Materials and Methods
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1 Preformulation studies

Prior to encapsulate the hepatitis B vaccine in to polymeric microspheres, an extensive preliminary preformulation study was carried out to establish the protocols for formulating an ideal vaccine loaded microspheres. Before studying the effect of process variables on vaccine loading in to polymeric microspheres, a through investigation of methods of preparation of polymeric microspheres was done by changing various parameters. To investigate the effect of process variables on vaccine loading into polymeric microspheres were carried out by changing one variable at a time keeping others constant. Thus, many batches were formulated, pooled and stored in a refrigerator. The process variables in this research work are

1.1. Effect of cross linking agent on microspheres formation

The need of cross linking agent in the formulation of vaccine loaded polymeric microspheres is the prime important for stabilization of microspheres. The selection of cross linking agent varies with polymer used. In the present study two different cross linking agents namely glutaraldehyde and dextran were employed for formulating PLGA [poly (lactide-co-glycolide)], PLA [poly (DL- lactide)] and Chitosan microspheres. However for preparing Albumin and Dextran microspheres glutaraldehyde was used as cross linker. The formation of hepatitis B vaccine encapsulated polymeric microspheres was greatly influenced by the concentrations of cross linking agent added during micro encapsulation process. The effect of change in concentration of cross linker was established by keeping the polymer concentration constant in order to ascertain the ideal concentration for respective polymer.
1.2. Effect of polymer on microspheres formation

The effect of change in concentration of polymer was studied by keeping the volume of cross linking agent constant and the polymer concentration was changed in order to establish the exact polymer concentration for obtaining ideal vaccine loaded microspheres. From the study on the effect of process variables on vaccine loading into microspheres, ideal variables were selected and ideal batches were formulated. The following techniques were used in this experiment.

- Hepatitis B vaccine encapsulated PLGA and PLA microspheres were formulated by **solvent evaporation technique**.
- Hepatitis B vaccine encapsulated chitosan, albumin and dextran were formulated by **emulsion cross linking technique**.

1.2. a. Grades of polymers

PLGA (50 : 50) and chitosan polymer of different viscosities grade i.e. 50 cps, 150 cps, and 300 cps were used in this research work to encapsulate hepatitis B vaccine.

1.3. Effect of continuous phase

Various continuous phases such as polyvinyl alcohol, sunflower oil, olive oil, linseed oil and paraffin oil were screened for respective microparticle formulation. The need for screening continuous phase was to establish their effect on the size, physical nature and vaccine pay load.
2 Preparation of microspheres

2.1 Preparation of hepatitis B vaccine encapsulated PLGA or PLA microspheres by solvent evaporation technique

1, 2 and 3% (w/v) of PLGA or PLA polymeric solution were prepared by dissolving in 1 ml of methylene chloride. The polymeric solution was kept in a cyclomixer for about 30 minutes and 2 ml (3mg/ml) of alum free hepatitis B vaccine was added to form an emulsion. The emulsion so formed was sonicated at 15000 rpm for 20 seconds and dispersed individually in a magnetic stirrer for 10 minutes in 1 ml of 0.5, 1, 1.5, 2, 2.5 and 3% (v/v) or w/v either in glutaraldehyde or dextran respectively to stabilize the microspheres. The pH of the emulsion was maintained at 7.4 in both the cases. The system was maintained under magnetic agitation for 30 minutes. The resulting emulsion was diluted individually in 400 ml of 3% (w/v) of polyvinyl alcohol solution. The system was maintained under magnetic stirrer for 8 hours to allow the solvent for evaporation. The microspheres were collected by centrifugation at 10,000 rpm for 20 minutes. Microspheres were suspended in 10 ml of distilled water and filtered through a membrane filter and dried in a vacuum desiccator. The hepatitis B vaccine encapsulated microspheres thus obtained were pooled and stored in a refrigerator.

2.2 Preparation of hepatitis B vaccine encapsulated chitosan microspheres

1, 2, and 3% (w/v) solutions of chitosan (medium molecular weight with 75 – 85 % deacetylation) of various viscosity grades were prepared in aqueous acetic acid (5% v/v). This solution was mixed with an emulsion containing sunflower oil and toluene to form water in oil (W/O) emulsion. To the individual batches, 2 ml of
hepatitis B vaccine was added and stirring was continued for half an hour. Finally, 1 ml of 0.5, 1, 1.5, 2, 2.5 and 3% either glutaraldehyde (v/v) or dextran (w/v) was added individually to their respective formulations. The emulsion was stirred at 3000 rpm for one hour. The pH of the emulsion was maintained at 6.2. The emulsion was centrifuged at 15000 rpm and the oil layer was removed. The remaining pellet was washed with petroleum ether, n-hexane and ethanol in order to remove excess oil. After washing, the hepatitis B vaccine encapsulated microspheres were collected, pooled and stored in refrigerator.

2.3 Preparation of hepatitis B vaccine encapsulated albumin / dextran microspheres

1, 2 and 3% (w/v) of albumin or dextran solution was mixed in an emulsion containing sunflower oil and toluene to form water in oil (W / O) emulsion. To the individual batches, 2 ml of hepatitis B vaccine was added and stirring was continued for half an hour. Finally, 1 ml of 0.5, 1, 1.5, 2, 2.5 and 3% (v/v) glutaraldehyde was added individually to their respective formulations and stirred for one hour. The pH of the emulsion was maintained at 6.8. The emulsion was centrifuged at 15000 rpm and the oil layer was removed. The remaining pellet was washed with petroleum ether, n-hexane and ethanol in order to remove excess oil. After washing the microspheres were collected, pooled and stored in a refrigerator.

3 Characterization of microspheres

3.1 Particle size and morphological features

The average diameter of hepatitis B vaccine loaded and unloaded microspheres were carried out by optical microscopy in which stage micrometer was employed. A minute quantity of microspheres was spread on the glass slide and the
size of 100 microspheres was determined in each batch. The average mean particle size was measured after performing the experiment in triplicate. In the evaluation, 3rd division of stage micrometer coincided with 2nd division of eyepiece micrometer and the 11th division of stage micrometer coincided with 8th division of eyepiece micrometer. One small division of stage micrometer is equivalent to 10 microns.

Therefore, magnification value \[ = \frac{11 - 3}{8 - 2} \times 10 \]

So one division of eyepiece micrometer \( = 13.33 \) microns

This method was repeated for each and every time for determination of particle size. The surface morphology of microspheres was investigated using JEOL JSM 5300, Japan Scanning Electron Microscope (SEM). Samples for SEM were mounted on to metal stubs and coated with gold palladium alloy to a thickness of 200 – 300 \( \AA \). The release of antigen from microparticle was greatly influenced by microparticle size. Therefore, it is necessary to control the size of the microspheres. The size of microspheres can be affected by polymer concentration, temperature and stirring process. However, the size of the microspheres was reduced by ultra sonification process. The ultra sonification was done for one minute in the earlier stage of microencapsulation process in order to reduce the particle size.

3.2 Determination of Vaccine entrapment

There are three different methods employed to determine the entrapment efficiency.

3.2.1 Extraction method

The amount of vaccine was measured by placing 50 mg of microspheres in 1.5 ml of dichloromethane and extracting the vaccine 3 times with 1.5 ml of phosphate
buffer saline containing tween 20 with the aid of cyclomixer and centrifuge. The total protein content of extraction solution was determined using Lowry's method and compared with standard curve of data obtained by assaying known concentration of vaccine. The encapsulation efficiency was calculated from the percentage of encapsulated vaccine with respect to the total amount of vaccine employed for preparation of microspheres.

3.2.2 Filtration method

This method involves the dissolution of microspheres in an organic solvent in which the hepatitis B vaccine is not soluble, followed by filtration and recovery of entrapped antigen in a filter. 50 mg of microspheres were dissolved in 1 ml of ethyl acetate with the aid of cyclomixer. Then the solution was filtered using 0.20 μm membrane filter, which was previously incubated with bovine serum albumin for 24 hours. The filtrate was washed with phosphate buffer saline tween (PBST) and incubated with buffer under magnetic stirring at 37°C for 1 hour. Therefore, the hepatitis B was eluted in the organic solvent. The antigenically active HBsAg was determined by enzyme immunoassay using Immunlite 2000 automated analyzer as described in section IV and total proteins was determined by Lowry's method. In both the methods the values were compared with standard curve of data obtained by assaying known concentration of vaccines.

3.2.3 Digestion method

This method involves alkaline hydrolysis of microspheres and determination of hepatitis B vaccine recovered. 50 mg of vaccine encapsulated microspheres were mixed with 10 ml of 5 % (w / v) SDS in 0.1 m sodium hydroxide solution with the aid of cyclomixer. The solution was kept in an ultrasonicator for about 1 minute. After
centrifugation, the antigenically active protein and total protein was determined as previously described.

3.2.4 Centrifugation method

The magnitude of loading of hepatitis B vaccine in microspheres was performed by mixing 50 mg of vaccine encapsulated microspheres with phosphate buffer saline (pH 7.3) under shaking at room temperature and kept for 3 hours. The suspension was centrifuged at 3000 rpm for 30 minutes to remove free unloaded vaccine. The loading degree was determined by quantifying the non bound hepatitis B vaccine in the supernatant with the Lowry's protein assay method. The loading capacity and efficacy was determined by

\[
\text{Loading Capacity} = \frac{\text{Total amount of hepatitis B vaccine} - \text{free hepatitis B vaccine}}{\text{Weight of microspheres}}
\]

\[
\text{Loading Efficacy} = \frac{\text{Total amount of hepatitis B vaccine} - \text{free hepatitis B vaccine}}{\text{Total amount of hepatitis B vaccine}}
\]

3.3 Compatibility studies using Infra Red spectrum

The interaction between vaccine and polymer was established by FTIR spectroscopy. Perkins Elmer spectrum one FTIR instrument was used whose resolution was 1.0 cm\(^{-1}\). Conventional KBR pellet method was used. In this experiment 50 mg of sample and 150 mg of potassium bromide (KBR) was taken in a mortar and triturated. The triturated sample was taken in to a pellet maker and was compressed at 10 Kg / cm\(^2\) using hydraulic press. The pellet was kept on to a sample holder and scanned from 4000 cm\(^{-1}\) to 450 cm\(^{-1}\). The FTIR spectrum of hepatitis B vaccine was performed by Neat method. In this method the vaccine sample was spread on the surface of a sodium chloride salt plate and was covered with another
similar plate. The sample taken was adjusted by rotating and pressing the plate together to squeeze out the excess material. Then the sample was scanned from 4000 cm\(^{-1}\) to 450 cm\(^{-1}\).

3.4 Water uptake of microspheres

100 mg of microspheres were suspended in 20 ml of PBS (pH 7.4) containing 0.05 \% (w/v) of tween 80 at room temperature for 24 hours to measure water uptake. Microspheres were filtered, collected, weighed immediately \((W_1)\) and dried. After drying the microspheres were weighed \((W_2)\). Water uptake of microspheres was calculated by applying the following method

\[
\text{Water up take (\%)} = \left( \frac{W_1 - W_2}{W_2} \right) \times 100
\]

3.5 Stability studies

There are two different methods employed to determine the stability of microspheres. They are

1. The stability of the formulated hepatitis B vaccine encapsulated microspheres and unloaded microspheres were determined over a period of 8 weeks. Both the vaccine loaded and unloaded microspheres were kept at 4\(^{0}\) C, room temperature and at 50\(^{0}\) C. The samples were taken and size of the microspheres were analysed at predetermined time interval 0, 1\(^{st}\), 2\(^{nd}\), 3\(^{rd}\), 4\(^{th}\), 5\(^{th}\), 6\(^{th}\), 7\(^{th}\) and 8\(^{th}\) week.

2. All the vaccine loaded microspheres were divided into three sets; they were stored at 4\(^{0}\) C, room temperature and 50\(^{0}\) C. After 15, 30 and 60 days, the vaccine content of the microspheres was determined using Immunlite 2000 automated analyzer as described in section IV.
4. *In vitro* release study

100 mg of hepatitis B vaccine encapsulated microspheres were suspended in 50 ml of phosphate buffer saline of pH 7.4 containing 0.2 % (w/v) tween 80 and 0.1 (w/v) of sodium azide in a 100 ml conical flask and incubated at 37°C by keeping the flask in a shaker cum incubator. The shaker was adjusted to 80 horizontal strokes per minute. At pre determined time intervals 0, 7th, 14th, 21st, 28th, 35th, 42nd, 49th, 56th, 63rd, 77th, 89th, 91st, 98th, and 105th day, 2 ml of vaccine releasing solution was removed and fresh medium was replaced. The samples were centrifuged at 4000 X g for 15 minutes and the supernatant solution was analysed by enzyme immunoassay using Immulite 2000 HBsAg assay kit (Diagnostica products corporation, CA).

4.1 Detection of HBsAg by Immulite 2000 automated analyser

Immulite 2000 automated analyser is a solid phase two step chemiluminescent enzyme immuno assay. It is a bead pack coated with antibody (anti HBs) directed against the HBsAg. The samples and protein based buffer introduced in reaction tube and incubated for approximately 30 minutes at 37°C. During this time the HBsAg in the sample binds to the anti HBs coated bead. Unbound serum is then removed by centrifugal wash. An alkaline phosphate - labeled anti HBs is introduced and the reaction tube is incubated for another 30 minutes cycle. The unbound enzyme conjugate was removed by centrifugal wash. Substrate was then added and the reaction was incubated for a further 5 minutes. The chemiluminescent substrate, a phosphate ester of adamantyl dioxetane, undergoes hydrolysis in the presence of alkaline phosphatase to yield an unstable intermediate. The continuous production of intermediate results in the sustained emission of light. The photon output of the unbound complex was measured by the luminometor and related to the presence of
HBsAg. The quantitative of HBsAg in the samples was calculated by comparing the standard curve.

5 Immunogenecity studies

The immunogenecity studies were carried out by antibody induction method. 78 healthy Wistar rats (150 - 250 gms) and divided into 13 groups. Each group containing 6 Wistar rats and housed in a separate microlon boxes (Torsons products, Kolkata) for the purpose of acclimatisation. The size of the animal box is 45 x 27 x 15 cm. They had free access to water and standard pellet diet. A constant day and night cycle was maintained.

5.1 Preparation of microparticulate injection

The microsphere samples were weighed diluted in sterile water, filtered (0.2 µm filter), vortexed and left to stand at room temperature for approximately one hour. Prior to immunization, particle suspensions were again vortexed and then aspirated using 21 gauge needles.

5.2 Dosage and immunization protocol

Vaccine dose (per inoculum) - 0.5 ml containing 1/10th of SHD (2 mcg)
Route of inoculation - intra muscular
Primary immunization - on the “0 day”
Booster dose (wherever applicable) - 4 weeks after primary immunization
Bleeding time - 45th, 90th, and 120th day

The groups were formed as follows

Group 1 - Immunized with a single dose of hepatitis B vaccine encapsulated PLGA microspheres
**Group 2** - Immunized with hepatitis B vaccine encapsulated PLGA microspheres as primary dose and booster dose.

**Group 3** - Immunized with hepatitis B vaccine encapsulated PLGA microspheres as primary dose and conventional alum adsorbed hepatitis B vaccine as booster dose.

**Group 4** - Immunized with a single dose of hepatitis B vaccine encapsulated PLA microspheres.

**Group 5** - Immunized with hepatitis B vaccine encapsulated PLA microspheres as primary dose and booster dose.

**Group 6** - Immunized with hepatitis B vaccine encapsulated PLA microspheres as primary dose and conventional alum adsorbed hepatitis B vaccine as booster dose.

**Group 7** - Immunized with a single dose of hepatitis B vaccine encapsulated Chitosan microspheres.

**Group 8** - Immunized with hepatitis B vaccine encapsulated Chitosan microspheres as primary dose and booster dose.

**Group 9** - Immunized with hepatitis B vaccine encapsulated Chitosan microspheres as primary dose and conventional alum adsorbed hepatitis B vaccine as booster dose.

**Group 10** - Immunized with a single dose of alum adsorbed hepatitis B vaccine.

**Group 11** - Immunized with alum adsorbed hepatitis B vaccine as primary dose and booster dose.

**Group 12** - Control animals (without vaccines).

**Group 13** - Control samples with dummy PLGA microspheres.
Group 14 - Control samples with dummy PLA microspheres
Group 15 - Control samples with dummy Chitosan microspheres

5.3 Collection of blood

Bleeding of immunized Wistar rats was done by retro orbital plexus at different time intervals by using a capillary tube. The blood samples were collected in a sterile glass tubes and kept in a slanting position so as to allow the serum to ooze out from the clotted blood. Finally the serum was separated by centrifugation process and stored at -20°C.

5.4 Evaluation of immune response

A study on antibody mediated immune response and cell mediated immune response explain the active participation of immune system against vaccines.

5.4.1 Quantitative measurement of antibodies to hepatitis B surface antigen (anti HBsAg) using chemiluminescent enzyme immunoassay

Immulite 2000 automated analyzer (Diagnostic products corporation, CA), is a two cycle, solid phase, chemiluminescent enzyme immuno assay for the detection of antibodies to hepatitis B surface antigen (anti HBs). The assay utilizes a polystyrene bead coated with surface antigen. The samples and protein based buffer was introduced in to the reaction tube containing HBsAg coated bead and incubated for 37°C. During this time the anti HBs in the sample binds to the HBsAg coated bead. The unbound serum was removed by centrifugal wash. Then an alkaline phosphatase-labeled HBsAg was added in to reaction tube for 30 minute cycle. Unbound enzyme was removed by centrifugal wash, chemiluminescent substrate was added and the reaction tube was incubated with agitation for an additional 5 minutes. Results are reported quantitatively.
5.4.2 Quantitative measurement of serum IgA, IgG and IgM

The Orion Diagnostica Turbox Ig G, Ig A and Ig M assay kits were used. It is a liquid phase immunoprecipitation assay with nephelometric end point detection. Antiserum to Ig G, Ig A and Ig M was diluted in buffer individually (annexure I) and added to aliquot rat's serum. The light scattering caused by antigen – antibody complex was measured after incubation. The resulting light scattering is directly proportional to the respective immunoglobulin concentration in the sample. Separate sample blank was prepared for each sample as well as a calibrated blank was prepared in duplicate for the calibrator. Both the sample and calibrators were taken in a separate cuvette and mixed well by gentle shaking and kept at room temperature to stand for 30 ± 5 minutes. The calibrator and samples were measured separately.

5.4.3 Quantitative measurement of serum IgE using Immulite 2000

Immunlite 2000 IgE analyzer is an automated third generation quantitative chemiluminescent enzyme immunoassay. The assay utilizes streptavidin coated bead, biotinylated and samples were incubated for 30 minutes. After a spin wash, an alkaline phosphatase – labeled monoclonal antibody specific for rat’s IgE was introduced and incubated for another 30 minutes. The bead was then washed again and enzyme label was measured with chemiluminescent substrate phosphoesters of adamantyl dioxetane. Results are reported quantitatively.

6. Data analysis

Differences in particle size and immunoglobulin titre values were analysed by statistical comparisons using one way ANOVA. The level of significance was taken as p< 0.05. Dunnet multiple comparison test was performed for immunoglobulin titre to compare test with control.