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Estimation of percent conjugation of cypermethric acid hapten to BSA and tetanus toxoid using 2,4,6 trinitrobenzenesulfonic acid method

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The use of immunoassays for analysis of small molecules has been extensive in endocrinology, clinical chemistry and other fields. However, small molecules such as pesticides are not able to induce immunogenic response by themselves but can generate immune response when conjugated to higher molecular weight compounds such as proteins. When the haptens are coupled to proteins the estimation of per cent conjugation is important as higher the concentration of hapten to protein more can be the specificity of developed antibodies. Here 2,4,6, trinitrobenzene sulphonic acid method was used to determine the per cent conjugation. Cypermethric acid, a metabolite of cypermethrin was used as hapten and was coupled to BSA and tetanus toxoid and the per cent conjugation was found to be 40% and 27 %, respectively.

Key words : Cypermethric acid, Hapten, Tetanus toxoid, Per cent conjugation.

INTRODUCTION

The use of immunoassays for analysis of small molecules has been extensive in endocrinology, clinical chemistry and other fields (Christopoulos and Diamondis, 1996). Immunoassays fro pesticides are the analytical methods, which make the use of antibodies as specific biochemical detector. Pesticides being small molecules are not immunogenic and are known as haptens. The antibodies are produced by immunizing mammals with analyte carrier. For development of a highly sensitive immunoassay, antigen recognition by the antibody is very essential. Many large molecules of higher molecular weight are able to generate immune response and produce specific antibodies. However, small molecules such as pesticides are not able to induce immunogenic response by themselves but can generate immune response when conjugated to higher molecular weight compounds such as proteins (Mellenburg *et al.*, 1995). Thus generation of antibodies to such small molecules becomes easy for the development of their immunoassay which ultimately helps in their analysis at very low concentration. The perfect haptens contains as much as of the structure of target molecule as possible plus a handle to facilitate recognition of target structure by antibodies. This is usually 3-6 carbon atoms long and contains a functional group such as $-NH_2$, -COOH, -SH, and –OH (Harrison *et al.*, 1988).

For this work Cypermethrin, a synthetic parathroid was selected. Cypermethrin is used against a wide range of insect pests particularly Lepidoptera in cereals, vegetables and fruits. Cypermethric acid, a metabolite of cypermethrin which contains most of the structure of cypermethrin was used as hapten and was coupled to 6 aminohexanoic acid as spacer arm.

Protein contains various functional groups (Table 1) at which small molecules can be attached through various methods.

The proteins can be conjugated to haptens by various methods like mixed anhydride, carbodiimide (Sheehan and Hess, 1995), glutaraldehyde (Holzapple *et al.*, 1994), n-hydroxysuccinimide ester (Anderson *et al.*, 1964) method etc. Various methods that can be used to estimate of

Table 1 : Functional groups of carrier proteins for hapten conjugation (Butler, 1977).

S. No.	Functional Groups	Amino acid (s)
1	Amino	Lysine ($-NH_2$ terminal amino acid)
2	Carboxyl	Glutamic acid, Aspartic acid (COOH-terminal amino acid)
3	Phenolic	Tyrosine
4	Imidazo	Histidine
5	Sulphhydril	Cysteine
6	Indolyl	Tryptophan
7	Guanidino	Arginine

coupling of proteins to haptens include direct chemical method (Dixon *et al.*, 1975) , spectrophotometric analysis (Little and Donahue, 1967), trinitrobenzene sulphonic acid method (Habeeb, 1966), ophthalaldehyde (Hermanson, 1994), radioactive labeling and acid hydrolysis.2,4,6 Trinitrobenzene sulphonic acid method is a simple, sensitive and direct spectrophotometric method, introduced by Habeeb, for the determination of e-amino groups of lysine present in carrier proteins, using free amino acids l-lysine and l-glutamic acid as reference standards and 2,4,6 trinitrobenezenesulphonic acid (TNBS) reagent. This simple method can be very effectively used for the estimation of per cent conjugation of hapten to protein.

Materials and Methods

Cypermethric acid coupled with 6 aminohexanoic acid hapten was prepared in the laboratory. Cypermethric acid was obtained from Gharada Chemicals, Mumbai as gift sample. BSA and 2, 4, 6 trinitrobenzenesulphonic acid were purchased from Sigma whereas Tetanus Toxoid was procured from Serum Institute Pune. All the other chemicals were purchased from Himedia or S.D. Fine chemicals. Spectrophotometric analysis was carried out on Genesys 5 spectrophotometer.

Preparation of hapten:
Cypermethric acid was coupled to 6-aminohexanoic acid which was used as a spacer arm via carbodiimide method and the hapten was characterized using various methods like TLC, Melting point, elemental analysis, FTIR and NMR.

Estimation of protein content of tetanus toxoid:
Protein content of the tetanus toxoid was estimated by Lowry method using BSA as standard protein.

Preparation and Purification of Hapten-tetanus toxoid conjugate:
18.2 mg (0.056 mmol) cypermethric acid hapten was dissolved in 0.5 ml dry dioxane and 17.48 ml (0.0731 mmol) tributylamine was added to it. The solution was cooled to 12-13^{0}C in a water bath. 7.4 ml (0.056 mmol) isobutylchloroformate was added to it and the mixture was stirred for 45 minutes at 12-14^{0}C. This was solution A. 3 ml tetanus toxoid (37.71 mg) was added to 1.5 ml of 1:5 dioxane: water and cooled to 4-6^{0}C. This was solution B. Solution A was added dropwise to a cooled (4-6^{0}C) solution B. This mixture was stirred for 5 hrs at 4-6^{0}C and pH was maintained using 0.1 N NaOH. The reaction mixture was dialysed four times to remove unreacted hapten and cypermethric acid hapten tetanus toxoid conjugate was purified using cellulose acetate membrane and phosphate buffer saline.

Preparation of hapten-BSA conjugate:
Cypermethric acid hapten (13.8 mg, 0.043 mmol) was dissolved in 0.5 ml dry dioxane. To it 13.15 ml (0.055 mmol) trybutylamine was added. The solution was cooled (12-14^{0}C) in ice bath. To the cooled solution 5.64 ml (0.043 mmol) dry isobutylchloroformate was added. The solution was stirred for 45 minutes at 12-14^{0}C. This solution was added dropwise to a cooled solution (4^{0}C) solution of BSA (108 mg in 1.5 ml 5:1 water:dioxane). The pH was maintained at 7.5 by 0.1N NAOH. The mixture was stirred at 4^{0}C for 5 hours. The hapten BSA conjugate was purified by dialysis using cellulose acetate membrane and phosphate buffer saline (4x500 ml).

Characterization of hapten protein conjugates:
The hapten protein conjugates were characterized by 2,4, 6 trinitrobenzene 1-sulphonic acid (TNBS) method.

Standard curve for L-lysine and L-glutamic acid:
To a 1 ml L-lysine and L-glutamic acid solution (4-20 mg), 1 ml of 4 % sodium bicarbonate (pH 8.5) and 1 ml of 0.01% freshly made 2,4,6 trinitrobenzene 1-sulphonic acid (TNBS) were added. The reaction was carried out at 42-44^{0}C for two hours. After two hours, 1 ml 10 % sodium docyl sulphate (SDS) and 0.5 ml 1N HCl were added. The absorbance of the solution was measured at 335 nm using Genesys 5 spectrophotometer.

Nonconjugated and conjugated protein analysis:
To a 1 ml solution of BSA, tetanus toxoid, hapten-BSA conjugate and hapten tetanus toxoid conjugate (each 100mg/ml), 1 ml of 4% 2,4, 6 trinitrobenzene 1-sulphonic acid (TNBS) were added. The reaction was carried out at 42-44^{0}C for two hours. After two hours, 1 ml 10% sodium docyl sulphate (SDS) and 0.5 ml 1N HCl were added. The absorbance of the solution was measured at 335 nm using Genesys 5 spectrophotometer. The per cent conjugation was obtained directly from the given formula.

Results and Discussion

Protein content of tetanus toxoid solution was measured by Folin Lowry method (Lowry *et al.*, 1951). BSA was used as standard protein. Serial dilutions of BSA were made from 0-100 mg/0.1 ml and a standard curve of absorbance versus protein concentration was plotted. Tetanus toxoid sample was diluted twenty times and the

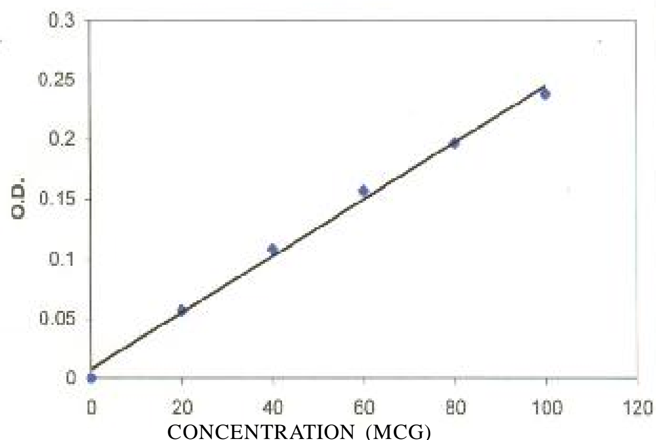

Fig. 1 : Protein estimation from tetanus toxoid.

absorbance was measured. The tetanus toxoid concentration in the solution was directly obtained from the graph (Fig. 1).

The concentration of tetanus toxoid in the solution was found to be 12.57 mg/ml.Hapten was conjugated to tetanus toxoid and BSA by mixed anhydriode method. Isobutyl chloroformate and tributylamine were used for the reaction. All the reagents used were dry. The mixed anhydride of the hapten was formed at low temperature (12-14^{0}C) by reacting hapten, isobutylchloroformate and tributylamine. After activation the mixed anhydride of the hapten was slowly added to cooled (4^{0}C) tetanus toxoid and BSA solution to obtain the desired conjugates.

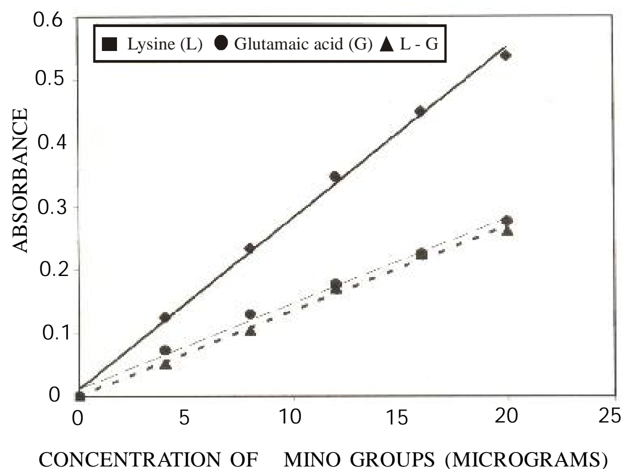

Fig. 3 : Estimation of ε-amino groups of L-lysine

The reaction is shown in Fig. 2.

Unreacted hapten was removed by dialysis against phosphate buffer saline using cellulose acetate membrane. Four changes were made, each change being after 6 hours. The hapten tetanus toxoid and hapten-BSA conjugate were then characterized by 2, 4, 6 trinitrobenzene L-sulfonic acid (TNBS) method. TNBS method is a simple, sensitive and direct spectrophotometric method for the determination of ε amino groups of L-Lysine present in the carrier proteins using free amino acids L-lysine and L-glutamic acid as reference standards. A standard curve of L-

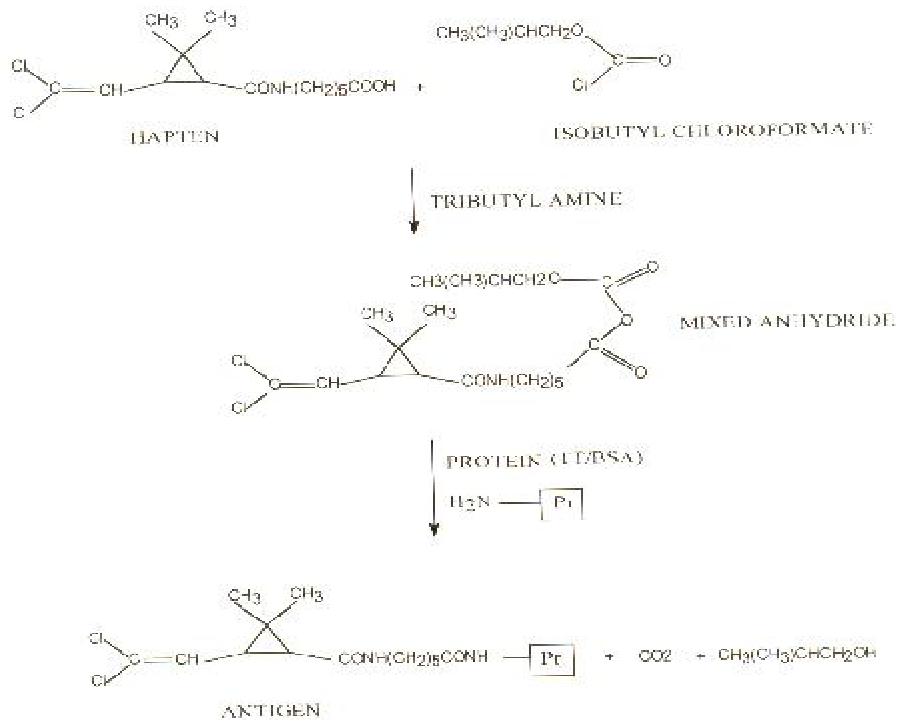

Fig. : 2 Conjugation of Cypermethric acid hapten to proteins.

Table 2 : Standard curve of L-lysine and L-glutamic acid.

S.N.	Concentration (μg/ml)	Absorbance		
		L-Lysine	L-Glutamic acid	L-Lysine – L-Glutamic acid
1.	4	0.124	0.073	0.051
2.	8	0.233	0.129	0.104
3.	12	0.347	0.176	0.170
4.	16	0.449	0.225	0.224
5.	20	0.537	0.276	0.261

Table 3: Analysis of protein samples by TNBS method.

S.N.	Sample	Concentration	Absorbance
1	BSA	100 μg/ml	0.032
2	Hapten-BSA conjugate		0.019
3	Tetanus toxoid	50.28 μg/ml	0.159
4	Hapten-tetanus toxoid		0.115

Lysine and L-glutamic acid was obtained using TNBS reagent. L-lysine has two amino groups, α-amino and ε-amino groups whileL-glutamic acid has only α-amino group. Spectral analysis of L-lysine and L-glutamic acid derivatives of TNBS at 335 nm showed that the difference between TNP-L-lysine and TNP-L-glutamic acid gives true estimation of ε-amino groups present in L-lysine (Table 3 and Fig. 3). The observations for the standard curve of L-lysine and L-glutamic acid and conjugated add non conjugated proteins are shown in Table 2.

Using the standard curve obtained for ε-amino groups per cent conjugation of hapetn to protein was calculated using the equation :

$$\% \text{ Conjugation} = \frac{\text{Concentration of } \varepsilon\text{-amino groups in carrier protein} - \text{Concentration of } \varepsilon\text{-amino groups in hapten-protein conjugate}}{\text{Concentration of } \varepsilon\text{–amino groups in carrier protein}} \times 100$$

From the given formula % BSA and % tetanus toxoid conjugation was calculated (Table 4)

Table 4: Percent Conjugation of Cypermethric acid hapten- protein conjugates.

S.N.	Conjugate	% Conjugation
1.	Hapten-BSA Conjugate	40
2.	Hapten-tetanus Toxoid Conjugate	27

Conclusion

2,4,6 Trinitrobenzene sulphonic acid method is a simple, sensitive and direct spectrophotometric method, introduced by Habeeb, for the determination of ε-amino groups of lysine present in carrier proteins. The per cent conjugation of hapten to protein was easily calculated from the simple formula and found to be 40% with BSA and 27 % with tetanus toxoid. The method can be effectively used for the confirmation and estimation of percent conjugation at ε-amino groups of L-lysine.

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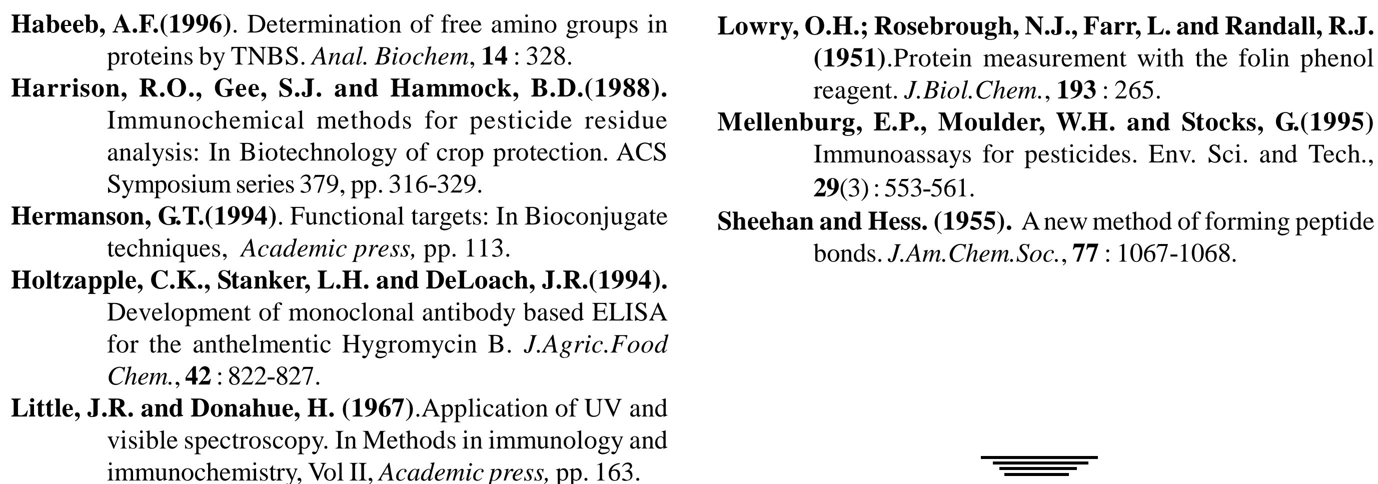

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