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Stabilization studies of working strength preparation of human albumin – HRP conjugate

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Abstract: To design an enzyme immunoassay-based method, the most important is conjugation of enzyme with antigens or antibodies and stability of labeled conjugate. In this study, human albumin was conjugated with horseradish peroxidase using periodate oxidation method. To stabilize biological activity and functional structure of different components of protein conjugate, special storage conditions are required. In this study albumin conjugate was stored using the different stabilizers i.e. biofax, protexidase and glycerol. Further working conjugates were made and stored at two different temperatures i.e. 4°C and 25°C. The stability of working conjugate was followed for 60 days. It was observed that stability of working Alb-HRP conjugate was maximum for 60 days with protexidase as stabilizer followed by the biofax and glycerol for 30 days at 4°C. While at 25°C, all working conjugates in different stabilizing solutions lost their activity only after 2 days. It was concluded that working Alb-HRP conjugate when stored in protexidase and diluted proved to be a better stabilizer for long time storage.

Keywords: Albumin-HRP conjugate, enzyme immunoassay, labeled conjugate, peroxidase.
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INTRODUCTION

Immunoassays have proved to be a great revolution and one of the most productive technical contributions in field of medicine and to fundamental life sciences as diagnostic tool. These are analytical tests based on the unique abilities of antibody/antigen[1].

Principles of immunoassay were first developed by Rosalin Yalow and Solomon Berson in 1959. These assays are not only bound to the medical diagnosis but also has been applied to veterinary, military, pharmaceutical, forensic and food sciences[2]. Enzyme-linked Immunosorbant assay (ELISA), also known as enzyme immunoassay (EIA) or immuno enzyme metric assay (IEMA). These are simple, sensitive and cost effective having long shelf life; these properties make them superior to other techniques and therefore are used for the antigen/antibody detection in sample of interest[3].

One of the important components of enzyme assay is conjugation of enzyme with antibody/antigen. Most commonly used enzyme is horseradish peroxidase (HRP), a 44,173.9-dalton glycoprotein isolated from horseradish. It has 4 lysine residues to be conjugated to antigen or antibody. The specificity to detect the protein of interest is provided by antibodies and a detectable signal is produced by HRP enzyme with its substrate[4]. Characteristics like stable nature, smaller volume and high turnover rate, make HRP an ideal enzyme for conjugation and to be used in different techniques for detection purposes. This is also an inexpensive enzyme as compared to other alternatives i.e. glucose oxidase, alkaline phosphatase. Strong signals are also generated in very short time duration due to its high turnover rate[5].

HRP-conjugates can lose their biological activity only within a small time period if not stored properly[6] therefore different stabilizers are used for the storage of conjugates. Most common are **Protexidase,** Biofax, Glycerol, Casein, Trehalose, Kathon CG, BSA, BycoA, Manitol, Dextran, Dry milk and Polyols etc. In this study Alb-HRP conjugate was prepared using periodate method[7] and stored in different stabilizers. Working conjugate were prepared and checked over a period for stability.

MATERIALS AND METHODS

HRP, BSA, Human Albumin, DEAE Sepharose and Glycerol were purchased from sigma. PD 10 and Sephadex G150 from Amersham Pharmacia biotech. Na bicarbonate and Na periodate from BDH. $CNBH_4$ and Methyl D-manno-pyranoside from Aldrich. K-blue from Neogen Co. Biofax from Flinn Scientific Inc. and Protexidase from Konto Chemicals Co. All other reagents were of analytical grade from Merck and BDH.

To purify horseradish peroxidase (HRP) Sephadex G-150 column was washed and equilibrated with 300 mls of 25mM of PO_4 buffer pH 7.4 followed by 150 mls of 2.5mM PO_4 buffer pH 8.0. (All washings were upwards for the better resolution of product). 100,000 units of HRP were dissolved in 2 ml of 2.5mM PO4 buffer pH 8 and centrifuged for 10 minutes at 5000 rpm in glass tubes. The supernatant was loaded on Sephadex G-150 column in flow of buffer and eluted with 2.5mM PO4 buffer pH 7.4 @ 60 mls/hr. two

minutes fractions were collected and profile for total protein concentration was checked at 280 nm and for enzyme activity at 403nm. Peak fractions were pooled and eluted again through the Sepharose DEAE column (30 ml) with 25 mM PO_4 buffer pH 8. Concentration of protein content was calculated from absorbance of the pool at 280 nm and 403 nm at 1:20 dilution in 25mM PO4 buffer pH 8 and same was used as blank to calculate the Rz value for purity and concentration of HRP product.

To conjugate human albumin 5mg of the HRP was conjugated with 3mg of human albumin using periodate method. Free enzyme was separated on Sephacryl column S200 and product was eluted with 25mM PBS at 30 mls/hr. The fractions were collected and read at 280 and 403 nm using PBS as blank for protein and enzyme content respectively.

To store the purified conjugate, it was divided into three fractions. Two fractions were diluted with biofax and protexidase at 1:1 dilution separately and stored at 4˚C, whereas to the third fraction 2mg/ml BSA was added, mixed and stored in 50% glycerol at -20˚C.

For stability of working Alb-HRP Conjugate at two different temperatures the conjugate stored in different stabilizers were diluted in the ratio of 1:1000K with diluent buffer and each was further divided into two portions and stored at two different temperatures i.e. 4°C and 25°C. Stability of these conjugate solutions was studied for 60 days for binding properties using albumin standards ranging between 0µg/ml to 500µg/ml.

Assay protocol

Anti albumin coated wells were allowed to react with 50µl of albumin standards i.e. 0 µg/ml, 3.9 µg/ml, 7.8 µg/ml, 15.62 µg/ml, 31.25 µg/ml, 62.5µg/ml, 250 µg/ml and 500 µg/ml and quality control pools with 200ml Alb-HRP conjugate. The wells were incubated for the competitive reaction to reach equilibrium. After washing the excessive reactants with PBSX (25mM PBS + 0.1% Triton X) 100 µl of TMB substrate was added and incubated for 20 minutes.

The reaction was stopped with 100µl of 1M H_2SO_4 and read at 450/620 nm within 30 minutes. Results were calculated using Stringray package for statistical analysis and were reported at less than 10% of CV. Sample handling and temperature conditions for all tests were strictly observed. For precision of assay calibrated micro/multi channel pipettes from Gilson were used throughout the assay.

RESULTS AND DISCUSSION

To design an enzyme immunoassay-based method, one of the most important components is conjugation of enzyme with antigens or antibodies for making a suitable conjugate for the detection of analyte of intetest[8].

Enzyme conjugation to antibody or antigen is basically formation of a linkage between enzyme e.g. urease, HRP, glucose oxidase, or alkaline phosphatase and antigen/specific antibody polyclonal or monoclonal. The linkage is stable and covalent in nature. In process of conjugation functional alteration occurs neither in the active site of enzyme nor in binding site of antigen or antibody for the analyte of interest. Scott E *et al* described methods of cross-linking of these enzymes to immune affinity-purified polyclonal or monoclonal antibodies (IgG)[9].

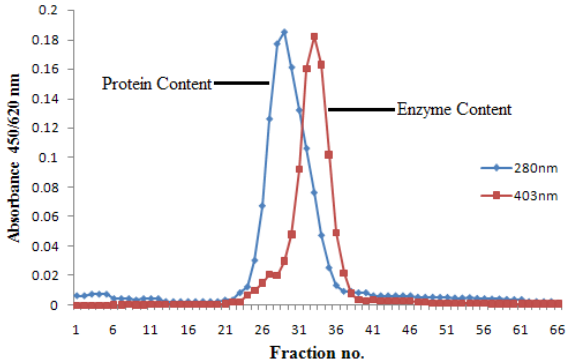

Figure 1: Elution profile of human albumin conjugate using Sephacryl S-200.

For use in ELISA, immune histochemistry or western blot etc. to detect the molecular targets. HRP can also be conjugated to antibody of interest directly or attached to a secondary antibody and then targets the antibody of interest. The HRP has also been used as colorimetric marker since very long time[10]. In such experiments, main function of HRP is to produce a detectable signal when antibody provides specificity for the location of protein of interest in presence of substrate[11].

Enzymes can also be modified to contain functional groups which are useful for conjugation with other proteins. In all methods of the conjugate preparation, avoidance of long oligomer generation and activity retention is the most important consideration. The extensive oligomer generation can cause the precipitation. Some of the common methods of producing the enzyme conjugates are: Glutaraldehyde-activated Enzyme, Periodate Oxidation Technique, SMCC-Activated Enzyme,

Hydrazide Activated Enzyme, SPDP-Activated Enzyme[4].

Purification of horseradish peroxidaes (HRP)

To prepare human albumin-HRP conjugate, purification of crude HRP was done on Sephadox G-150 column. The fractions with reasonable activity were pooled and eluted through Sepharose DEAE column. The RZ value which is the ratio of absorbance at 403nm to 280nm was taken into the consideration for activity of product. For ELISA mostly the Rz value above 0.5 is appropriate for conjugation. In this study activity of the purified HRP when read at 280nm and 403nm using bicarbonate buffer as blank. The Rz value was 3.11 which indicated that it has very high activity[7, 12].

Conjugation of human albumin

In current study, human albumin was conjugated with horseradish peroxidase for the use in assay for estimation of microalbuminuria using periodates oxidation method[7]. Unconjugated enzyme was separated on Sephacryl S-200 and eluted with 25mM PBS. The functional and enzymatic activity of eluted fractions was checked using uncoated microtitre plate and anti-albumin coated microtitre plate. Fig. 1 shows that fraction no. 24-31 has the maximum protein content coupled to HRP. These fractions were pooled and free albumin was separated on Con-A column with 0.4M methyl D- manno-pyranoside. The Rz value of the purified conjugate was 3.38.

Stability of working Alb–HRP conjugate at two different temperatures

To stabilize biological activity and functional structure of different components of protein conjugate, special storage conditions are required[8,13]. The conjugate stability in solution and lyophilisation affects its enzymatic activity[14]. In the current study only stability studies were carried out on conjugate in its liquid form and no lypholysation was done. It has been observed in other studies that conjugates which contained Kathon CG, BSA and calcium chloride in tris-HCl buffer maintained activity of HRP-AFB1 diluted at 1:1000 for 1 year at room temperature but the reference conjugate in which no any additive was used lost its activity after only a few days[6]. Studies elsewhere have also used stabilizers for the stability of conjugate e.g. BSA[15] polyols[16] potassium phosphate and Tris-HCl[17] dextran[18] glycerol, kathon CG, ethanol, trehalose[19] etc. In a study effect of different stabilizers on stability of HRP conjugated enzyme of afllatoxin were checked for 45 days in accelerated and for 10 months in normal procedures and found out that stability of HRP-BSA-AFB1 conjugate was best in trehalose and casein[20].

In current study the conjugate was stored in three different stabilizers i.e. biofax, protexidase and glycerol available at NHRC labs. The purified conjugate was divided into three parts and then added to three different stabilizers in ratio of 1:1 dilution. Working conjugates were prepared and stored at two different temperatures i.e. 4°C and 25°C for stability studies for 60 days.

Figure 2 shows the stability of standard curve using working conjugates in different stabilizers at different time intervals stored at 4°C.

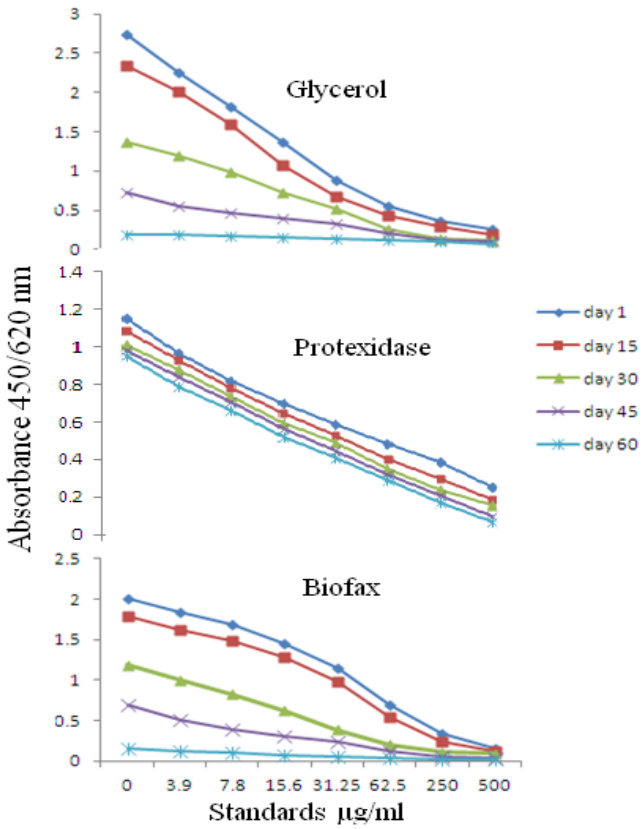

Figure 2: Stability of working conjugate at 4ºC over 60 days.

Absorbance was plotted against the no. of days. Table 1 shows the values of quality control pools run in these assays. QC values of assay batches using working conjugate in protexidase remained consistent even after 60 days. Insignificant difference was observed in the binding properties and slope of standard curve.

QC values also remained within 2SD. Using working conjugate with biofax and glycerol as stabilizer; QC values were consistent during first four weeks of preparation of working conjugates. Inspite of very low binding on day 45 (Figure 2) the QC values were not very different and remained within 2SD (Table 1). Whereas on day 60 the

binding of standard points dropped to great extent and was reflected in values of QC pools (Table 1). Figure 3 shows the stability of standard curve using working conjugates in different stabilizers at different time intervals stored at 25°C. Absorbance was plotted against the no. of days.

Table 1: Stability of quality control pools (known values) at 4˚C over a period of 60 days.

Time (days)	Preservatives								
	Biofax (µg/ml)			Protexidase (µg/ml)			Glycerol (µg/ml)		
	QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3
1	34.2	64.5	505	33	64.05	495	34	66.5	507
7	34	63.9	501	32.3	64	492	33.7	64.9	503
15	32.7	62.6	496	31	63.9	508	32.2	62.5	495
30	31.2	58.12	493	30.2	63.5	496	31.9	59.2	491
45	27.9	46.5	409	30	63.4	503	27.4	45.5	404
60	11.3	20.2	150	29.2	63.1	501	10.3	12.4	160

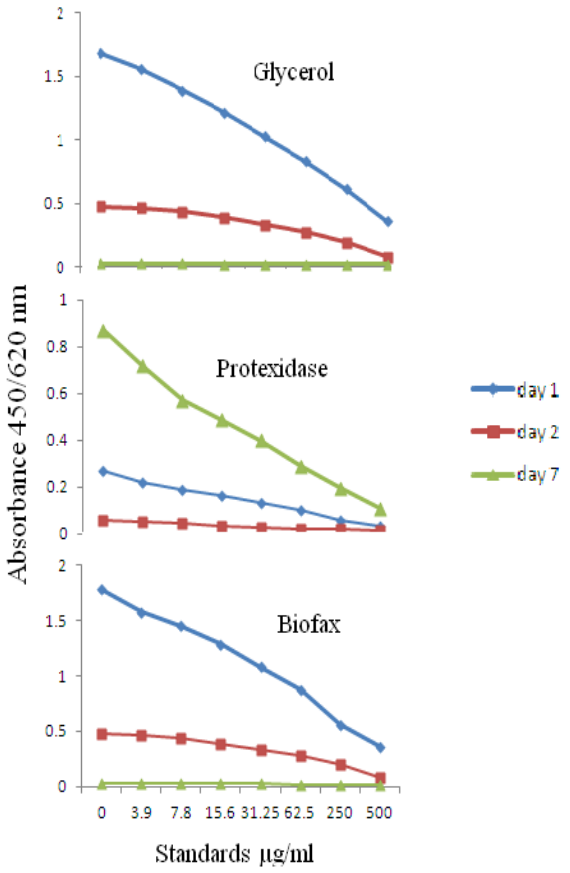

Figure 3: Stability of working conjugate at 25°C over 7 days.

Table 2 shows the values of quality control pools run in these assays. It is clear from figure 3 and also table 2 that the working conjugate stored at 25°C showed, on day one the binding was appropriate in all working conjugates in their respective stabilizers, whereas on day 7, the binding was dropped significantly and there was no displacement in standard points. Table 2 shows the instability of QC values.

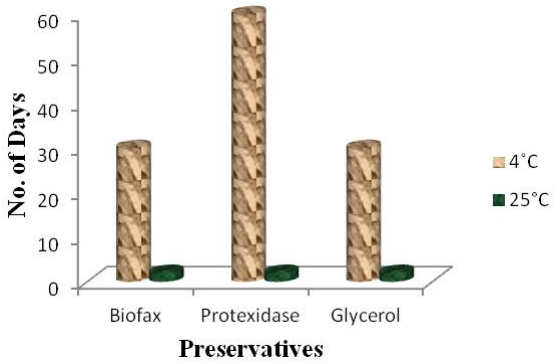

Figure 4: Comparison of different stabilizers used for storage of working Alb-HRP conjugate

This stability study showed that conjugate with glycerol and BSA as stabilizer was not so good as compared to other stabilizers. The main reason could be damage of binding sites because of proteolytic activity of BSA. Taylor LS *et al* used the conjugate for studying the effect of BSA on its stability. The conjugate had BSA in its own molecule. In his study it was observed that interaction between the BSA present in molecule and BSA used in stabilizer was not favorable[15]. In our study it was also observed that stabilizers containing BSA in their composition were not suitable for conjugate stability as compared to other stabilizers. It might be due to the changed conformation induced by BSA.

Table 2: Stability of quality control pools (known values) at 25˚C over a period of 7 days.

Time (days)	Preservatives								
	Biofax (µg/ml)			Protexidase (µg/ml)			Glycerol (µg/ml)		
	QC 1	QC 2	QC 3	QC1	QC2	QC 3	QC 1	QC 2	QC 3
1	35	67	505	36.2	53.7	508	33	62	505
2	25	48	430	20.25	38.12	174	22	45	430
7	5	7	30	9	11	40	6	8	30

On comparing the stability of Alb-HRP conjugate in three different stabilizers. It was observed that protexidase was more stable at 4°C

followed by the biofax and glycerol. While at 25°C, all working conjugates in different stabilizers were not stable for a longer period as shown in Figure 4. Therefore it was concluded that for the storage of conjugate, similarity between the components of stabilizer and conjugate must be considered.

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