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Design and synthesis of immunoconjugates and development of competition inhibition enzyme-linked immunosorbent assay (CIEIA) for the detection of *O*-isopropyl methylphosphonofluoridate (sarin): An organophosphorous toxicant

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ABSTRACT

Three haptens of the organophosphorus (OP) toxicant 'sarin' having different spacer arm were designed and synthesized. Haptens were conjugated with BSA (bovine serum albumin) and ovalbumin (OVA) for raising antibody and coating antigen. High antibody titer with higher specificity was obtained from 4-(4-(isopropoxy(methyl)phosphoryloxy)phenylamino)-4-oxobutanoic acid (hapten B) having reasonable long spacer arm. For the standard curve, an IC_{50} (inhibitory concentration) of free antigen was found to be 0.415 μg mL^{-1} on the basis of indirect competitive ELISA. The study revealed that heterology in competition inhibition enzyme immunoassay (CIEIA) produced remarkable improvement in the sensitivity and specificity of the assay. Under the optimized conditions, the quantitative working range was found to be 0.19–1.56 μg mL^{-1} with a limit of detection (LOD) of 0.05 μg mL^{-1}. The antibodies showed negligible cross reactivity (CR) with other OP toxicants and pesticides, which makes the assay suitable for the selective detection of sarin.

1. Introduction

Organophosphorous (OP) compounds particularly phosphates and phosphonates have been widely used as pesticides in the agricultural industry and as chemical warfare agents [1]. *O*-Isopropyl methylphosphonofluoridate ($C_4H_{10}FO_2P$) popularly known as 'sarin' (GB) is one of the highly toxic OP nerve agent with LD_{50} 75–100 mg min^{-1} m^{-3} [2]. Additionally, sarin and related phosphonates (Fig. 1) are chemical warfare agents and there is a possibility of their use during a military or terrorist attack [3]. Therefore, the detection of such deadly nerve agents in battlefield conditions presents a major challenge to the researchers and continues to be of national security interest.

Analysis of environmental samples for the presence of markers has now been regarded as a key component, in the event of the alleged use of CW (chemical warfare) agents. Ideally, a marker should be present in an easily obtainable matrix, should be relatively long-lived, and provide unequivocal identification of the agent. Phosphonofluoridates rapidly degrade into *O*-alkyl alkylphosphonic acids and ultimately to alkylphosphonic acids (APAs) [4,5]. These APAs have negligible industrial applications therefore; their presence in any environmental matrix indicates the probable prior presence of nerve agents which is an important aspect of verification analysis of CWC (chemical weapon convention) related chemicals. Sarin rapidly degrade to its hydrolysis products, *i.e.*, the corresponding *O*-isopropyl methylphosphonic acid (IMPA), thus it is considered as a marker for the retrospective detection of sarin in environmental sample. Various analytical methods have been developed for rapid and high-throughput screening of large numbers of samples in case of nerve agent exposure [6,7]. Unfortunately, the majority of current monitoring protocols for OP exposures [8] still suffer from some intrinsic disadvantages of either low detection sensitivity and specificity (*i.e.*, Ellman colorimetric assays) [9,10] or expensive analysis settings entailing well-trained personnel and not convenient for field applications [11]. Thus simple, sensitive, selective, and field-deployable tools are still highly required for biomonitoring and diagnostic evaluation of OP exposures. Immunoassays such as enzyme-linked immunosorbent assays (ELISAs) [12] have the potential to become an alternative or a complementary method to the traditional ones, because they have proven to be fast, sensitive, cost-effective tools for on-site monitoring [13]. Several ELISA methods for the detection of OP pesticides [14] have been developed and some of them have been validated by applying them to residue analysis in environmental waters or agricultural products. However, to the best of our knowledge very few immunoassays for the detection of OP toxicants like sarin, soman, and VX using polyclonal and monoclonal

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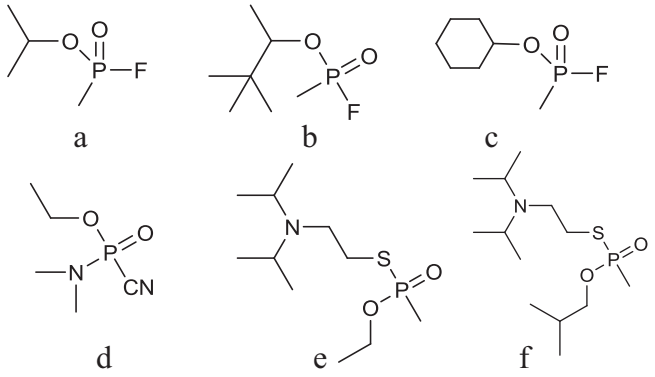


Fig. 1. Chemical structures of nerve agents (a) sarin (GB) (b) soman (GD) (c) cyclosarin (GF) (d) tabun (GA) (e) VX (f) Russian VX.

antibodies have been developed and reported so far [15–17]. The initial step in the development of an immunoassay is the production of specific antibodies. However, small molecules (popularly called as hapten) like sarin seldom stimulate an immune response, therefore it is necessary to design appropriate chemical structure [18] which can be covalently coupled to a carrier protein and presented to the animal's immune system. The structure of the hapten [19] used as an immunogen can influence the characteristics of the antibodies against a toxicant as well as the sensitivity and specificity of the analytical method. Previously Zhou et al. reported the production of antibody of sarin using two artificial antigens, N^{α},N^{ε}-di(*O*,*O*-diisopropyl)phosphoryl L-lysine (DIP)–BSA conjugate and DIP–KLH (keyhole limpet hemocyanin) [20]. Similarly, the role of immunogen design in induction of soman specific monoclonal antibodies was excellently illustrated by Johnson et al. [15]. In view of that, herein, we report the design and synthesis of haptens for the development of specific ELISA for the detection of sarin based on polyclonal antibodies.

2. Experimental

2.1. Reagents and materials

Reagents were obtained from Sigma–Aldrich (India) (unless stated otherwise), including bovine serum albumin (crystalline, fraction V) (BSA) and OVA. Solvents used were of analytical grade for synthesis. The reaction progress was monitored by thin-layer chromatography (TLC) using GF_{254} silica gel on glass plates with 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 Igs-HRP conjugate secondary antibody was obtained from Dako (Denmark).

2.2. Instruments

The single beam scanning UV–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 high-resolution mass spectrometer equipped with electrospray ionization (ESI) on Masslynx 4.0 data acquisition system, MALDI-MS data were recorded on MALDI 4700 TOF Applied Biosystems. The GC–MS analysis was performed in electron ionization (EI) mode with a Thermo Trace DSQ Mass-Spectrometer 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. Injected volume was 2 μL, and injection was done with 5 μL microsyringe. EI source was kept at 230 °C and 70 eV ionization energy, and temperature was kept at 150 °C. Quantization 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 Bruker AVANCE 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), m (multiplet). Coupling constants are reported in Hertz (Hz).

2.3. Synthesis of hapten (A–C)

The haptens used for immunization and coating antigens are described in Fig. 2.

2.3.1. Synthesis of 4-aminophenyl isopropyl methylphosphonate (*3*, hapten A)

4-amino phenol (1.3 g, 11.9 mmol) was dissolved in dry THF (15 mL) and sodium hydride (95%) (336 mg, 14 mmol) was added into it in small portions at 0 °C. The mixture was stirred for 0.5 h followed by drop wise addition of **2** (1.8 gm, 12 mmol) [21] at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, and further stirring was done for 2 h at RT. The solution was filtered, the solvent was evaporated under reduced pressure and the residue was chromatographed on silica gel (hexane: ethyl acetate, 8:2) to afford **3** as viscous oil (1.2 g, 50% yield). ESI-MS 230 $(M+H)^{+}$. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.4 (t, J = 5.6 Hz, 5.6 Hz, 6H), 1.9 (d, J = 17.6 Hz, 3H), 2.1 (4.9 (m, 1H), 7.20 (d, J = 8.1 Hz, 2H), 8.09 (d, J = 8.5 Hz, 2H). ^{31}P NMR (100 MHz, $CDCl_3$) δ (ppm) 31.1

2.3.2. Synthesis of 4-(4-(isopropoxy(methyl)phosphoryloxy)phenylamino)-4-oxobutanoic acid (*4*, hapten B)

A solution of **3** (1 g, 4.3 mmol) in pyridine (10 mL) was added dropwise to a stirred solution of succinic anhydride (430 mg, 4.3 mmol) dissolved in pyridine (10 mL). After stirring for 3 h at room temperature, 20 mL saturated K_2CO_3 was added to the reaction mixture and extracted with ethyl acetate. The water layer was acidified by adding 6 mol L^{-1} HCl and extracted with ethyl acetate. Combined organic layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to afford **4** as brown viscous liquid (1.1 g, 52%). ESI-MS 330 $(M+H)^{+}$. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.4 (t, J = 5.6 Hz, 5.6 Hz, 6H), 1.9 (d, J = 17.6 Hz, 3H), 2.65 (s, 4H), 4.9 (m, 1H), 7.20 (d, J = 8.1 Hz, 2H), 8.09 (d, J = 8.5 Hz, 2H), 9.1 (s, br, NH). ^{31}P NMR (100 MHz, $CDCl_3$) δ (ppm) 26.1.

2.3.3. Synthesis of 4-(isopropoxy(methyl)phosphorylamino)butanoic acid (*5*, hapten C)

To a stirred solution of **2** (1 g, 6.4 mmol) in 5 mL of cooled MeOH, a solution of KOH (896 mg, 16 mmol) and 4-amino butyric acid (922 mg, 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

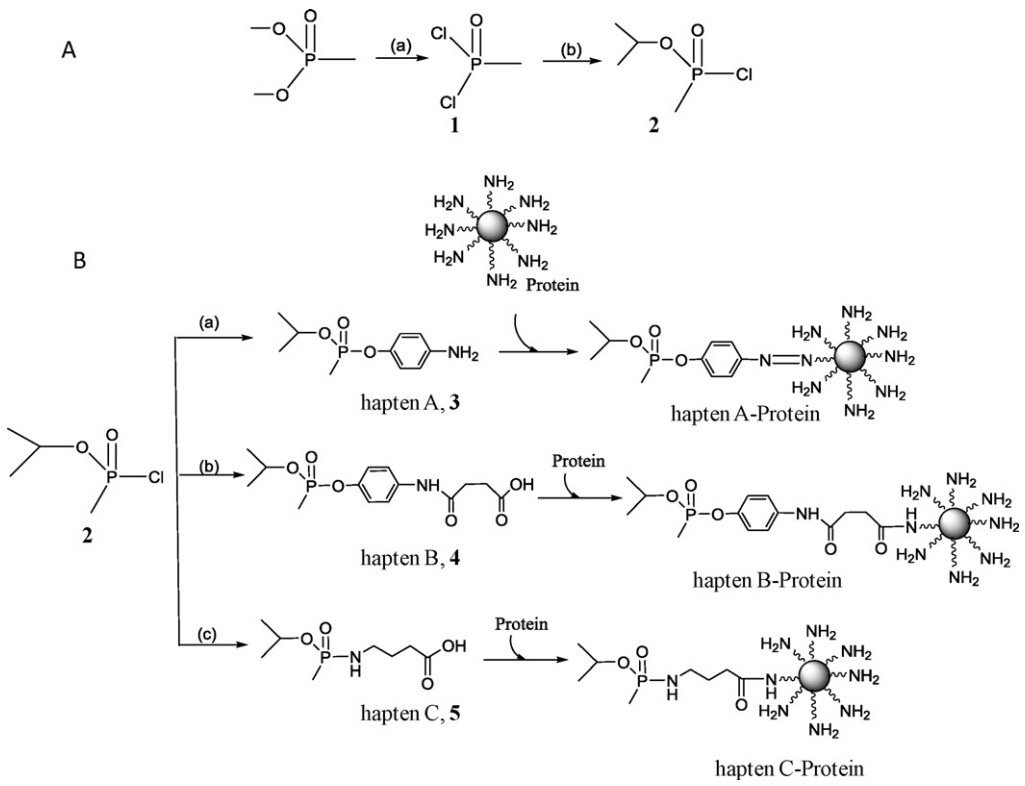


Fig. 2. A: (a) $SOCl_2$, toluene, 5 h at reflux temperature; (b) isopropyl alcohol, DCM, 4 h rt. B: (a) 4-amino phenol, NAH, dry THF, 2 h at rt.; (b) **3**, succinic anhydride, dry pyridine; (c) 4-amino butyric acid, CH_3OH, KOH, 1 h rt.

gel (hexane:ethyl acetate, 8:2) to afford **5** as white viscous liquid (500 mg, 50% yield). ESI-MS 224 $(M+H)^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 1.4 (t, J = 5.6 Hz, 5.6 Hz, 6H), 1.9 (d, J = 17.6 Hz, 3H), 2.0 (s, br, NH), 1.87 (m, 2H), 2.4 (m, 2H), 4.9 (m, 1H). ^{31}P NMR (100 MHz, $CDCl_3$) δ (ppm) 30.8.

2.4. Conjugation of hapten to proteins

To conjugate haptens (A, B and C) to carrier proteins, the carboxylic acid group of haptens was directly employed in the binding of the amino groups of the carrier proteins, using 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide (EDC) as an activating reagent. Thus, N-hydroxy succinamide (5.7 mg, 50 μmol) and EDC (9.5 mg, 50 μmol) were added to a solution of hapten (A/B/C, 50 μmol) in DMF. The reaction mixture was incubated for 1 h at RT. The protein stock solution (10 mg mL^{-1}; 0.15 μmol mL^{-1}) was prepared in borate buffer (pH 9.0). The activated hapten (A/B/C, 120 μL, 6.0 μmol) was then added dropwise to protein solution (0.13 μmol, 8.8 mg, 880 μL) using hapten: protein molar ratio equal to 46:1. The final reaction volume of the protein–hapten conjugates was kept constant at 1 mL for each preparation. The conjugates were incubated overnight at room temperature and centrifuged for 5 min at 10,000 rpm to remove the precipitate. It was further purified by passing through a P10 gel filtration column (Pharmacia, Sweden). Fractions with the highest protein concentration were determined by absorbance measurements at 280 nm using a molar extinction coefficient of 43,824/M/cm on a UV spectrometer. All hapten–protein (hapten–BSA and hapten–OVA) conjugates were prepared using the same procedure. The final protein concentration of the conjugates was determined using a Micro BCA™ protein assay kit (Pierce).

2.5. Characterization of conjugates

Hapten–protein conjugates were characterized by spectrophotometric analysis and MALDI-MS according to the reported method [22,23].

2.6. Preparation of polyclonal antiserum

Female New Zealand white rabbits weighing 1–1.5 kg were used for raising polyclonal antibodies against conjugated sarin. The rabbits were initially immunized by the subcutaneous route with 500 μg of hapten–BSA conjugate (one rabbit per immunogen) and Freund's complete adjuvant at four different sites. All the animals subsequently received the booster dose of 500 μg of hapten–BSA conjugate along with Freund's incomplete adjuvant intramuscularly at 15-days interval for 45 days. The animals were bleed from the heart, and the sera were separated and stored at −20 °C.

2.7. Purification of immunoglobulins (IgGs)

Five milliliters of saturated ammonium sulfate was slowly added dropwise to 5 mL of hyperimmune serum diluted 1:1, and the mixture was stirred for 30 min. The precipitated proteins (IgGs) were removed by centrifugation (Sigma 4K15 centrifuge) at 5000 rpm for 10 min at 4 °C. The precipitated IgGs were washed twice with 1:1 (vol/vol) saturated ammonium sulfate to remove remaining soluble proteins. The precipitate was dissolved in 1 mL of 0.1 M PBS, pH 7.2, and desalted by dialysis. IgGs from dialysed product was affinity purified using Proetin-A column (Ab purification kit-Sigma) as per their instruction manual.

2.8. Indirect ELISA

2.8.1. Titration of antisera

The titers of antisera were determined by measuring the binding of serial dilution of the antisera to the coating antigen using chequerboard titration. All incubations were performed at 37 °C except for the coating antigen. Flat-bottom polystyrene ELISA plates (Nunc-Maxisorp) were coated with hapten–OVA (200–10 ng mL^{-1}, 100 μL well^{-1}) in coating buffer (pH 9.6) overnight at 4 °C. The wells were washed 3 times with PBST (PBS containing 0.05% Tween 20) solution, and then blocked with 1% OVA in 10 mM PBS buffer (200 μL well^{-1}) for 2 h at 37 °C. After washing 3 times with PBST solution, the wells were incubated with (1/500–1/128,000, 100 μL well^{-1}) diluted antibody in PBST for 1 h and washed 5 times with PBST solution. HRP-conjugated goat anti-rabbit IgG diluted 1:5000 in PBS was added (100 μL well^{-1}). After incubation for 1 h and washing 5 times with PBST solution, developing solution was added to the wells (100 μL well^{-1}) and incubated for 10 min. The reaction was stopped by addition of 50 μL well^{-1} of 2 mol L^{-1} H_2SO_4, and the absorbance was recorded at 490 nm.

2.8.2. Competition inhibition enzyme immunoassay (CIEIA)

In a CIEIA, all three conjugates *i.e.*, hapten A/B/C–OVA were used as coating antigens to investigate the effect of hapten heterology on sensitivity of the CIEIA. Flat bottom polystyrene ELISA plates were coated with hapten–OVA (200 ng mL^{-1}, 100 μL well^{-1}) in 10 mM PBS buffer (pH 7.4) overnight at 4 °C. The wells were washed three times with PBST solution, and blocked with 1% OVA in 10 mM PBS buffer (200 μL well^{-1}) for 2 h at 37 °C. Sarin was dissolved in 10 mM PBS containing 10% methanol (50 μL well^{-1}) ranged from 0.003 μg mL^{-1} to 250 μg mL^{-1} and purified IgG OP-B diluted with PBS (1/500, 1/1000, 1/2000, 100 μL well^{-1}) were added, incubated for 1 h, and washed 3 times with the PBST solution. HRP-conjugated goat anti-rabbit IgG's diluted 1:5000 in PBS was added (100 μL well^{-1}). To study the effect of solvent on CIEIA sensitivity, standard analyte solutions were prepared in various concentrations of methanol (10, 20, 40, and 80% in PBS which became 5, 10, 20, and 40% respectively after combination with equal volumes of diluted antisera). After incubation for 1 h and washing 3 times with PBST solution, developing solution was added to the wells (100 μL well^{-1}) and incubated for 10 min. The reaction was stopped by addition of 50 μL well^{-1} of 2 mol L^{-1} H_2SO_4 and the absorbance was recorded at 490 nm. Similarly, the effect of pH and the effect of the phosphate ion concentration were also studied. Competitive curves were obtained by plotting the normalized signal (B/B_0) against the logarithm of analyte concentration. The IC_{50} and LOD were obtained from a four parameter logistic equation [24] of the sigmoidal curves.

2.8.3. Determination of cross-reactivities

Various organophosphorus toxicants, pesticides and the metabolite of sarin *i.e.*, *O*-isopropyl methylphosphonic acid were tested for cross-reactivity using the CIEIA procedure described above. The cross-reactivity values were calculated as follows: (IC_{50} of sarin/IC_{50} of compound) × 100.

2.8.4. Analysis of water sample

Standard solution of actual sarin was prepared of 50 μg mL^{-1} concentration in 10% methanol in PBS (10 mM). The assay parameters showed no noticeable difference between sarin standard curve obtained in water and in PBS. Consequently, it seemed applicable to perform direct determination of actual sarin in environmental sample without further treatment. Tap water was spiked with appropriate aliquots of actual sarin standard solution to get the final concentration of sarin 0.2, 0.5, 1 and 1.5 μg mL^{-1}. The samples and control were allowed to stand overnight at room temperature. Samples were divided in two portions for ELISA and GC–MS analysis. ELISA was carried out after filtering the water sample over non-sterile 0.2 μ filters. No further sample treatment was done for ELISA. For the GC–MS analysis, samples were derivatized by *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by the reported method [25].

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

2.9. GC–MS analysis

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 GC–MS response (to construct the calibration curve) of trimethylsilyl derivatives of IMPA the serial concentration of derivatized analytes were prepared in concentration range from 0.1 μg mL^{-1} to 10 μg mL^{-1}. Samples were subjected to GC–MS analysis and six point calibrations of trimethylsilyl derivatives of IMPA were drawn with r^2 value 0.980. Prior to GC–MS analysis, spiked samples 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 volume was finally adjusted to 2.0 mL with dry acetonitrile. Finally, 2 μL of each sample was injected, by pulsed split-less, on the GC column.

3. Results and discussion

3.1. Synthesis and characterization of the hapten–protein conjugates

Hapten design and synthesis plays a key role in developing immunoassays. The molecular structure of sarin is made up of *O*-isopropyl methyl phosphate and a fluoride group, the former having a big and complicated space structure, which may stimulate animal to produce a recognizable antibody. Therefore, we preferred to choose the structure of phosphate as the determinant group. In order to project *O*-isopropyl methyl phosphate epitope outward to the protein the fluoride group was altered and a spacer arm introduced to couple with the carrier protein. Based on these considerations, three haptens were synthesized as described in Fig. 2. Hapten A was synthesized by reacting **2** with 4-amino phenol under basic conditions. Reaction of hapten A with succinic anhydride in the presence of pyridine lead to hapten B with four carbon atom spacer arm and a terminal carboxylic group. Hapten C was obtained by the displacement of the chlorine atom of **2** by an amino carboxylic acid. The facile substitution of Cl by an aminocarboxylic acid could be attributed to the polar nature of methanol (reaction solvent) which would stabilize the polar transition state developed in the S_N2 reaction. The synthesized haptens were characterized by ^1H, ^{31}P NMR and ESI-MS. Two different approaches were used to couple the hapten (A, B, and C) to carrier protein. Hapten A was linked to carrier protein by diazotization reaction *via* a diazo bond, formed between amino group of hapten A and free lysine amino group of carrier protein. Hapten B and C were covalently attached through their carboxylic acid moiety to the lysine amino group of carrier protein utilizing the active succimidyl ester method [26].

Table 1
Determination of the hapten density on hapten–protein conjugates using chemical (TNBSA) and physical (MALDI-MS) methods.

Conjugate	Chemical TNBSA method[a]		MALDI-MS		
	% of amino groups consumed	Average no. of amino groups used	Observed mass (m/z)/Da	Change in mass (ΔM)/Da	$\Delta M/M_n$[b] (hapten density)[c]
Hapten A–BSA	75	19.5	72,758	5304	23.1
Hapten B–BSA	70	18.2	75,621	8167	24.7
Hapten C–BSA	72	18.7	71,454	4000	15.8
Native BSA			67,454	n/a	n/a
Hapten A–OVA	34	6.8	45,465	1263	5.5
Hapten B–OVA	30	6.0	45,493	1291	5.4
Hapten C–OVA	21	4.2	45,201	999	3.9
Native OVA			44,202	n/a	

[a] The number of lysine groups on the protein that were utilized in conjugates before and after conjugation.
[b] Mass of the hapten M_n.
[c] Number of hapten molecules per BSA molecule.

The free amino groups in the protein before and after the conjugation were determined by reaction with 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 carrier protein was calculated from the absorbance values at 335 nm (which is the characteristic absorption peak of the TNP group) using the following equation: substitution (%) = $[A_{control} - A_{conjugate})/A_{control}] \times 100$. Control experiments were performed in parallel and consisted of protein mixed with hapten without EDC. The resulting conjugates were additionally characterized by MALDI-MS. As shown in Fig. 3 spectra were successfully obtained for all three hapten–BSA conjugates. The molecular weight (MW) of native BSA was determined to be 66,451, while the MW determined on the basis of the published protein sequence was 66,432.9 [27]. 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. 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. The observed value for BSA was used in the following equation to determine the hapten density: number of haptens = (conjugate MW − BSA MW)/hapten MW. Few spectrum contains two peaks that represent singly $(M+H)^+$ and doubly $(M+2H)^{2+}$ charged states. The molecular weight of each conjugate was calculated from the peak centroid using the software provided with the MS instrument. The hapten density in hapten–BSA conjugate was in the range of 15–24 molecules per BSA protein, whereas in case of hapten–OVA conjugate the hapten density was found to be 4–5 per OVA protein. The large difference in the coupling ratio between hapten–BSA and hapten–OVA might be caused by the different number of reaction sites on BSA and OVA proteins. Considering the three-dimensional structure of BSA, only 26 ε-NH_2 groups of the total 59 lysines [22] residues in BSA are present at the protein surface and therefore readily available for coupling to haptens while OVA contains only 20 lysine ε-amine groups capable of conjugation. Theoretically, there are fewer sites to interact with the antibody when OVA is used as the coating antigen, thereby producing better sensitivity than using a coating antigen with a higher load of hapten. The result of the TNBSA and MALDI-MS analyses of all the conjugates is shown in Table 1. The findings of MALDI-MS analysis in terms of hapten density were in close agreement with the data obtained from the TNBSA spectrophotometric method of analysis.

3.2. Indirect ELISA

Maxisorp ELISA plates were used for the screening of antisera. Antisera from all the rabbits injected with hapten A–BSA, hapten B–BSA and hapten C–BSA were collected one week after the third booster injection and coded as OP-A, OP-B and OP-C. Absorbencies were measured for all the antisera against each of the three coating haptens coupled to OVA in various concentrations using checkerboard pattern. All the three antisera obtained showed reasonably high recognition for the coating antigen however, they showed different degrees of inhibition by the analyte for binding to the coating antigen which were derivatized to different structure and conjugated with carrier protein. The titer values after the third booster were about 1.0 at 1/16,000 and 1/32,000 dilutions for antiserum OP-B. Fig. 4 shows the antibody titers in sera of rabbit immunized against hapten A–BSA, hapten B–BSA and hapten C–BSA. Titer values are the means of triplicates. The relative standard deviations (CV) were all below 10%. The result suggests that a hapten with a longer spacer arm for coupling to carrier protein was better to use as an immunogen for antibody production. Additionally, from the present studies it was also revealed that a small molecule like sarin covalently linked to a carrier protein may suffer a considerable amount of masking in the region of hapten. The lower titer value of antisera OP-A and OP-C possibly because of steric hindrance by the phenyl ring in hapten A and small spacer arm length of hapten C as a result hapten structure was getting masked by the carrier protein and thus resulted into low immune response.

3.3. CIEIA

The effect of spacer heterology between immunogen and coating antigen on the sensitivity and specificity of the obtained (polyclonal antibodies) OP-B was evaluated by performing the indirect competition assays. The antiserum OP-B was selected as the most suitable one on the basis of its titer value which is the highest. As evident from Table 2 in case of CIEIA higher sensitivity was observed in the heterologous system when hapten B–BSA was used as immunogen and hapten C–OVA conjugate as the coating antigen. In view of that, hapten C–OVA conjugate was used as a coating hapten which was heterologous in terms of the spacer arm to the immunizing hapten structure and carrier protein. To rule out any effect seen for binding to the carrier protein OVA was used as a

Table 2
The IC_{50} value for sarin in homologous and heterologous CIEIA.[a]

Coating antigens	OP-B obtained from hapten 2–BSA	
	IC_{50} ($\mu g\,mL^{-1}$)	LOD ($\mu g\,mL^{-1}$)
Hapten A–OVA	10.4	2.2
Hapten B–OVA	24.2	1.7
Hapten C–OVA	0.4	0.05

[a] ELISA conditions: antiserum to hapten B–BSA, diluted 1/2000 with 10 mM PBS; coating antigen concentration 200 ng $well^{-1}$; goat antirabbit IgG-HRP diluted 1/5000. Data were obtained from the four parameter sigmoidal fitting. Each set of data represents the average of three replicates.

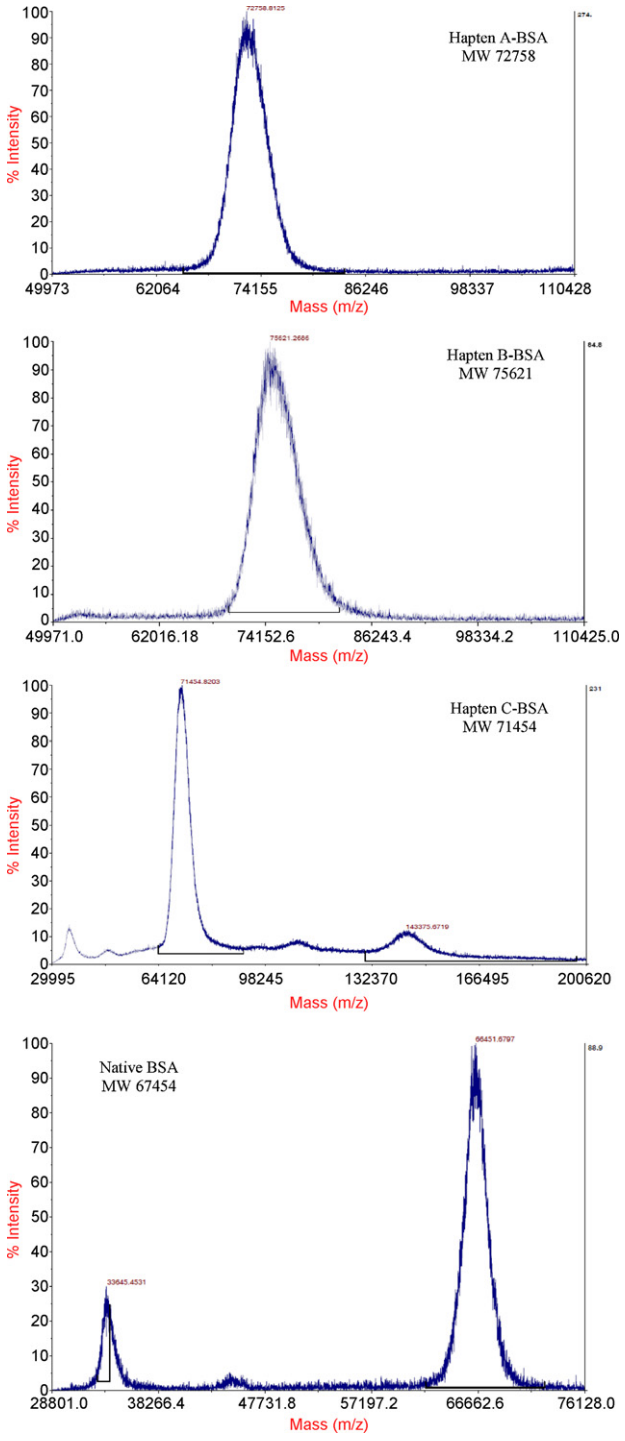


Fig. 3. MALDI-MS spectra of hapten–BSA conjugates and native BSA. On each graph *X*-axis represent the mass (*m*/*z*) and *Y*-axis count relative intensity (%).

carrier protein for the preparation of the coating antigens. Hapten heterology is commonly used to eliminate problems associated with the strong affinity of the antibodies to the spacer arm that leads to no or poor inhibition by the target compound [28].

It usually results in weaker recognition of plate-coating antigens compared with recognition of the target compound, allowing analyte at low concentrations to compete with coating antigens. The *in situ* formation of *O*-isopropyl methylphosphonic acid was also confirmed indirectly by the formation of *O*-isopropyl methyl methylphosphonate using GC–MS (gas chromatography–mass spectrometry) studies. The optimum dilution of OP-B was preferred

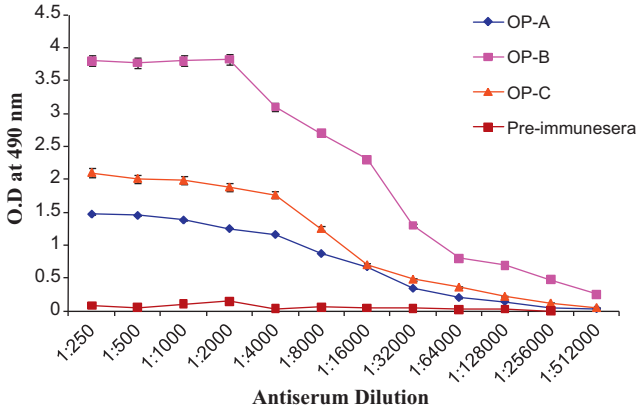


Fig. 4. Chequerboard titration of polyclonal antisera OP-A, OP-B and OP-C. Microtitration plates were coated with 200 ng mL^{-1} of hapten C–OVA conjugate Data represent the mean of three experiments. Vertical bars indicate ±SD about the mean. The relative standard deviations were all below 10%.

at 1/2000 and the concentration of coating antigen hapten C–OVA was chosen at 200 ng well^{-1}. Since organic solvents are used for the extraction of non-polar toxicants or their degradation products from various biomedical or environmental samples, it is desirable to assess the effect of organic solvents on CIEIA performance. Therefore, the effects of methanol on the ELISA system were evaluated by preparing standard curves in buffers containing various amounts of methanol (5, 10, 20, and 40% in PBS). The results are presented in Table 3. Several other workers reached the same conclusion as ours in that methanol caused the least negative effect on the performance of the assay [29,30]. Table 3 also presents the effect of pH on CIEIA performance. The physiological pH (pH 7.4) was selected as the best one. The effect of the phosphate ion concentration at the competition step on CIEIA characteristics was also studied. As evident from Table 3 increasing the concentration of the phosphate caused a large improvement in the sensitivity of the assay, which is in agreement with the results of previous studies [31]. Due to the nonpolar nature of sarin, it seems reasonable to assume that hydrophobic interactions are important in the antibody–analyte binding. Therefore, increased ionic strength of the medium would enhance the antibody–analyte binding, which could explain the behavior observed. Although 40 mM concentration provided the highest sensitivity, it was considered inappropriate because of the very slow color development. The optimum concentration selected was 10 mM where A_{max}/IC_{50} value is the highest.

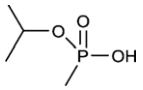
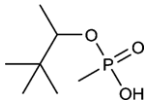
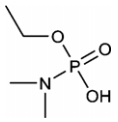
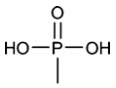
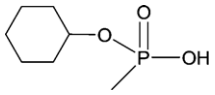
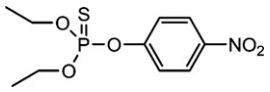
Table 3
Effects of methanol concentration, pH and buffer concentration on assay parameters of the indirect ELISA.[a]

		Abs_{max}	Slope	IC_{50} (μg mL^{-1})
Methanol (%)	10	0.814	0.578	0.4
	20	0.752	0.764	12.2
	30	0.873	0.674	18.5
	40	0.953	0.664	21.6
pH	6.5	0.685	0.874	28.5
	6.0	0.798	0.856	17.3
	7.4	0.881	0.734	0.5
	8.5	0.954	0.983	0.9
Buffer[b]	10 mM	1.56	0.654	0.5
	20 mM	1.53	0.632	0.3
	30 mM	1.29	0.692	0.4
	40 mM	0.97	0.767	0.2

[a] CIEIA conditions: antiserum to hapten B–BSA, diluted 1/1000 with 10 mM PBST; coating antigen, hapten C–OVA, 200 ng well^{-1}; goat antirabbit IgG-HRP diluted 1/5000. Data were obtained from the four parameter sigmoidal fitting. Each set of data represents the average of three replicates.

[b] Incubation time required for color development; 10 mM = 8 min, 20 mM = 13 min, 30 mM = 17 min, 40 mM = 25 min.

Table 4
ELISA[a] cross-reactivity for sarin against OP-B.

Entry	Toxicant/pesticide	Structure	IC_{50} ($\mu g\,mL^{-1}$)	Cross reactivity[b] (%)
1.	*O*-Isopropyl methylphosphonic acid		0.4	100
2.	Pinacolyl methylphosphonic acid		4	10
3.	*N*,*N*′-Dimethyl *O*-ethyl phosphonic acid		64	0.6
4.	Methylphosphonic acid		8	5
5.	*O*-Cyclohexyl methylphosphonic acid		32	1.25
6.	Diethyl parathion		21	1.9

[a] Determined by indirect ELISA using antiserum to hapten B–BSA and coating antigen hapten C–OVA. All reactions were performed in triplicate on microtitration plates coated with coating antigen hapten.
[b] Crossreactivity (%) = (IC_{50} of sarin/IC_{50} of other compound) × 100.

On the basis of these results, the optimal conditions for the sarin CIEIA are summarized as follows: a quantity of 200 ng mL^{-1} of the hapten C–OVA conjugate as a coating antigen was coated onto the plate and placed at 4 °C for overnight incubation. The plate was blocked with 0.1% OVA incubated for 2 h at 37 °C. The antiserum OP-B was diluted 2000-fold with PBS (10 mM, pH 8.0) and competed with the target analyte dissolved in PBS (10 mM, 10% methanol, pH 8.0) for 1 h at room temperature. Fig. 5 shows a typical calibration curve obtained under the optimum conditions of heterologous CIEIA. The IC_{50} value of the assay was 0.415 $\mu g\,mL^{-1}$ with a detection limit of 0.05 $\mu g\,mL^{-1}$. The linear working range of the ELISA was between 0.19 and 1.56 $\mu g\,mL^{-1}$. It was evident from Fig. 5 that there was a concentration dependent inhibition of antibody activity by the free analyte over a concentration range from 6.25 $\mu g\,mL^{-1}$ to 0.05 $\mu g\,mL^{-1}$. The coefficient of variation (CV) among replicates was less than 10%.

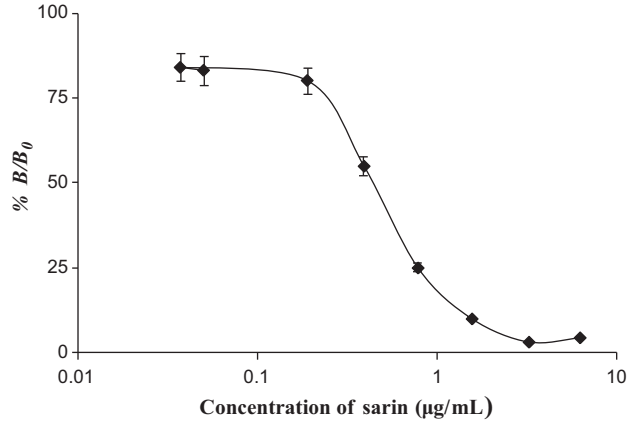


Fig. 5. Inhibition curve for sarin by CIEIA using antiserum to hapten B–BSA, diluted 1/2000, the coating antigen hapten C–OVA (200 ng $well^{-1}$) and 0.1% OVA as blocking agent. $\%B/B_0 = (A - A_{xs}/A_0 - A_{xs}) \times 100$, where A is the absorbance, A_0 is the absorbance at zero dose of the analyte, and A_{xs} 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 were calculated as the smallest concentration of analyte that produced a signal statistically different from the blank (Student's *t*-test).

3.4. Cross-reactivity studies

To determine the specificity of the optimized CIEIA, organophosphorus toxicants, pesticides and the degradation products of nerve agent were tested for cross-reactivity. Table 4 shows the cross-reactivity that was determined by the assay. The highest interference was obtained for soman, which showed a cross-reactivity of 10%. The cross-reactivity of the antibody for soman is understandable, because it has very similar hydrophobic structure as sarin and only the isopropyl group was substituted with pinacolyl group in its structure. Methyl phosphonic acid also showed a very small cross reaction (5%). It is interesting to note that other OP toxicants like *O*-cyclohexyl methylphosphonic acid (cyclosarin), *N*,*N*′-dimethyl *O*-ethyl phosphonic acid (tabun) and pesticide like ethyl parathion demonstrated crossreactivity less than 5%.

3.5. Recovery

Sarin spiked water samples were analyzed by the optimized inhibition ELISA. Extraction procedure from the water samples was carried out according to the reported method [25]. Each spiked sample was analyzed in triplicates by CIEIA. Due to the non-volatile nature of IMPA (forms by the hydrolysis of sarin) derivatization was performed to generate the volatile *tert*-butyl dimethylsilyl species and GC–MS analysis was performed. The recovery of IMPA from water samples (Table 5) by the optimized ELISA was in the range of 91–94% (mean recovery 92.5%). A control sample without sarin was also analyzed and the value was lower than the assay detection limit (0.05 $\mu g\,mL^{-1}$). The assessment of the matrix effect indicated that there was no significant matrix effect by the tap

Table 5
Analysis of sarin-spiked environmental water by ELISA[a] and GC–MS analysis.

Sample	Sarin spiked ($\mu g\,mL^{-1}$)	ELISA			GC–MS		
		IMPA found ($\mu g\,mL^{-1}$) ± SD[b]	R (%)	CV (%)[c]	IMPA found ($\mu g\,mL^{-1}$) ± SD[b]	R (%)	CV (%)[c]
Tap water	0.2	0.18 ± 0.14	91	0.1	0.19 ± 0.56	92	0.6
	0.5	0.46 ± 0.32	92	0.3	0.44 ± 1.23	88	1.3
	1	0.94 ± 0.40	94	0.4	0.89 ± 0.40	89	0.4
	1.5	1.38 ± 0.42	92	0.4	1.39 ± 0.70	92	0.7

[a] Determined by inhibition ELISA using antiserum to hapten B–BSA and coating antigen hapten C–OVA (200 ng well^{-1}).
[b] Standard deviation (SD, $n = 3$).
[c] Coefficient of variation (CV).

water on the performance of the assay. The IMPA recovery findings by ELISA format are in good agreement with those obtained through GC–MS analysis (mean recovery 90.25%) (Table 5). These findings clearly validated the usefulness of the developed ELISA for the determination of IMPA, a marker of sarin in environmental sample.

4. Conclusion

In conclusion, we have successfully generated a polyclonal antibody response against sarin utilizing multiple hapten approach. A high antibody titer with fine specificity was obtained with hapten B–BSA having hapten density around 24 molecules per carrier protein. An IC_{50} of free antigen was found to be 0.4 $\mu g\,mL^{-1}$ in heterologous CIEIA. Under optimized conditions, the quantitative working range was 0. 19–1.56 $\mu g\,mL^{-1}$ with a LOD of 0.05 $\mu g\,mL^{-1}$. There was negligible CR with other OP toxicants and pesticides except soman. Analysis of the IC_{50} values of all the inhibitors suggests that OP-B has high specificity for *O*-isopropyl methyl phosphonate. These results suggest that OP-B could be used in an immunoassay to distinguish GB agent in the presence of other organophosphorous toxicants or pesticide. The mean recovery of IMPA using ELISA was found to be 92.5%. Thus, by the presence of IMPA in environmental matrix the probable prior presence of sarin can be detected. The presented CIEIA does not require any sample pretreatment other than filtration. We conclude that OP-B could be used in general diagnostics test for organophosphorous chemical warfare agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.07.011.

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