CHAPTER 6
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As preformulation tool for estimation and compatibility studies, with excipients, modified Rocket electrophoresis method was developed, optimized and validated for accuracy, precision and repeatability. Linear relationship in range of 2.5 to 30Lf/ml between concentration of standard TT and height of precipitin rocket in gel was obtained which was used for quantitative determination of TT as well as for compatibility assessment in preformulation studies for preparing microparticle system for TT. Compatibility of various formulation additives with toxoids was tested and additives found to be compatible were selected for the preparation of microparticles.

Analytical methods like DC protein Assay for estimation of Protein Content, Flocculation test for Lf Determination of toxoids, HPLC and SDS-PAGE for Molecular weight determination and Isoelectric point focusing for pI of toxoids were used for their characterization.

Quantitative estimation of TT and DT was done using HPLC and ELISA method. Chitosan (CS) was used as a polymer for the preparation of microparticles and was characterized using FITR. Viscosity of 1% solution was determined using Brookefield Rheometer and found to be 132 cps. pH of 2mg/ml solution of CS was 4.6. Sodium tripolyphosphate (STPP) was used as crosslinking agent for CS. pH of 1mg/ml solution of STPP was found to be 9.7.

Lf was found to be 3840Lf/ml for TT and 4500 Lf/ml for DT using flocculation test. Molecular weight of TT and DT using HPLC and SDS-Page was found to be 160-170 kDa for TT and 60-65 kDa for DT. Retention time was found to be 7.24 min for TT and 8.12 min for DT, when analyzed using HPLC.

Quantitative estimation of TT and DT, using HPLC was performed in the range of 6.5 Lf to 100 Lf and using ELISA in the range of 0.001207 to 0.0386 Lf/ml for TT and 0.005 to 0.156 Lf/ml for DT

The pI was calculated by comparing the Rf of the sample band with the Rf of pI markers using Isoelectric Focusing technique and found to be in between 4.8-5.0 for TT and 4.65–4.8 for DT.

Optimization of a amount of STPP required to crosslink CS was done by measuring change in %T with addition of STPP in CS solution. Optimum ratio of CS : STPP was found to be 1.16: 1. Change in pH was also monitored. Fourier Transform Infra Red Spectroscopy of CS and CS-STPP complex was performed for the confirmation of the complexation of CS using STPP.

CS, microparticles were prepared by Coacervation-Phase Separation method induced by ionic interactions in aqueous system. TT loaded microparticles were prepared using optimized ratio of CS and STPP. Various experimental conditions were optimized to achieve maximum TT loading and desirable product characteristics, like Viscosity of the reaction medium, Volume of water, pH of reaction medium, Rate of STPP addition. Maximum %EE was found in water which was having least viscosity among the solvent compositions studied. %EE was found maximum in 60ml volume of reaction medium having initial pH of 4.5 and STPP addition of rate of 1ml per 5min. Similar conditions were used for DT loading in chitosan microparticles.

After optimizing the formulation variables, maximum possible entrapment of TT and DT in the CS microparticles was studied by loading the vaccines up to 10,000Lf. A very high (97-99%) entrapment was found for all toxoid loadings up to 10,000Lf/batch for both, TT and DT. Unentrapped TT and DT were estimated using ELISA and also confirmed using HPLC. However, batch containing 1500Lf was taken for final studies in both cases as these batches showed least aggregation and 99.52 and 98.25% entrapment for TT & DT, respectively.

As the desirable particle size for the Peyer's patch targeting is 10 µm, batches were processed for particle size control using Emulsiflex C-5 and CAT-560 Silverson type homogenizer, either alone or in combination. Particle size of the batch containing 1500 Lf TT (Batch 27TT) was having Dmean of 15.154 µm and for batch containing 1500Lf DT (Batch 28DT) was having Dmean of 14.623 µm. Individual discrete particles were clearly visible in the E-SEM of the optimized batches. TEM showed discrete, non aggregated, solid and spherical shaped particles measuring less than 1µm.

The effect of high shearing rate as well as pressure of compressed air which may affect the conformations of TT and DT was studied using SDS-PAGE and HPLC. The results of SDS-PAGE and HPLC of processed TT and DT, showed no changes as compared to control.

Release study of the optimized batches was performed in 0.1N HCl and PBS (pH 7.4). There was only 2.4% & 2.1% release of the TT and DT respectively from loaded chitosan microparticles in 0.1N HCl. This amount can be attributed to the surface bound toxoids. Study in PBS (pH 7.4) showed no release of TT and DT up to 2 hours, which is desirable as it will ensure that the toxoids will be released only after the uptake of the microparticles by the PPs and M-cells of the intestine.

Bioadhesion testing of the final batches Batch 27 TT and 28DT was performed by measuring force required to break the adhesive bond between membrane and the formulation digitally. The bioadhesion force was higher for 27TT (22.65 dynes) and 28DT (18.967 dynes) compared to 26 Plain CS (13.167 dynes) values.

Rheological Studies of the final suspension product studied using Brookefield Rheometer, showed desirable thixotropic as well as pseudoplastic characteristics, which is desirable for the stability of suspension.

The sedimentation profiles of plain and loaded chitosan microparticles were studied. The particles showed a very slow sedimentation profile over 8 days. There was no difference in sedimentation profile of plain and loaded chitosan microparticles after 8 days.

Stability of the Batch 27TT and 28 DT was studied at 4^0 and 25^0C for three months and examined for leaching of the toxoid. There was very negligible leaching of TT and DT in both conditions, indicating a good shelf life even at RT.

Optimized batches, Batch 27TT and Batch 28 DT, were used for in vivo studies, which were carried out in BALB/c mice by administering formulation through oral route. Systemic immune response was determined by measuring the IgG level in serum and Local immune response was determined by measuring IgA level in Intestinal Lavage, Fecal Matter extracts and Intestinal Washings by ELISA method.

Dose response study was carried out to know the optimum dose required to produce IgG level similar to that of IgG levels produce by the i.m. administration of 10Lf TT and 25 Lf DT. CS-TT 40Lf and CS-DT 40 Lf were found to give nearly same serum IgG levels as that of corresponding i.m. vaccine dosing.

In another experiment, Serum IgG levels were determined on 14^{th}, 22^{nd} and 29^{th} day and compared with IgG levels obtained after administration of TT or DT in PBS. IgG levels

were significantly higher of CS-TT and CS-DT as compared to TT and DT in PBS, respectively.

Local IgA levels in Intestinal Lavage and fecal matter extract was determined on 4th, 8th, 14th and 22nd day and on 22th day in Intestinal washings. IgA levels produced after administration of batch 27TT and 28DT were significantly different when compared statistically using Student's t-test with TT-PBS and DT-PBS, respectively.

Conclusions:

The developed Rocket Electrophoresis method was quite effective and useful technique during preformulation studies. RE could be easily standardized for the required antigen concentration by changing antitoxin concentration. Results of RE are less prone to operator's bias and can be documented by taking photograph and/or scanned by densitometer. It was concluded that the developed Rocket electrophoresis technique is a sensitive, reproducible and economical method, which can be used for the qualitative and quantitative estimation of TT. The method is also a good indicator of antigenicity of TT and can accurately predict compatibility with excipients, making it a very useful tool in preformulation studies.

Flocculation test was found to be useful for the estimation of TT and DT, when the concentration of toxoids is high. The observed values of Limes flocculation units of toxoids were same as that of the labeled claims, indicating accuracy of the method.

Values of molecular weight obtained by both methods, SDS-PAGE and HPLC, were almost similar, indicating that both methods complement each other. However, Molecular weights of TT and DT were slightly higher as compared to the reported molecular weight of the toxins, which are 150 kDa for tetanus toxin and 58kDa for Diphtheria toxin. This shift may be due to toxoidation using formaldehyde, which leads to crosslinking between molecules of toxin as well as crosslinking with proteins in the culture medium.

The pI value was found to be nearly same when estimated using IEF, for both the toxoids, indicating that the reaction medium pH requirements will be same for both the systems.

The chitosan microparticles were prepared by Coacervation-Phase separation induced by ionic interaction with STPP in a completely aqueous system which does not involve

use of organic solvents. This will ensure the stability of the antigens and will also be cost effective, environment friendly procedure. The crosslinking reaction between CS and STPP was monitored by change in %T and pH measurement. The reduction in %T with increasing concentration of STPP was attributed to the crosslinking of the protonated amino groups of chitosan by phosphate ions of STPP. The pH of the system was below the isoelectric point of chitosan (pH 6.5) indicating that the decrease in %T was only due to ionic interaction of the protonated amino groups of chitosan and phosphate groups of the STPP and not due to the coacervation-phase separation reaction. The FTIR studies indicated that the ionic interactions between phosphate groups of STPP and amino groups of CS were responsible for formation of complex between STPP and CS.

Modified ELISA method which was used for the estimation of TT from supernatant for the %Entrapment Efficiency (%EE) study, was reproducible, sensitive and accurate.

In maximum loading capacity study, the high entrapment was probably due to very strong ionic interaction between the TT/ DT and CS at acidic pH. Capable of attracting several cationic species, the STPP may have functioned as a crosslinker that can bind the positively charged CS-TT/CS-DT intermediate into an electrostatically stabilized CS-STPP-TT or CS-STPP-DT ionic complex. Moreover, this had not changed the antigenicity of the TT or DT as we could detect TT and DT using ELISA. This proves the very excellent protein loading capacity of chitosan microparticles, which can be advantageous in administering large doses of toxoids / vaccines in a small volume like in case of polio drops.

Both ELISA and HPLC methods were found to complement each other when used for the unentrapped TT and DT estimation and in determining maximum loading capacity of CS microparticles.

The final optimized batch had particle size from 4 μm to 25 μm with mean particle size of 15 μm. It was assumed that there will be immediate absorption of the small particles (10-40%) and remaining particles will remain lodged in PPs wherein it will release the antigen slowly, giving sustained effect. It was also expected that chitosan, which is having very strong mucoadhesive properties will enhance the residence time of the microparticles in the intestine leading to maximum contact time for the delivery system.

E-SEM and TEM study confirmed the role of SCMC and particle size reduction treatment, as the individual particles were clearly visible in the E-SEM and TEM.

The results of both SDS-PAGE and HPLC studies shows that the high pressure homogenization procedures adopted for particle size reduction had not affected the conformation of the toxoids, indicating that the processing has not adversely affected the antigenicity of the vaccines.

Release study in 0.1 N HCl showed negligible amount of TT and DT release indicating the stability of chitosan microparticles in gastric pH (0.1N HCl) which shows that entrapment afforded protection to the toxoids in acidic environment. Study in intestinal medium pH (PBS 7.4) showed no release of TT and DT upto 2 hours which is also important from the delivery point of view of entrapped TT to PPs, as the soluble TT and DT are incapable of formation of memory cells indicating that entrapment of TT and DT in microparticles will abrogate tolerance to soluble TT and DT.

The sedimentation and redispersion studies showed that developed product had slow sedimentation rate and it was easily redispersed indicating that the cake formed is not hard. This is an important feature of the chitosan microparticles formulation which is in the form of suspension. The suspension showed thixotropic as well as pseudoplastic properties. The chitosan TT and DT microparticle suspension, were found to be stable at 4^0 and 25^0C for three months. This is of great significance from the transportation and administration point of view. As it is a oral delivery system, it can be self administered by the patients thus eliminating the need of medical supervision and storage in refrigerator conditions is also not required as it is stable at RT. This will be of great help in increasing immunization coverage and avoiding the lapses in booster dose administration.

The results of the oral vaccination studies in mice showed that the systemic and local mucosal immune response was strongly enhanced after incorporating the vaccine into chitosan microparticles. Systemic immune response was dose dependent. Induction of immune response showed that the antigenicity of the TT and DT was maintained during the microencapsulation and size reduction process.

In addition, significantly higher levels of specific anti-TT and anti-DT IgA in the gastrointestinal tract were detected. The IgA levels were not significantly different on 4^{th} day but the difference was significantly different in subsequent sampling days indicating the ability of chitosan microparticles to induce formation of IgA and memory cells. Besides increased patient compliance and reduction of costs, the induction of local

memory cells is an important feature of chitosan microparticles as oral vaccine carriers. Because of the production of IgA at mucosal sites, pathogens can be neutralized during the invasion through the mucosa. This will be a great advantage over parenteral formulations which provide only systemic immunity which is ineffective in preventing pathogens entering the body through peripheral mucosal route or sites and hence may not be able to prevent infection.

Toxoid loaded chitosan microparticles would be probably taken up by the M-cells of the PPs as nearly 40-50% particles are below 10μm which is required for uptake by PPs. TEM study also showed presence of large number of nanoparticles which must have played important role in induction of systemic as well as local immune response. Also, the fact that systemic and mucosal immunity was developed upon oral administration of the CS-TT and CS-DT microparticles, indicates that they must have been taken up by the M-Cells of the PPs.

As TT or DT was not found to be released in PBS during in-vitro studies, it is most likely that TT or DT is released only after biodegradation of the particles in vivo probably by lysozymes and chitinases, which are present in M cells of the PPs.

Thus, these oral immunization studies clearly demonstrate that the prepared chitosan microparticles were able to enhance the local immune response as well the systemic immune response which was comparable with the parenterally delivered vaccine. Indicating that oral administration of entrapped TT and DT is viable alternative to the parenteral administration. As it is a oral delivery system it can be self administered by the patients thus eliminating the need of medical supervision and storage in refrigerator conditions is also not required as it is stable at RT. This will be of great help in increasing immunization coverage and avoiding the lapses in booster dose administration

Thus, the outcomes of the entire research project prove that the aims, objectives and hypothesis of conducting the research work were fulfilled to a great extent. The work could not only achieve nearly 99% vaccine loading but also ensure retention of conformation and antigenicity of the vaccines. Oral administration of the vaccine loaded microparticles could induce both systemic as well as local immunity which proves that they were bale to transport the vaccines across the gastrointestinal epithelium (mucosa).

The results conclusively prove that the developed chitosan based oral vaccine delivery systems could be effectively used as a more convenient, cost effective and patient friendly alternative to the conventional parenteral formulations.

However, results obtained in this study needs to further substantiated by carrying out following studies: Oral immunization using the chitosan entrapped toxoids in a dosage so as to obtain equivalent IgG titres as that of the parenteral immunization, Quantification of IgG and IgA levels using monoclonal antibodies against Tetanus or Diphtheria Toxoids as primary antibodies in ELISA, Quantification of IgA levels in the IU/ml, Estimation of Toxin neutralizing antibodies using Toxin challenge method in animals, Scale up to Pilot and Production level batches, Long term stability studies for predicting shelf life, Combination vaccine of the Tetanus and Diphtheria toxoids and clinical studies in Human volunteers.