nuclear magnetic resonance (NMR) (400 MHz, $CDCl_3$) δ 0.95 (s, 9H), 1.35 (d, 3H, $J = 7.6$ Hz), 1.94 (d, 3H, $J = 7.2$ Hz), 2.49 (m, 2H), 2.72 (m, 2H), 2.76 (m, 2H), 3.66 (m, 2H), 3.37 (m, 1H), 4.0 (br, NH). ^{13}C NMR (100 MHz, $CDCl_3$) 16.9,



Scheme 1 Synthesis of hapten A, B and hapten A–BSA/OVA conjugate. (a) 4-(2-Aminoethylamino)-4-oxobutanoic acid, CH_3OH, KOH, 1 h, rt; (b) *tert*-butyl 2-aminoethylcarbamate, CH_3OH, KOH, 2 h, rt; HCl (4 M), ethyl acetate, 1 h, rt.

22.4, 25.3 (*t*-Bu, CH_3), 29.1, 30.2, 37.6, 39.0, 40.9, 90.4, 173.3, 173.8. ESI-MS 323 $(M + 1)^+$.

Synthesis of *N*-2-aminoethyl-*O*-pinacolyl methylphosphonate (hapten B). To a stirred solution of *O*-pinacolyl methylphosphonochloridate (2.37 g, 12 mmol) in 5 mL of cooled MeOH, a solution of KOH (896 mg, 16 mmol) and *tert*-butyl 2-aminoethylcarbamate (1.9 g, 12 mmol)[21] in 15 mL of methanol was added drop wise. After stirring for 10 min, the reaction mixture was filtered and extracted with chloroform. The extract was dried over anhydrous sodium sulfate and the solvent was evaporated. The residue (500 mg, 3.1 mmol) was dissolved in ethyl acetate (5 mL) and treated with aq. HCl (4M, 1.2 equiv.) and stirred for 1 hour at room temperature (rt). The reaction mixture was then cautiously alkalinized to pH 14 with 25% aq. NaOH solution and the aqueous phase was vigorously extracted with DCM (3×) and ethyl acetate (2×). The combined organic phase was washed with brine and dried ($MgSO_4$), and evaporated *in vacuo* to give 2-aminoethyl-*O*-pinacolyl methylphosphonate (hapten B) as brown oil. Yield 64%. ^{1}H nuclear magnetic resonance (NMR) (400 MHz, $CDCl_3$) δ 0.95 (s, 9H), 1.35 (d, 3H, J = 7.6 Hz), 1.92 (br s, 2H), 1.94 (d, 3H, J = 7.2 Hz), 2.80 (t, 2H, J = 6.0 Hz), 3.14–3.19 (q, 2H, J = 6.0 Hz,), 3.37 (m, 1H), 5.13 (br s, 1H).^{13}C NMR (100 MHz, $CDCl_3$) 16.9, 22.4, 25.3, 37.6, 41.7, 42.3, 90.4. ESI-MS 223 $(M + 1)^+$.

Preparation of immunogen and coating antigen

In order to conjugate hapten A to BSA and OVA, the carboxylic acid group of hapten was directly employed in the binding to the amino groups of carrier proteins, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as an activating reagent.[16]

Characterization of conjugates

Hapten–protein conjugates were characterized by spectrophotometric analysis and MALDI-MS.[22]

Antibody production and purification

Female New Zealand white rabbits weighing 1–1.5 kg (two rabbits) were used for raising polyclonal antibodies against conjugated PMPA. The rabbits were initially immunized by the subcutaneous route with 500 μg of hapten A–BSA conjugate (two rabbit) and Freund's complete adjuvant at four different sites. They subsequently received booster doses of 500 μg of hapten A–BSA conjugate along with Freund's incomplete adjuvant intramuscularly at 15 day intervals for 45 days. The rabbits were bled from the heart, and the sera was separated and stored at −20 °C. The antiserum was coded as OP-A for further studies.

Screening of antisera

Hapten B was directly coated on an ELISA plate as described previously.[23] Briefly, standard microtiter plates were pretreated with a dilute solution of GA (6.25%) in carbonate-buffer followed by direct linkage of the aliphatic amino terminated PMPA derivative (hapten B) to the GA network. The remaining GA binding sites had been blocked with 0.5 mol L^{-1} of aminohexanoic acid in PBS-buffer (10 mMol). Optimum concentrations of hapten B (100 to 0.045 μg mL^{-1}) and antisera (1 : 500 to 1 : 128 000) were chosen to produce absorbance values of approximately 0.4–0.9 U in the absence of the analyte in a noncompetitive indirect ELISA format by the checkerboard titration. Similarly, the optimum coating concentration of hapten A–OVA conjugate (0.5 to 0.0125 μg mL^{-1}) was also determined. The extent of the binding of the primary antibody to the hapten B coated and hapten A–OVA coated surface was then determined with a 1 : 5000 dilution of the peroxidase labelled secondary antibody (HRP-conjugated goat anti-rabbit IgG diluted) in PBS. The plates were incubated for 1 h at room temperature (25 °C) followed by the addition of 100 μL $well^{-1}$ of OPD (*O*-phenylenediamine) solution (10 mg of OPD and 10 μL of 30% H_2O_2 diluted with 25 mL of phosphate–citrate buffer, pH 5.4). The reaction was stopped after 15 minutes by adding 50 μL $well^{-1}$ of 2 M H_2SO_4, and the absorbance was measured at 490 nm.

Competitive inhibition assay

All the incubations were performed at room temperature except coating antigens. ELISA plates were coated with hapten B (10 μg mL^{-1} $well^{-1}$) and also with hapten A–OVA conjugate (0.2 μg mL^{-1} $well^{-1}$) in 0.05 M carbonate–bicarbonate buffer (pH 9.6) overnight at 4 °C. The following day, the coated plates were washed four times with PBST and were blocked by incubation with 1% OVA in PBS (200 μL $well^{-1}$) for 1 h. After another washing step 50 μL $well^{-1}$ of serial dilutions (0.0004 μg mL^{-1} to 200 μg mL^{-1}) of PMPA in 10% methanol–PBS (10 mM) was added, followed by 50 μL $well^{-1}$ addition of antiserum OP-A diluted at 1 : 2000 with PBST. After incubation for 1 h and washing step, 100 μL $well^{-1}$ of HRP-conjugated goat anti-rabbit IgG diluted 1 : 5000 with PBST was added to the plates and incubated for 1 h. The plates were then washed again and developed as described above. The absorbance was recorded at 490 nm and data analysis was performed by normalizing the absorbance using the $\%B/B_0 = (A - A_{ex}/A_0 - A_{ex}) \times 100$, where A is the absorbance, A_0 is the absorbance at zero dose of the analyte, and A_{ex} is the absorbance at an excess of the analyte.

Optimization of ELISA

To determine the PMPA in environmental samples, it is essential to develop an ELISA with optimum sensitivity. Therefore, the effects of coating concentration, blocking buffer, antibody dilution, solvent, incubation buffer and pH of the buffer were also studied. Assay optimization was performed using PMPA as the competitor analyte in CIELISA. The main criterion used to evaluate immunoassay performance was IC_{50} (inhibited concentration by 50%).

Cross-reactivity studies

Cross-reactivity of related analogues was calculated on the basis of standard calibration curves. The data were normalized by $\%B/B_0$ transformation and the specific hapten concentration yielding 50% inhibition was used to calculate the cross-reactivity according to the formula: cross-reactivity (%) = (IC_{50} of PMPA/IC_{50} of other compound) × 100.

Standard spiking solutions

The standard stock solution of PMPA (1 mg mL^{-1}) was prepared in methanol separately by accurately weighing 10 mg of analyte into a 10 mL volumetric flask. Using this working standard (50 μg mL^{-1}) of PMPA was prepared by drawing 100 μL aliquot from stock standard solutions of the analyte and adjusting to 2.0 mL with 10% methanol in PBS (10 mM). Tap water (pH 7.4) was spiked by the appropriate aliquots of PMPA to get the final concentration of 1, 2, 4 and 6 μg mL^{-1}. The samples were coded as samples A, B, C and D respectively. The water sample with no PMPA was used as the control. All the samples and control were allowed to stand overnight at room temperature and analyzed further by the optimized ELISA and GC-MS analysis.

Spiking of real soman

The standard solution of soman was prepared of 50 μg mL^{-1} concentration in 10% methanol in PBS (10 mM). Tap water (pH 7.4) was spiked with appropriate aliquots of soman standard solution to get the final concentration of soman 2 and 5 μg mL^{-1}. Both the samples and control were allowed to stand overnight at room temperature and analyzed by ELISA and GC-MS. For GC-MS analysis, samples were derivatized by *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by the reported method.[24]

Caution: soman is a highly toxic chemical. It should be prepared and handled by trained professionals in an efficient fume hood equipped with an alkali scrubber. Individuals handling them must wear facemasks, gloves and protective suits. To avoid any accident a sufficient amount of decontamination solution should be available at the working place.

GC-MS analysis

The standard stock solution of internal standard (IS), tri-*n*-propyl phosphate (TPP), was prepared at the concentration of 1 mg mL^{-1} in dry acetonitrile (dried over molecular sieves). To ascertain the linearity of the GC-MS response (to construct the calibration curve) of trimethylsilyl derivatives of PMPA the serial concentration of derivatized analytes was prepared in the concentration range from 0.5 μg mL^{-1} to 10 μg mL^{-1}. Samples were subjected to GC-MS analysis and six point calibrations of trimethylsilyl derivatives of PMPA were drawn with an r^2 value of 0.986. Prior to GC-MS analysis of spiked samples A to D, they were homogenized for 60 s with 20 mL of acetonitrile and 20 mL of dichloromethane. After filtration, the organic phase was transferred to a conical flask; the solvent was evaporated to dryness and reconstituted with 1 mL of hexane. To this 50 μL of BSTFA was added and solutions were heated at 70 °C for 1 h in polytetrafluoroethylene (PTFE) capped (air tight) vials. After cooling to room temperature 10 μL of stock standard solution of IS was added, and the volume was finally adjusted to 2.0 mL with dry acetonitrile. Finally, 2 μL of each sample was injected, by pulsed splitless, on the GC column for PMPA quantification.

Results and discussion

Hapten synthesis

A suitable hapten derivative and its conjugated form to be used in animal immunization should preserve the structure of the target compound as much as possible. To obtain a high selectivity and sensitivity of an antibody against a given OP toxicant, it is required to synthesize hapten having a spacer arm with a suitable functionality, such as a free carboxyl or amino group for the binding of the hapten to a protein at the phosphate group and preserve the *O*-pinacolyl methyl group of the molecule. Based on these considerations haptens A and B were synthesized by the displacement of the chlorine atom of *O*-pinacolyl methylphosphonochloridate by an amino carboxylic acid and *tert*-butyl 2-aminoethylcarbamate. The synthesized haptens were characterized by ^{1}H, ^{31}P NMR and ESI-MS. For the direct immobilization of hapten B, microtiter plates were pre-treated with GA in aqueous buffer solutions at pH 9.6. It has been found that during this pre-coating step a polyGA network is created and bound to the surface. Subsequently, hapten B is linked to the surface-immobilized polyGA at pH 8.0 by formation of stabilized imino bonds as described in Fig. 2.

Hapten A was covalently attached through a carboxylic acid moiety to the lysine amino group of the carrier protein utilizing the active succinimidyl ester method.[25] The free amino groups in the protein before and after the conjugation were determined by reaction with trinitrobenzene sulfonic 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 using the following equation: substitution (%) = $[(A_{control} - A_{conjugate})/A_{control}] \times 100$. Finally the conjugation was also confirmed by MALDI-MS. The hapten coupling ratio of hapten A : BSA was found to be 11.5 : 1. The molecular weight (M_w) of native BSA was determined to be 67 454,[26] the relative increase in the molecular weight of the conjugates was manifested as a gradual mass peak shift as a function of hapten to protein ratio (Fig. 3). This is interpreted as an increase in the hapten density of the conjugates and therefore provides a method for determining the number of haptens per protein molecule. Similarly, the hapten A : OVA coupling ratio was found to be 4 : 1 obtained at an initial hapten to protein molar ratio of 40 : 1.

Selection of antisera and coating concentration

The hapten A–BSA conjugate having the hapten density of 11.5 per protein molecule was used as an immunogen in rabbits and two rabbits were employed for single immunogen. Considerably high optical density (O.D.) values of anti-PMPA antisera were observed in both the rabbits. The titer values of antisera obtained from both the rabbits were presented in Table 1.

Titer values are the means of triplicates. The standard deviations (SD) were all below 10%. Because of high titer, antisera from rabbit A was preferred for further purification step. Antisera were first purified by protein-A sepharose followed by the BSA-sepharose-4B column to remove anti-carrier (BSA)

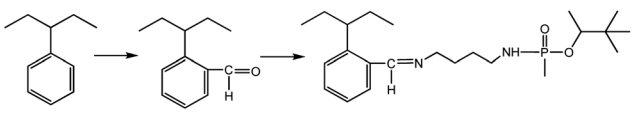


Fig. 2 Schematic representation of direct attachment of hapten B to a GA network on the PS surface.

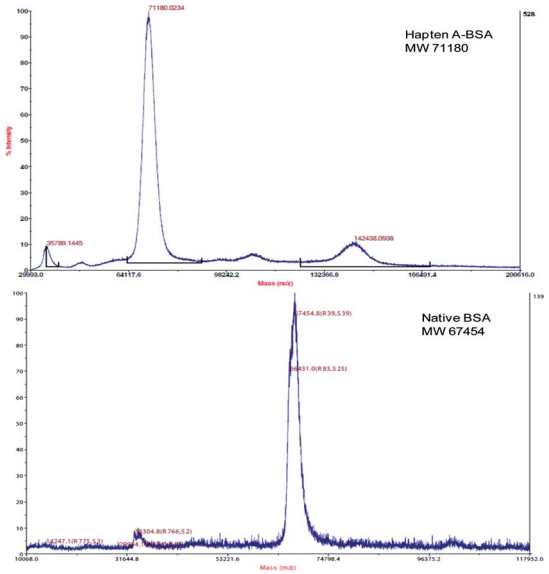


Fig. 3 MALDI-MS spectra of hapten A–BSA conjugates and native BSA. On each graph the *X*-axis represents the mass (*m*/*z*) and the *Y*-axis counts the relative intensity (%).

Table 1 Titer values of antisera[a] from rabbit A and B

Rabbit A		Rabbit B	
Antisera	O.D. (490 nm)	Antisera	O.D. (490 nm)
Pre-bleed	0.095 ± 0.01	Pre-bleed	0.072 ± 0.04
1st Bleed	0.295 ± 0.03	1st Bleed	0.171 ± 0.01
3rd Bleed	0.677 ± 0.01	3rd Bleed	0.525 ± 0.02

[a] Absorbance was measured by a checkerboard pattern, with several coating concentrations and several antibody dilutions, and measured after 15 min incubation with OPD. For convenience, only data from a coating antigen concentration of 0.2 μg mL^{-1} well^{-1} of hapten A–OVA conjugate are shown. Hapten A–BSA was used as an immunogen. Antibody dilution was 1 : 2000. Titer values are the means of triplicate.

antibodies. Anti-PMPA antibodies (OP-A) displayed good reactivity against the hapten A–OVA conjugate and demonstrated a significantly low reactivity with carrier proteins (BSA). The optimum dilution of OP-A was preferred at 1 : 2000 and the concentration of coating antigen hapten A–OVA was chosen at 0.2 μg mL^{-1} well^{-1}.

Binding of OP-A with the target molecule on hapten B coated ELISA plates was checked by confirming the reactivity of OP-A at different concentrations of hapten B (0.045 μg mL^{-1} to 100 μg mL^{-1}) (Fig. 4). On this basis the coating concentration of hapten B was optimized to be 10 μg mL^{-1} and 100 μL well^{-1} for the direct attachment. The absorbance value initially increases with increasing coating concentration of hapten B but run through a maximum value at approximately 25 μg mL^{-1}. This indicates that the amount of hapten B immobilized increases with the coating concentration. It has been found that when the GA-pretreatment is omitted the absorbance value with the zero dose of analyte (A_0) and in the presence of excess of analyte (A_{ex}) was nearly indistinguishable from the background, thus indicating that the analyte was not immobilized at all on the PS plate.

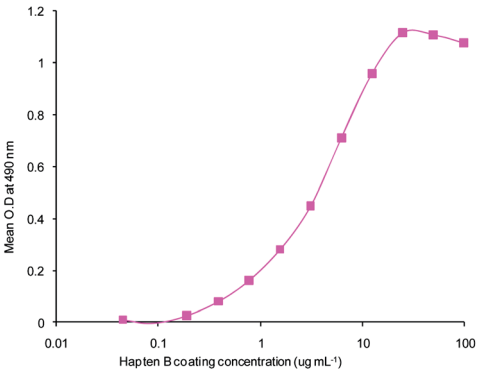


Fig. 4 Effect of concentration of the coating hapten B (0.045 μg mL^{-1} to 100 μg mL^{-1}) on the binding of anti-PMPA antibodies (OP-A, dilution 1 : 2000).

Competitive curve of PMPA standards

The typical standard curve of the PMPA immunoassay is given in Fig. 5. The IC_{50} values and limit of detection (LOD) for PMPA were found to be 0.101 μg mL^{-1} and 0.024 μg mL^{-1} in the case of hapten A–OVA coated CIELISA with the dynamic linearity range between 0.012 μg mL^{-1} and 1.56 μg mL^{-1}. However, the level of signal increased with a high degree of reproducibility in the case of direct hapten coated plate and the IC_{50} and LOD were found to be 0.027 μg mL^{-1} and 0.006 μg mL^{-1} with the dynamic linearity range between 0.012 μg mL^{-1} and 0.098 μg mL^{-1}. This was mainly because of retention of functional activity of hapten molecules on polystyrene plates. The observed retention of functionality is believed to be a result of the direct hapten immobilisation on the polystyrene surface without the use of a protein carrier; previous studies report the loss of functionality when immobilisation *via* protein conjugation is employed.[16b]

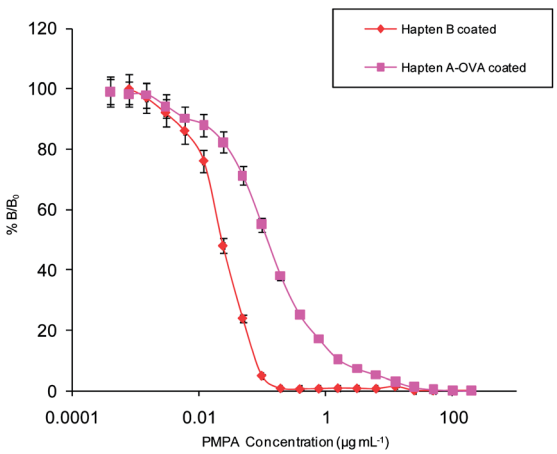


Fig. 5 Inhibition curve for PMPA by inhibition ELISA using antiserum to hapten A–BSA, diluted 1 : 2000, the coating antigen hapten A–OVA (0.2 μg mL^{-1} well^{-1}), hapten B (10 μg mL^{-1} well^{-1}) and 0.1% OVA as the blocking agent. $\%B/B_0 = (A - A_{ex}/A_0 - A_{ex}) \times 100$, where A is the absorbance, A_0 is the absorbance at zero dose of the analyte, and A_{ex} is the absorbance at an excess of the analyte. Data represent the mean of three experiments. Vertical bars indicate ±SD about the mean. The LOD was calculated as the smallest concentration of the analyte that produced a signal statistically different from the blank (Student's *t*-test).

Optimization of ELISA

Optimization studies were carried out in the CIELISA format. Since methanol was used as a solvent to dissolve the PMPA in the CIELISA format therefore it was desirable to assess the effect of methanol on ELISA performance. Consequently, the effect of methanol on the ELISA system was evaluated by preparing standard curves in buffers containing various amounts of methanol (10, 20, 30, and 40% in PBS). The results are presented in Table 2. Increasing the concentration of methanol affected significantly the sensitivity of the assay but caused little effect on the speed of the color development. A larger decrease in the sensitivity was observed with an increasing amount of methanol. The optimum concentration selected was 10% where IC_{50} was the lowest. The influence of the blocking reagent was investigated by using three blocking buffers *i.e.* 5% skimmed milk powder, 1% BSA and 1% OVA and increased sensitivity was found when 1% OVA (in 10 mM PBS buffer) was used as a blocking buffer. The effect of the phosphate ion concentration at the competition step on ELISA characteristics was also studied.

As evident from Table 2 40 mM concentration provided the highest sensitivity, however, it was considered inappropriate because of the very slow color development.

The optimum concentration selected was 10 mM. To determine the effect of pH on the assay, the phosphate buffer was used in the range of pH 6.5 to pH 8.5. As shown in Table 2 the ELISA was more sensitive under neutral and slightly alkaline conditions. This means that the interaction between the antibody and the target analyte was most favored at pH 7.5–8.5. Consequently, the optimum pH range of the assay buffer was set at 7.5 for the ELISA.

Cross-reactivity (CR) studies

In immunoassay based toxicant detection, it is important to have the use of an antibody that demonstrates very high sensitivity as well as specificity. Therefore, to determine the specificity of the optimized ELISA, organophosphorus toxicants, pesticides and the degradation products of nerve agent were tested for cross-reactivity. Table 3 shows the cross-reactivity that was found by the assay.

The cross-reactivity of OP-A for IMPA is understandable, because it has very similar hydrophobic structure as PMPA and only the pinacolyl group was substituted with the isopropyl group in its structure. Methylphosphonic acid (MPA) also showed cross-reactivity (21%). It is interesting to note that other OP compounds like *O*-cyclohexyl methylphosphonic acid, *N*,*N*′ dimethyl *O*-ethylphosphonic acid and diethyl parathion demonstrated cross-reactivity less than 5%.

Recovery

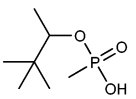
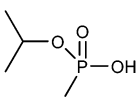
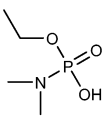
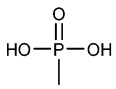
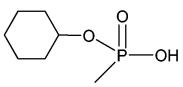
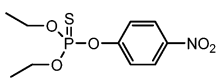
Water samples, which were spiked with PMPA and real soman, were analyzed by the optimized inhibition ELISA. The assay

Table 2 Effects of methanol concentration, pH and buffer concentration on assay parameters of the CIELISA[a]

		Abs_{max}	Slope	IC_{50} (μg mL^{-1})
Methanol (%)	10	0.644	0.668	0.024
	20	0.762	0.644	12.21
	30	0.865	0.784	18.53
	40	0.933	0.774	21.64
Buffer[b]	10 mM	1.51	0.684	0.02
	20 mM	1.43	0.672	0.70
	30 mM	1.20	0.682	0.42
	40 mM	0.92	0.787	0.01
pH	6.5	0.680	0.854	28.52
	6.0	0.788	0.886	17.38
	7.4	0.861	0.764	0.05
	8.5	0.924	0.923	0.91

[a] *CIELISA conditions*: antiserum to hapten A–BSA, diluted 1 : 2000 with 10 mM PBS; coating antigen hapten B (10 μg mL^{-1} well^{-1}); goat anti-rabbit IgG-HRP diluted 1 : 5000. Data were obtained from the four parameter sigmoidal fitting. Each set of data represents the average of three replicate. [b] Incubation time required for color development: 10 mM = 9 min, 20 mM = 15 min, 30 mM = 20 min, and 40 mM = 25 min.

Table 3 ELISA cross-reactivity of the anti-PMPA antibodies with other OP compounds and pesticide[a]

Entry	Toxicant/pesticide	IC_{50} (μg mL^{-1})	Cross-reactivity[b] (%)
1.	 *O*- Pinacolyl methylphosphonic acid	0.027	100
2.	 *O*-Isopropyl methylphosphonic acid	0.054	50
3.	 *N,N*′ Dimethyl *O*-ethylphosphonic acid	78	0.03
4.	 Methylphosphonic acid	0.124	21.7
5.	 *O*-Cyclohexyl methylphosphonic acid	32	0.084
6.	 Diethyl parathion	21	0.12

[a] Determined by CIEIA using antiserum to hapten A–BSA on the hapten B coated microtiter plates. All reactions were performed in triplicate on microtitration plates. [b] Cross-reactivity (%) = (IC_{50} of PMPA/IC_{50} of other compound) × 100. The highest interference was obtained for isopropyl methylphosphonic acid (IMPA), which showed a cross-reactivity of 50%.

Table 4 Comparison of PMPA and soman spiked environmental water by ELISA and GC-MS analysis

		ELISA[a]				GC-MS			
Tap water sample	PMPA/soman spiked (μg mL^{-1})	PMPA/soman found (μg mL^{-1})	Mean recovery (%)	SD[b]	CV (%)[c]	PMPA/soman found (μg mL^{-1})	Mean recovery (%)	SD[b]	CV (%)[c]
Spiked with PMPA	1	0.84	80.0	3.26	4.0	0.87	83.35	3.57	4.2
	2	1.80	89.0	5.03	5.6	1.84	87.81	6.85	7.8
	4	3.54	84.1	4.25	5.0	3.92	91.19	2.88	3.1
	6	5.24	81.7	4.89	5.9	5.29	86.66	3.05	3.5
Spiked with real soman	2	1.82	87.5	3.97	4.5	1.70	84.33	4.04	4.7
	5	4.70	89.4	5.22	5.8	4.57	87.66	3.51	4.0

[a] Determined by inhibition ELISA using antiserum to hapten A–BSA on the hapten B coated microtiter plates. [b] Standard deviation (SD, $n = 3$). [c] Coefficient of variation (CV). Data obtained from triplicate of the spiked sample.

parameters showed no noticeable difference between the PMPA standard curve obtained in water and in PBS. Consequently, it seemed applicable to perform direct determination of PMPA in tap water without further treatment. Tap water was spiked with PMPA at the concentration of 1, 2, 4 and 6 μg mL^{-1} and with soman at the concentration of 2 and 5 μg mL^{-1}. Extraction of PMPA and soman from the water samples was carried out according to the reported method.[23] Each spiked sample was analyzed in triplicate by the direct hapten attached immunoassay method. The recovery of PMPA from PMPA spiked water samples and also from soman spiked water (Table 4) by the optimized ELISA was in the range of 80–89%. Validation of the method was carried out by GC-MS analysis. Due to the non-volatile nature of PMPA, derivatization was performed to generate the volatile *tert*-butyl dimethylsilyl species and GC-MS analysis was performed. The PMPA recovery findings by the ELISA format are in good agreement with those obtained through GC-MS analysis (83–91% recovery of PMPA) (Table 4). These findings clearly validated the usefulness of the developed ELISA for the determination of PMPA in the water sample.

Conclusion

In conclusion, we have conjugated PMPA with carrier proteins and successfully generated polyclonal antibody response in rabbits. A high antibody titer with moderate antibody specificity was obtained with the hapten A–BSA conjugate having hapten density around 11 molecules per carrier protein. The sensitivity of the assay was enhanced by the direct immobilization of PMPA derivative (hapten B) with glutaraldehyde on a polystyrene surface of a microtiter plate. The IC_{50} value for the detection of PMPA was found to be 0.101 μg mL^{-1} and 0.027 μg mL^{-1} in the case of hapten A–protein conjugate coated and direct hapten B coated microtiter plates. The mean recovery of PMPA from water samples was 83.7%. Thus, by the presence of PMPA in the environmental matrix the probable presence of soman can be detected. The important advantages of the presented ELISA format lies in its simplicity; as it does not require any sample pretreatment, general applicability for high throughput screening and suitability for onsite analysis. Additionally no tracer or coating antigen synthesis is necessary, which often lacks reproducible conjugate preparation and therefore results in less-well characterized and standardized assays.

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