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Research Paper

Synthesis of hapten for immunoassay of chlorpyrifos pesticide

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Abstract

The production of chlorpyrifos specific antibodies requires presence of a conjugated form of the pesticide to develop an immune response in animals. Thus in turn demands the design of an appropriate chemical structure (a hapten) which can be covalently coupled to a carrier protein. Chlorpyrifos being a low molecular weight compound (M.W.350.4 gm) which is below the threshold for a molecule to be antigenic so, unable to elicit an effective immune response to generate anti chlorpyrifos antibodies. Hence to make it antigenic it needs covalently linkage with suitable carrier molecules. This was carried by replacing chlorine atom at 6th position of pyridyl ring in the chlorpyrifos molecule with thiopropanate group. Characterization of prepared hapten by thin layer chromatography (TLC) showed the appearance of two bands one for standard chlorpyrifos having 0.67 Rf value and an another (unknown) band for chlorpyrifos antigen showing the attachment of thiopropanate group with 0.41Rf value. It was further analyzed by nuclear magnetic resonance (NMR) and spectral data of hapten was recorded in terms of peaks at 400.13 Mhz. NMR study indicated the highest peak with 7.94Mhz representing the aliphatic chain with sulphur group and showing the presence of thiopropanate group thereby confirming the formation of hapten. While the other small peaks with 4.39, 4.14, 3.36, 1.41 and 1.39 Mhz values represent benzene groups in the NMR spectrum. After that hapten containing active carboxyl group was then coupled with carrier protein i.e. BSA for its further usage as antigen.

Keywords: Antigen, Chlorpyrifos, Hapten, Immunoassay, Residue analysis.

Introduction

Due to the widespread use of pesticides, there is an increasing concern over food and environmental contamination caused by their use. The techniques such as gas chromatography [1,2] liquid chromatography [3,4] and high-performance liquid chromatography [5] have been used successfully with great sensitivity and reliability for detection of chlorpyrifos and its metabolites. However, these classical methods require a high capital expenditure and skilled analysts and involve time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Immunoassays have recently been emerging as an alternative to traditional methods to meet such demands. Immunochemical techniques that have been used extensively in clinical laboratories began recently to gain fast acceptance, sensitive and cost-effective tool for environmental analysis .These can even detect at nano-gram scale among targeted compounds *in situ* [6-8]. As immunoassay is a novel and promising analytical technique with high specificity, sensitivity, simplicity and suitability for the analysis of a large number of samples in a short period of time, while only low sample volumes are necessary. These advantages of immunoassays utilized for their high throughput

capacities and ability to detect the analyte in complex samples such as whole blood, plasma, serum or urine, without prior extensive sample preparation, no extraction steps and clean-up procedures. Though ELISA's have been developed for the detection of various pesticides, a few attempts have been made for chlorpyrifos[5]. Even in those, the detection assays previously developed had low sensitivity limits.

Chlorpyrifos [*O*, *O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] being a broad spectrum organophosphorus insecticide, and used extensively in both agricultural and domestic condition. Chlorpyrifos can enter the animal/human body by all routes, including inhalation, ingestion and dermal absorption [9]. It moderately toxic to mammalian species, but extremely toxic to bees and a wide range of aquatic species [10]. Therefore, there was an increasing demand for its more comprehensive monitoring. Chlorpyrifos may remain in the body for several days or weeks in severe cases and may promote an occurrence of severe clinical effects. Chlorpyrifos decreases the synthesis of DNA in the developing brain, leading to a drop in the number of brain cells. If these findings are extrapolated to humans, it may mean that early childhood exposure to chlorpyrifos may lead to lasting effects on learning, attention and behavior [11]. Since chlorpyrifos pesticides are small molecules therefore pesticide derivatives namely haptens, must be synthesized and coupled to proteins to induce antibody production[12]. Chlorpyrifos lacks a reactive functional group, so its couplings to the proteins need the prior introduction of reactive amino or carboxyl group. Therefore the present study was planned with the objective of hapten synthesis for immunoassay of chlorpyrifos detection by using the sera against the prepared conjugate.

Materials and Methods

a) Reagents used

Chlorpyrifos (Eldrin EC-20), 3-Mercaptopropanoic acid, absolute ethanol, KOH, N-hydroxysuccinimide, N,N-dimethylformamide , dicyclohexylcarbodiimide, silica gel G, BSA (A-3059) , borrate buffer, lypholizer, rotary evaporator, reflux apparatus, hexane, ethyl acetate and acetic acid, chromatography tank and dialysis bag.

b) Hapten synthesis

Hapten was prepared by replacing chlorine at 6^{th} position in the pyridyl ring of chlorpyrifos molecule with a thiopropanoate group. Ten grams of 3-mercaptopropanoic acid was dissolved in 250 ml of absolute ethanol, to which 7.5 g KOH was added and heated until dissolved. Ten grams of technical grade chlorpyrifos was dissolved in 250 ml of absolute ethanol and added to the solution prepared above. After reflux for 1 h, the reaction mixture was filtered and the solvent was removed under reduced pressure. The residue was again dissolved in 250 ml of 5% $NaHCO_3$, followed by three washings of hexane (100 ml each). Aqueous layer was acidified to pH 2 and extracted with three washings of dichloromethane (DCM) 250 ml each. The extract was dried over sodium sulphate (Na_2SO_4) and concentrated on rotary vapour.

c) Characterization of the hapten

Product thus obtained in the form of white powder was further characterized by thin layer chromatography (TLC) and Nuclear magnetic resonance (NMR) spectral analysis.

(i) Thin layer chromatography analysis

In the present work, plates for TLC (each measuring 20 x 5 cm with 0.25 mm layer thickness) were prepared by spreading a uniform layer of silica gel G in the form of aqueous slurry (10g SiO_2/100 ml H_2O) on glass plates. The plates were dried at room temperature followed by activation at 120 ˚C for 45 mintue. Then 50 ml of hexane: ethyl acetate (3:2) with 1% acetic acid was added to a round glass chromatography tank (25 cm X 10 cm) lined with filter paper and covered tightly. Vapours in tank were allowed to equilibrate for at least 2 hours. The plate was spotted with chlorpyrifos and prepared hapten after that developed in the pre-saturated tank. After developing the plates, the extra solvent was evaporated in fume hood, and again the plate was developed in another pre-saturated tank with iodine crystals for 30 seconds. The excess of iodine was removed in fume hood for about 45 minutes. The

distance travelled by solvent and eluted compounds was noted. The location of spot is calculated through an Rf (retention factor) by using the following formula:\

$$Rf = \frac{\text{Distance travelled by sample from origin}}{\text{Distance travelled by solvent front from origin}}$$

R_f values of hapten was calculated and compared with chlorpyrifos (Eldrin -20) considering it as as standard

(ii) Nuclear Magnetic Resonance Imaging

Structural modification of chlorpyrifos was confirmed by Proton-Nuclear Magnetic Resonance (^{1}H NMR) using dimethyl sulphoxide (DMSO) as the solvent. About 700 µL of deuterated solvent (for a 5-mm tube) was used to dissolve 5mg-10mg of sample. It was further filtered by using glass wool. Wash glass wool with a small amount of deuterated solvent to remove glass particles. The height of liquid should be about 4.5 cm. Tube labeled (on the cap or topmost 2 cm of the tube) with permanent marker. NMR tube caps kept tightly to prevent evaporation of solvent while waiting in the queue. Paramagnetic compounds have broad peaks or missing signals on NMR spectra that may not be identifiable. Therefore typical concentrations like 10-mg (H1)/50 mg (C13) for 300 MHz, 5 mg (H1) /20 mg (C13) for 400 MHz were used to obtained NMR spectra with different chemical shifts. After chemical shifts were expressed in MHz and the results were compared with the one result obtained earlier for the same hapten [12].

d) Preparation of hapten protein conjugate

Hapten containing an active carboxyl group was coupled with the carrier protein by N-hydroxysuccinimide active ester method. Hapten was dissolved in N, N-dimethylformamide (DMF) to prepare a 25 mM solution.50% molar excess of N-hydroxysuccinimide (NHS) and dicyclohexyl carbodiimide (DHC) were added to the above solution and the mixture was activated for 2 h at 37°C. Mixture was centrifuged at 5000 rpm for 20 minutes and supernatant were collected. Ten mg/ml BSA was prepared in 0.2 M borate buffer (pH 9.0) and added to the supernatant with vigorous stirring. Mixture was stirred at 37 °C for 2 h to complete the conjugation.

e) Purification of conjugate

Conjugate prepared above was separated from uncoupled hapten by dialysis against 0.01 M phosphate buffered saline, (PBS) pH 7.2. Conjugate was aliquotted and stored at -20 °C until use.

Results and Discussion

a) Structural modification of chlorpyrifos

Out of the two approaches that could be used to modify the structure of chlorpyrifos are:
a) Spacer coupling through the pyridyl ring by substitution of chlorine at the 6th position (ortho- to nitrogen)

b) Through replacement of the o-ethyl group with a suitable spacer arm keeping the pyridyl ring moiety intact; the former one was successfully followed in the present attempt. In the present study, chlorine present at the 6th position in the pyridyl ring moiety was replaced with the nucleophilic mercaptopropanoate group to generate a free carboxylic functional group at the terminus of the molecule (Figure 1). Basic synthetic ways for preparation of the hapten derivatives (hapten design) have been explored [7,13]. Hapten immunochemistry represents a consistent area for the development and preparation of conventional antibodies.

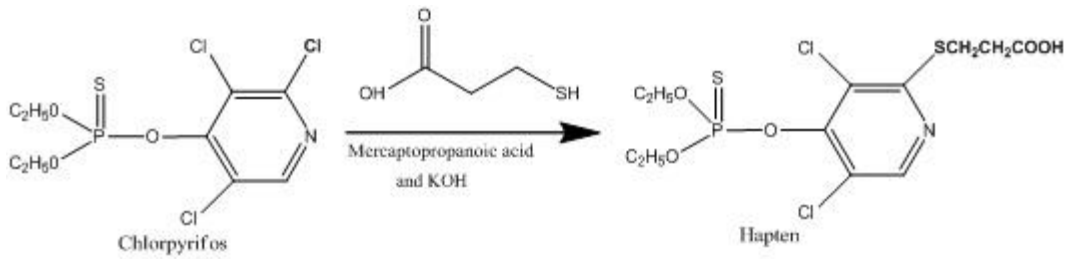

Figure 1: Synthesis of chlorpyrifos hapten (Introduction of thiopropanoate group in the presence of mercaptopropanoic acid and KOH)

Earlier reports are available regarding the synthesis of different types of chlorpyrifos haptens by replacing the o-ethyl group with spacer arms of different lengths [6, 14]. or replacement of chlorine in the aromatic ring [12]. In the present study, hapten was synthesized by derivatizing chlorpyrifos through its aromatic ring moiety since it involves only a minor modification of the analyte chemical structure thereby retaining its structural recognition and properties besides preventing any undesirable antibody response as has been observed [14]. Moreover, for the synthesis of hapten by the method of replacing the o-ethyl group, one key ingredient ethyl dichlorothiophosphate, which are highly toxic, inflammable and can cause severe burns and liberates toxic gases on coming in contact with acids. Further, by present attempt the yield of the hapten was significantly higher beacuse of 3-mercaptopropanoic acid reaction with chlorpyrifos yields 3.024 g hapten starting with 10 g of the technical grade chlorpyrifos. This accounts to 30.24 % yield. Earlier, 9.28% yield was obtained[14], which was quite low as compared to that obtained in the present study.

b) Characterization of the hapten:

The hapten, thus synthesized was characterized by thin layer chromatography (TLC) and Nuclear Magnetic Resonance (NMR) spectral analysis.

(i) Thin Layer Chromatography (TLC)

Lane-1 showed the TLC analysis of chlorpyrifos and lane-2shows the TLC analysis of hapten samples with unknown marked run with the solvent (ethyl acetate/tetrahydrofuran/acetic acid: 75:25:1). The Rf value of hapten was found to be 0.41 and that of chlorpyrifos 0.67(Fig.2). Similar observation has been recorded for the same hapten [14] and compared with the result obtained in present study.

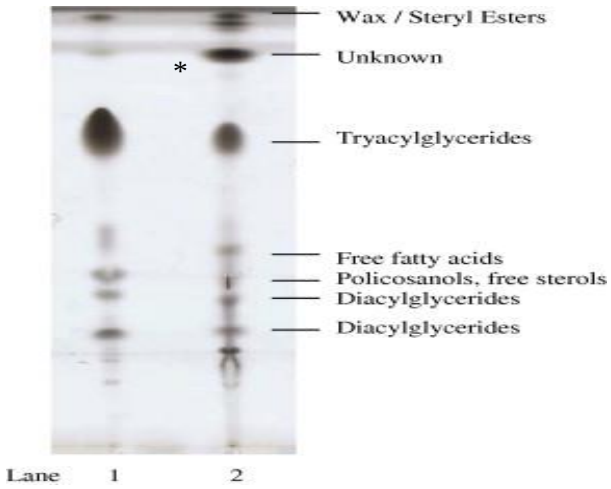

Figure 2: Thin Layer Chromatography Plate showing the presence of bands for chlorpyrifos and chlorpyrifos antigen

***Unknown**: chlorpyrifos antigen, **Lane 1:** Chlorpyrifos, **Lane 2:** Chlorpyrifos antigen.

(ii) Nuclear Magnetic Resonance Imaging

Structural modification of chlorpyrifos was further confirmed by Proton-Nuclear Magnetic Resonance (^{1}H NMR) using dimethyl sulphoxide (DMSO) as the solvent [15].

^{1}HNMR studies show the characteristic signal at (400.13 MHz in DMSO^6) 7.94 (s, 1H), 4.39(q, 2H), 4.14 (q, 2H), 3.36 (m, 4H), 1.41 (t, 3H), 1.39 (t, 3H). The chemical shifts were expressed in 400.13MHtz and the abbreviations s, d, t, q, m represent singlet, doublet, triplet, quartlet and multiplet, respectively. NMR study indicated the highest peak with 7.94 MHz representing the aliphatic chain with sulphur group and including the presence of thiopropanate group while the other small peaks with 3.36, 1.41, and 1.39MHz values represent benzene groups in the NMR spectrum. The result yielded in the present study (Fig.3) matched with the one obtained earlier [12] thereby confirming the purity and confirmation of the hapten.

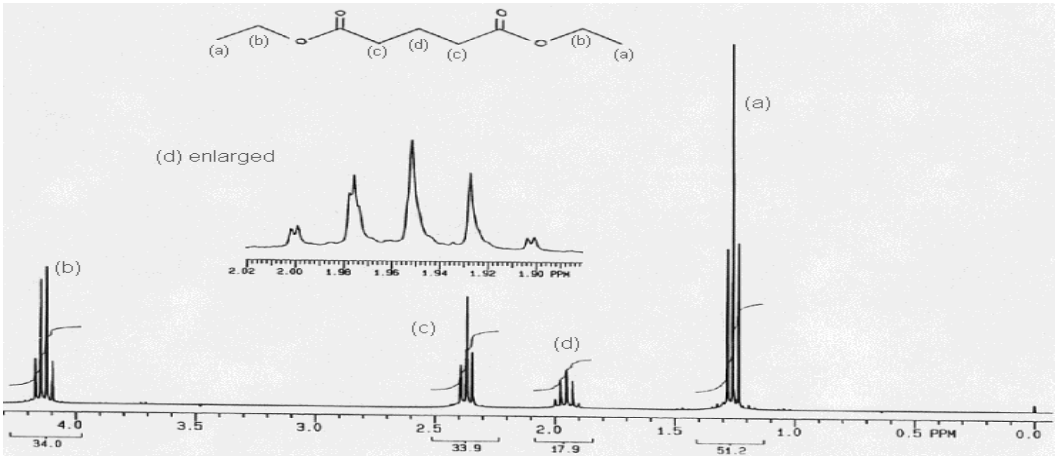

Figure 3: The NMR spectral data of the synthesized chlorpyrifos hapten showing the highest peak for the presence of thiopropanate group

c) Coupling of hapten to carrier protein

Various proteins have been used by a number of analysts with ease and effectiveness, as carrier molecules with a variety of pesticides [6, 16, 17]. In the present study, BSA was choosen as the carrier protein since a single polypeptide chain of BSA has 59 lysine groups containing side chain of amino groups, out of which 30-35 are available for coupling to carboxyl group of hapten, which makes it an excellent carrier. OVA has 20 lysines and KLH has a very high number of lysines (300-600 are usually available for binding). It always best to choose the carrier protein containing the optimum number of lysine residues for binding, since very high number of binding sites will reduce immunogenicity because of either the change in tertiary structure of the protein caused by masking of the essential free amino groups or the removal of critical determinant sites on the carrier by haptenic block. Moreover, the easy availability of BSA and ability to solubilize in organic solvents under various pH range and ionic strength makes BSA a popular carrier protein. The conjugate was obtained in dilute form after dialysis and hence, lyophilized at -40 º C for 2 hours to concentrate it.

d) Purification of conjugate

The conjugate was separated from the uncoupled haptens by dialysis since it results in a well purified antigen and a simple process. Dialysis has earlier been successfully used for separation of hapten-protein conjugates from uncoupled haptens [16, 18]. Bromophos hapten-protein conjugates were separated from the uncoupled haptens by gel filtration (Sephadex G-25), the same has been applied for the separation of chlorpyrifos-protein conjugates [12, 14]. Reports are also available describing the separation of Cyanophos-protein conjugates by gel filtration followed by Dialysis [19].

Conclusion

Hapten of chlorpyrifos was prepared by nucleophilic substitution of chlorine present at the C-6 position of the aromatic ring of chlorpyrifos, with a mercaptopropanoic group. For this, chlorpyrifos was made to react with mercaptopropanoic acid in the presence of potassium hydroxide (KOH). The purity of the hapten/chlorpyrifos-derivative containing the carboxylic group attached to its terminus was confirmed by TLC analysis and the hapten was characterized by proton-magnetic resonance imaging (^{1}H-NMR). Subsequently, the hapten was conjugated with bovine serum albumin (BSA). The yield of the conjugate founded during the current study was 30.24%.

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