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# Improved Method For Hepatitis B Vaccine In-Vitro Potency

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***ABSTRACT :*** *Aluminum salts were most successful and widely used adjuvant for vaccines. Analysis of antigen quality and stability after addition to adjuvant is of great importance in selection of adjuvant for vaccine formulation. Methods to determine the quality of the vaccine in presence of adjuvant needs to be developed to characterize the antigen adjuvant interaction and to control the vaccine formulation quality during routine manufacturing and stability. Some manufacturers use aluminum hydroxide and some use aluminum phosphate as adjuvant for same antigen vaccine. For analysis of antigen adsorbed to aluminum salt separation from aluminum salt particles is required. Separation of aluminum salts and antigen can be achieved by desorbing the antigen. In this research work method for desorption of antigen from aluminum adjuvant before estimation of antigen was evaluated. Desorption of hepatitis B surface antigen in Hepatitis B vaccine prepared using aluminum phosphate and aluminum hydroxide was tried using desorbing agents. Combination of salts with metal chelator found suitable for desorbing hepatitis B surface antigen without effecting antigenicity from both aluminum phosphate and aluminum hydroxide.*

***KEYWORDS :*** *Adjuvant, Aluminum salts, Desorption, Hepatitis B vaccine, Potency*

## I. INTRODUCTION

The word 'Adjuvant' derived from Latin word Adjuvare which means to help. Adjuvants are used to boost the immune response and stability of the vaccines. [1] Many vaccine adjuvants were studied over 70 years for different types of vaccines. Aluminum salts were most successful and widely used adjuvant for vaccines. Analysis of antigen quality and stability after addition to adjuvant is of great importance in selection of adjuvant for vaccine formulation. Methods to determine the quality of the vaccine in presence of adjuvant needs to be developed to characterize the antigen adjuvant interaction and to control the vaccine formulation quality during routine manufacturing. Various desorption methods can be used to desorb the protein from adjuvant. Some are mild and some are harsh agents. Mild agents may cause under estimation owing to incomplete desorption, harsh conditions may denature the protein. Salts and surfactants can be used for desoring proteins from aluminum salts. Sometimes combination of desorption agents needed to desorb the protein completely from adjuvant. Typical salts used for desorption of proteins from aluminum salts include citrates, carbonates, sodium di hydrogen phosphate, tri pottasium phosphate, dipotassium phosphate, ammonium phosphate , sodium phosphate. Salts concentrations up to 1000 mM is used to desorb the proteins completely. Desorption salt should remain soluble at the concentrations used for desorption. Surfactants of any natures ie. non ionic, ionic or zwitterionic can be used for desorption. surfactants like triton X, TDAP can be used. surfactants like tweens helps in protecting proteins during the desorption process.

Recombinant hepatitis B vaccine was introduced in 1986. First WHO requirements for hepatitis B vaccines made by recombinant techniques in yeast were published in 1987 and revised in 1989 to include all vaccines produced by recombinant DNA techniques. The active substance in recombinant hepatitis B vaccine is HBsAg that has been produced in yeast or mammalian cells into which the HBsAg gene (or HBsAg/pre-HBsAg genes) has been inserted using plasmids. [2] The transformed cells are grown in large vessels, and the expressed HBsAg self assembles into spherical particles that expose the highly immunogenic determinant. Following thorough purification from host-cell components, alum is added to make final vaccine for immunization. A new recombinant hepatitis B vaccine that is intended for adult patients with renal insufficiency uses alum and lipid A as adjuvants. Hepatitis B vaccine is available as monovalent formulations or in fixed combination with other vaccines, including diphtheria–tetanus–pertussis (DTP), Haemoeuxphilus influenzae type b, hepatitis A and inactivated polio. In monovalent hepatitis B vaccine aluminum hydroxide is the preferred adjuvant and in combination vaccine either aluminum phosphate is used or combination of aluminum hydroxide and phosphate is used. All the Indian manufacturers are using aluminum hydroxide for monovalent hepatitis B vaccine and aluminum phosphate for combination vaccines containing hepatitis B component.

Potency testing of hepatitis B vaccine is carried out by either in-vivo or in-vitro method. In-vitro method of estimation includes mostly ELISA based. Use of in-vivo potency for routine testing require large number of animals and not cost effective. For in-vivo potency detailed procedure was defined in pharmacopoeia and for in-vitro no procedure was detailed.[3, 4, 5]. When WHO requirements for recombinant hepatitis B vaccines were published, it was considered that assays of antigenicity of adjuvanted vaccines would be difficult to standardize. The potency requirement stated 'that an appropriate quantitative test for antigen content and an immunogenicity assay be performed on samples representative of the final filling lot. Several factors must be considered when validating an in-vitro assay for an individual vaccine. as vaccines from different manufacturers contain different forms of adjuvant. These include the need for a pre-treatment step to disaggregate the antigen, eg with detergent, to ensure consistent responses in vaccines of all ages, the HBsAg test kit used, the diluent in which dilutions of the vaccine are prepared and the reference preparation [6].The FDA-approved monovalent HBsAg vaccine with the highest mass ratio of HBsAg to aluminum, Engerix-B®, contains only 10 µg HBsAg per 250 µg aluminum from aluminum hydroxide. This ratio, 0.04 mg HBsAg/mg aluminum, is likely far in excess of the adsorptive capacity of the adjuvant for this antigen in the vaccine, ensuring complete adsorption. Investigations into the direct effects of antigen adsorption on antigen conformation and stability have only recently begun. The work of Hem and colleagues demonstrated that the environment of the protein adsorbed to a mineral salt adjuvant can be significantly different from the bulk environment in which the protein stability is normally studied. In one of the earliest such studies published, [7] used SDS-PAGE, denaturing size exclusion chromatography (SEC) and microscopy to investigate the structural perturbations of HBsAg following adsorption. On storage adsorbed antigens also showed a sharply decreased ability to desorb from the adjuvant. Consequence of chemical and physical alterations is the inability to adequately desorb proteins from the adjuvant, strong adsorption may negatively impact the immunogenicity of the vaccine[8].

## II. MATERIAL AND METHODS

### 2.1. Material

Hepatitis B surface antigen (HBsAg) produced in yeast Pichia Pastoris was provided by Biological E Ltd, Hyderabad. Aluminum phosphate and Aluminum hydroxide adjuvants were purchased from Brentag Biosector, Denmark. AxSYM HBsAg V2 kit for antigen estimation was purchased from Abbott . All the chemicals for buffers preparation, salts used for desorption and bovine serum albumin were of analytical grade and purchased from commercial suppliers.

### 2.2. Methods

#### 2.2.1. Preparation of Hepatitis B vaccine formulations with Aluminum adjuvants

Formulations with three different concentrations of HBsAg protein (10, 20 & 30 µg/mL) were prepared with aluminum hydroxide and aluminum phosphate. Both aluminum hydroxide and aluminum phosphate were used at 0.5mg/mL of aluminum. 100 mL of each formulation was prepared.

#### 2.2.2. Desorption of Antigen from Adjuvant

Desorption of adjuvant is carried out using sodium hydrogen ortho phosphate and Sodium edetate (EDTA). Desorption reagent was prepared by mixing 1 mL of 56 g per liter solution of sodium edetate and 49 mL of sodium hydrogen ortho phosphate 96 g per liter solution. Sample and standard were serially diluted with phosphate buffer saline pH 7.4 having 0.2% bovine serum albumin. Four two fold dilutions ranging in 500 to 16000 were made for each formulations such that signal falls within linear range of the assay. Desorption was carried out by mixing equal quantities of desorption reagent and vaccine formulation diluted and incubated for 3 hours at 37ºC in 96 well plate under constant shaking on a plate shaker.

#### 2.2.3. In-vitro potency testing (IVRP)

In-vitro potency of the formulations was estimated by using Abbott AxSYM HBsAg V2 kit on AxSYM automated enzyme immunoassay system. Total content of desorbed sample and standard dilutions, anti-HBs monoclonal antibody coated microparticles and biotinylated anti-HBs polyclonal antibody were mixed together and incubated in a reaction vessel. This reaction mixture was later dispensed onto a matrix cell. The anti-biotin-alkaline phosphatase conjugate was then dispensed onto the matrix cell, followed by buffer washes. The alkaline phosphatase activity was determined by the addition of a substrate, 4-methylumbelliferyl phosphate, which was converted to methylumbelliferone, and the fluorescent signal was measured by the AxSYM instrument. S/N values were calculated using an automated analyzer against the stored AxSYM HBsAg index calibration curve [9]. Relative potency was calculated using parallel line statistical program F.R.Marsman, RIVM, Bilthoven, The Netherlands (version: 2000-1) using S/N values obtained for different

dilutions of vaccine. HBsAg antigen diluted to 20µg/mL was used as standard for estimation of in-vitro potency.

## III. RESULTS

Table 1 showing in-vitro potency of the formulations tested without desorption

| HBsAg concentration | With aluminum Hydroxide | With aluminum phosphate |
|---|---|---|
| 10 µg/mL | 0.32 | 0.38 |
| 20 µg/mL | 0.64 | 0.71 |
| 30 µg/mL | 0.94 | 1.1 |
| Correlation coefficient | 1.00 | 1.00 |

Figure 1 showing graph and regression equation for obtained IVRP Vs Theoretical IVRP for formulations tested without desorption

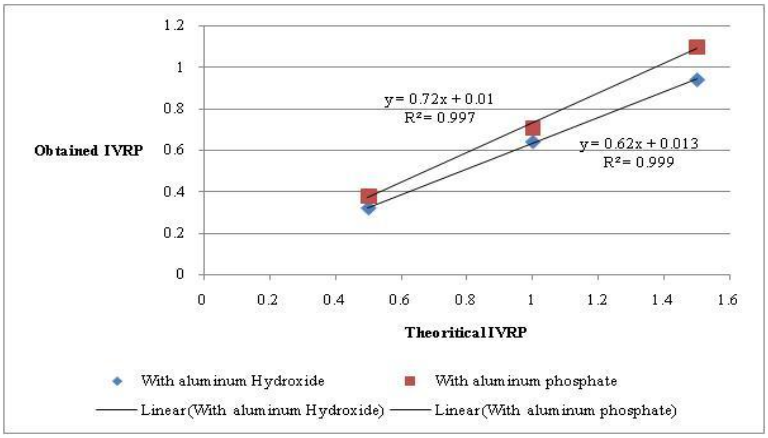


Table 2 showing in-vitro potency of the formulations after desorption

| HBsAg concentration | With aluminum Hydroxide | With aluminum phosphate |
|---|---|---|
| 10 µg/mL | 0.44 | 0.48 |
| 20 µg/mL | 0.9 | 0.93 |
| 30 µg/mL | 1.3 | 1.4 |
| Correlation coefficient | 0.999 | 1.00 |

Figure 2 showing graph and regression equation for obtained IVRP Vs Theoretical IVRP for formulations tested without desorption

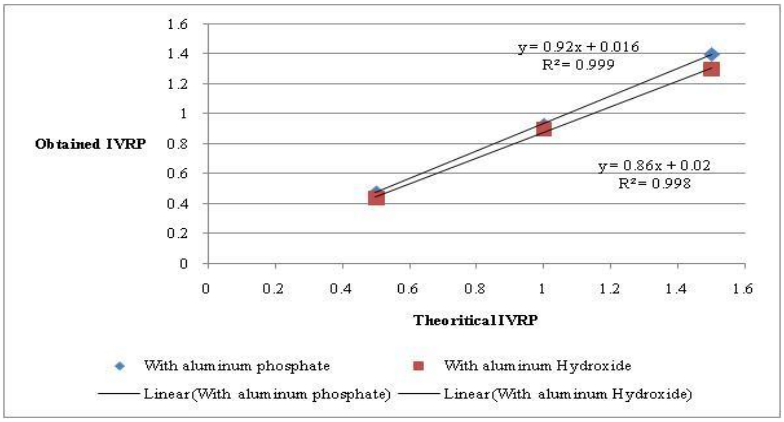

Table 3 % recovery against standard antigen 20µg/mL

| Formulation | % recovery | | | |
|---|---|---|---|---|
| | Aluminum hydroxide formulation | | Aluminum phosphate formulation | |
| | without desorption | with desorption | without desorption | with desorption |
| 10µg/mL | 64 | 88 | 76 | 96 |
| 20µg/mL | 64 | 90 | 71 | 93 |
| 30µg/mL | 63 | 87 | 73 | 93 |

## IV. DISCUSSION

In-vitro potency of different formulations estimated before and after employment of desorption step before putting in to the assay. Correlation coefficient values obtained by plotting In-vitro potency values obtained for three formulations made of aluminum hydroxide and three formulations made of aluminum phosphate against formulated protein concentration (Table 1 and 2) indicate linear increase in relative potency with increase in concentration. Enzyme immunoassay method is showing good linearity (Correlation coefficient above 0.99) with or without desorption of antigen from adjuvant. In-vitro potency values were slightly higher for aluminum phosphate formulations compared to aluminum hydroxide formulations. Desorption of antigen yielded higher in-vitro potency values and close to theoretical values considering antigen diluted to 20µg/mL as standard and its potency as 1.0. Graph plotted against theoretical relative potency against obtained relative potency showed regression ($R^2$) values above 0.99 for both sets of formulations (Fig. 1 and 2). Slope values in linear curve equation obtained for formulations (Fig. 1 and Fig. 2) explains higher response in the desorbed formulation compared to formulations tested without desorption. Percentage recoveries of formulations with aluminum hydroxide, without desorption were about 60% and with desorption were above 85%. Percentage recoveries of formulations with aluminum phosphate, without desorption were about 70% and with desorption were above 90% (Table 3). Lower recovery when tested without desorption indicate masking effect of adjuvant. The recoveries against unadjuvanted standard indicate minimal loss of antigen with desorption process.

## V. CONCLUSION

Improvement of method of in-vitro potency estimation in hepatitis B vaccine made of aluminum salts was achieved by inclusion of desorption step before estimation of antigen by enzyme immunoassay. Desorption of hepatitis B surface antigen from aluminum adjuvants without effecting the antigen quality can be achieved by using combination of sodium phosphate salt and sodium edetate. Desorption method studied is working slightly better with aluminum phosphate compared to aluminum hydroxide. Further evaluation of efficiency of desorption on storage of vaccine is required to declare suitability of this improved method on stability. No interference of desorption agents observed in enzyme immunoassay used for IVRP estimation. Method improvement with inclusion of desorption step resulted in complete recovery of antigen compared to direct estimation of vaccine by enzyme immunoassay.

## VI. ACKNOWLEDGEMENTS

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