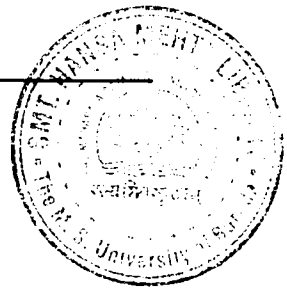
CHAPTER 4
PREFORMULATION



Chapter 4

Preformulation

4.1. Introduction:

The trend towards the use of peptides and subunit proteins in modern vaccine design has necessitated the use of immunological adjuvants to achieve effective immunity (***Dunn, M.J., 1990***). In vivo and in vitro assays are used to determine the antigenic potency of adjuvanted vaccines (***Katz, J.B., 1989***) such as vaccines adsorbed on aluminium hydroxide or phosphate, developmental vesicular adjuvants such as liposomes (***Weiner, A.L., 1990***), niosomes (***Brewer, J.M., 1992***), particulate adjuvants such PLGA microparticles with stabilizers (***Gupta, R.K., 1996***) and chitosan microparticles (***van der Lubben, I.M., 2003***).

TT and DT are large proteins with a molecular weight of 150,000 and 58,000 daltons, respectively, prepared by detoxification of tetanus toxin and diphtheria toxin, respectively, using formaldehyde. TT and DT are prone to denaturation by various conditions such as microencapsulation (interaction with polymer and solvents), storage, and rehydration of microspheres (***Gupta, R.K., 1996***). To understand the effect of additives on the stability and antigenicity of the TT and DT during preparation of microparticles compatibility study should be carried out as a part of preformulation study.

TT and DT can be quantified by electrophoresing them into antibody containing gel in the technique termed Rocket Electrophoresis (RE). This technique relies on the antigen and antibodies having different charges at the selected pH. The pH of the gel is chosen so that antibodies are immobile and the antigen is negatively charged. Precipitin rockets form at the equivalence point of antigen and antibody concentration and the height of the rocket being proportional to antigen concentration (***Roitt, I., 1993***).

4.2. Materials and Reagents:

4.2.1. Materials:

Purified Tetanus toxoid (Batch I 3000 and Batch II 3840 Lf/ml), and Equine tetanus antitoxin (500 IU/ml) was obtained from Serum Institute of India, Pune, India as a gift sample. Chitosan was obtained from Central Institute of Fisheries Technology, Cochin and used as it is without purification and modification. Agarose, Tris, Coomasie Brilliant

Blue R-250, Ascorbyl Palmitate were purchased from HiMedia Lab, Mumbai. Sodium tripolyphosphate was purchased from National Chemicals, Baroda. Calcium Chloride, Glycerin, Propylene Glycol, Acetic acid, Methanol, Glycine, Sodium Alginate, Isopropyl Alcohol, Diethyl Ether were purchased from S.D. Fine chemicals, Mumbai. Glutaraldehyde was purchased from E. Merck India Ltd, Mumbai. Submarine gel electrophoresis apparatus with Constant supply Power unit, Bangalore Genei, Bangalore was used. All the Gels were photographed using Bio-Rad Gel Documentation system, Bio-Rad, USA and analyzed using Quantity One™ software. All the chemicals were of Analytical Reagent grade unless otherwise specified. MilliQ water was used wherever required.

4.2.2 Reagents:

A. Tris-Glycine Gel and Tank Buffer:-

Tris-Glycine Gel and Tank Buffer (pH 8.6) were prepared by dissolving Tris (3gm) and Glycine (14.2 gm) in MilliQ water and volume was made upto 1000ml after adjusting pH to 8.6 with 0.1N NaOH.

B. Staining Solution:-

Staining Solution was prepared by dissolving Coomasie Brilliant Blue R-250 (300 mg) in Methanol (45ml), and adding Acetic acid (10ml) and MilliQ water (45ml).

C. Destaining solution:-

Destaining solution was prepared by mixing Methanol (45ml) with Acetic acid (10ml) and MilliQ water (45ml).

D. Chitosan Solution:-

Chitosan Solution was prepared by adding Chitosan (2 gm) to 100ml MilliQ water containing 1ml Glacial acetic acid under continuous stirring. Chitosan solution obtained was filtered through sterile Dacron cloth.

4.3. Experimental:

In evaluating the results of rocket electrophoresis, plain, highly purified and concentrated TT batches were used. In order to estimate the contents of toxoid in Lf units, the TT batches were first assayed using flocculation test. Batch I was found to have 3000 Lf/ml and used for the optimization, linearity, validation and compatibility studies.

4.3.1. Optimization of RE run Conditions:

Rocket electrophoresis (RE) run was optimized by studying the effect of variables like agarose gel amount, ATS amount, run environment temperature and run duration on the precipitin rocket height, as shown in **Table *1***.

The final optimized conditions for the RE run were as follows:- Rocket electrophoresis was performed using 1% Agarose gel prepared in Tris-Glycine buffer (pH 8.6). Required amount of agarose was dissolved in 20 ml buffer. At 50^{0}C the required amount of Equine Tetanus Antitoxin (ATS) was added with continuous stirring, to give concentration of 1 IU/ml in agarose gel. The agarose solution containing ATS was poured evenly on 10x7 cm gel casting tray along with sample well preparation comb to give sample well of 10 µl capacity. The gel was allowed to solidify for 30 min at room temperature, after which the sample comb was removed and 8 µl of Tetanus toxoid solution of different concentrations was added into each sample well. Sample wells loaded with samples were kept on the cathode side of the submarine gel electrophoresis apparatus and connected with Tris-Glycine tank buffer using filter paper wicks. The gel was electrophoresed at 4-8^{0}C for 18 hrs across electrical potential difference of 180V, in Tris-Glycine tank buffer (pH 8.6). The precipitin rocket was formed at the equivalence zone of TT and IgG in ATS. Gels were then stained with Coomasie brilliant blue R-250 staining reagent for 30 min and destained with destaining reagent for 4 hours. The heights of the rocket shaped precipitate were measured from the top of the well to the top of the precipitate using digital caliper.

4.3.2. Determination of linearity for TT in RE

Different TT concentrations were loaded into the sample wells and RE was run as per above described optimized procedure. The process was repeated 6 times and was photographed using Gel Doc system, Bio-Rad. The linear relationship between standard TT concentration in Lf/ml and height of precipitin rocket was used for qualitative and quantitative estimation of TT in different samples.

4.3.3. Validation of RE:

4.3.3.1 Accuracy of method:- Solutions of known concentration of TT in MilliQ water were prepared and loaded in sample well as unknown along with standards. RE was run as per the above described optimized procedure. The concentration of TT was calculated

from relationship between height of rocket and concentration of TT and checked against actual value. Student's t-Test was applied to the set of values.

4.3.3.2 Precision of the method:- Precision of the method was determined by carrying out the analysis at different time intervals on successive days. The data obtained was compared using Student's t' test.

4.3.3.3 Repeatability of the method:- Repeatability of the method was studied by using another batch of TT having 3840 Lf/ml concentration which was diluted to get the concentration in the range of 2.5 Lf/ml to 30Lf/ml.

4.3.4. Application of RE in compatibility studies with different additives:

Chitosan, Calcium Chloride, Ascorbyl Plamitate, Glycerine, Sodium Alginate, Isopropyl Alcohol, Diethyl ether, Propylene Glycol, Sodium tripolyphosphate were tested for compatibility with TT. The process details are shown in **Table 14.** The processed sample was loaded into the sample wells along with standards and RE was carried as per optimized conditions. The rocket height was measured and concentration was calculated using the height of standards.

4.3.5. *In -silico* Studies of Interaction

4.3.5.1 *In -silico* Study of Interaction between CS and TT

Formation of any ionic bonds between CS and TT was checked *in-silico* using molecular modeling software. Though CS was found compatible with TT there may be ionic interaction between the $-NH_2$ group of the CS and –COOH group of side chains of the amino acids of TT. The interaction between CS and TT was studied *in-silico* using Hyper Chem™ Version 5.02 Molecular Modeling Software. Amino acid sequence of Tetanus Toxin was used to form α-helix of 20 amino acid ***(http://au.expasy.org/cgi-bin/get-sprot-entry?Q9LA13)***. This fragment of Tetanus toxin was subjected geometric optimization using Steepest Descent method as a force field to obtain geometrically optimized minimum energy structure. Similarly, 20, β (1-4) linked glucosamine units of CS were treated to obtain geometrically optimized minimum energy structure. TT fragment and Chitosan fragment were kept together and subjected to Molecular mechanics optimization using Steepest Descent method to obtain geometrically optimized minimum energy structure. Hydrogen bonds formed between the TT and Chitosan fragment were recomputed.

4.3.5.2 *In –silico* Study of interaction between CS and DT:

Similar study was carried out using α-helix of 20 amino acid of Diphtheria Toxin (*Delange, R.J., 1976*) instead of Tetanus toxin.

4.4 Results and Discussion:

4.4.1. Optimization of run conditions:-

Rocket electrophoresis run conditions were optimized by studying the effect of variables as shown in **Table 9.**

Table 9: Optimization of RE run conditions.

Parameter	Levels	Remarks
Agarose gel amount (ml)	30	Gel thickness was not uniform. Rockets formed were not clear
	20	reproducible and clear rockets were obtained
ATS amount (IgG IU/ml)	1.5	Rocket height was less and was not properly resolved
	1.0	Rocket height in between 2 to 40 mm for standard curve of TT
	0.5	Rockets were not fully developed
Run Environment Temperature (^{0}C)	20-25	Gels were distorted and incompletely developed
	4-8	There was no distortion of the gels and found to give better results.
Run duration (hrs)	6	Rockets were not fully developed
	12	Rockets were not fully developed, difference in rocket height was not proportional to the concentration of the TT
	16	Rockets were not fully developed, rocket height for higher concentration was not proportional to the concentration of the TT
	18	18 hrs was found to give reproducible rocket height which was proportional to TT conc

Agarose gel, 20ml of 1%, was found to give reproducible and clear rockets as compared to 30 ml, probably due to the free movement of TT in the presence of lesser amount of agarose per surface area which may be impeded at a higher quantity and thickness of gel. Run Environment Temperature of 4-8^{0}C was found to give better results whereas the gel was often found to be distorted at 20-25^{0}C, probably due the generated heat during the run. Run duration of 18 hrs at 4-8^{0}C was found to give reproducible rocket height. At

lower run duration, the rocket formation was not complete as there was no proportionate difference in height of rocket formed for different loaded concentrations.

The present RE method optimized uses less amount of agarose, safe materials like Tris-Glycine instead of controlled substances like sodium barbital buffer *(Ljungqvist, L., 1987)*, with sample volume of only 8µl. The minimum detectable concentration which was found to give reproducible rocket was 2.5 Lf which is useful when the available sample volume as well as concentration is less. RE method is easy to perform and the cost of run is less as compared to SDS-PAGE and ELISA. The method also gives information about the antigenicity of the antigen as the formation of rocket entirely depends on the reaction with antibodies present in the gel, which indicate that antigenic epitopes of antigen are preserved, which is not obtained using SDS-PAGE, where antigen is separated based on the molecular weight in reducing conditions. The final results can be documented by taking gel photographs using Gel Doc system making the method beneficial in validation procedures and quality control audits.

4.4.2. Standard curve for TT by RE

The relationship between Concentration of TT in the sample well and height of the precipitin rocket was found to be linear in range of 2.5 Lf/ml to 30Lf/ml. Standard calibration curve prepared **(Table 10)** is represented graphically in **Figure 20.** The regression equation is given as

$$y = 1.3034 x - 0.4246, R^2 = 0.9921 \ldots\ldots\ldots \textbf{Equation 1}$$

Unknown concentration was calculated using regression **Equation 1**

Gel photograph was taken using Bio-Rad Gel Documentation system, USA, **(Figure 21)** which also clearly indicates the sensitivity of the developed method.

Table 10: Standard curve for Tetanus Toxoid by RE

Std concentration of TT (Lf/ml)	Height of Precipitin rocket (mm) Mean ± S.D. (n=6)
02.50	02.82 ± 0.32
05.00	06.23 ± 0.29
10.00	13.81 ± 0.77
15.00	18.58 ± 1.10
20.00	24.49 ± 0..69
25.00	30.72 ± 1.47
30.00	40.50 ± 2.12

Figure 20: Standard Curve for Tetanus Toxoid by RE

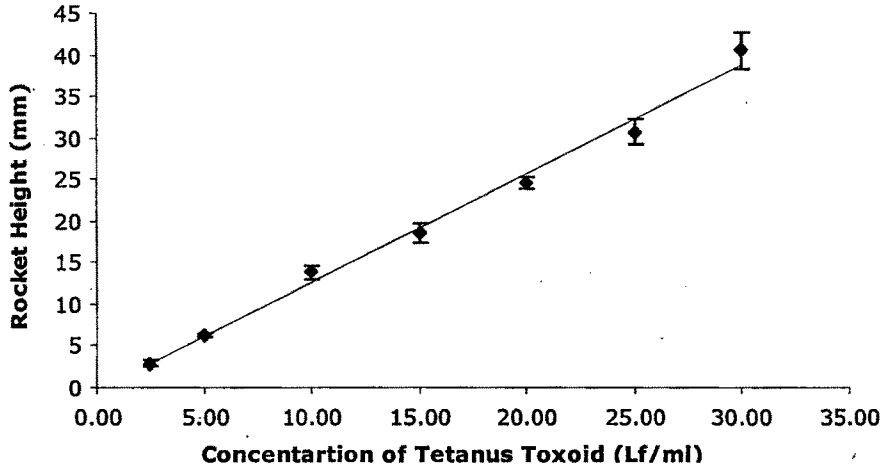
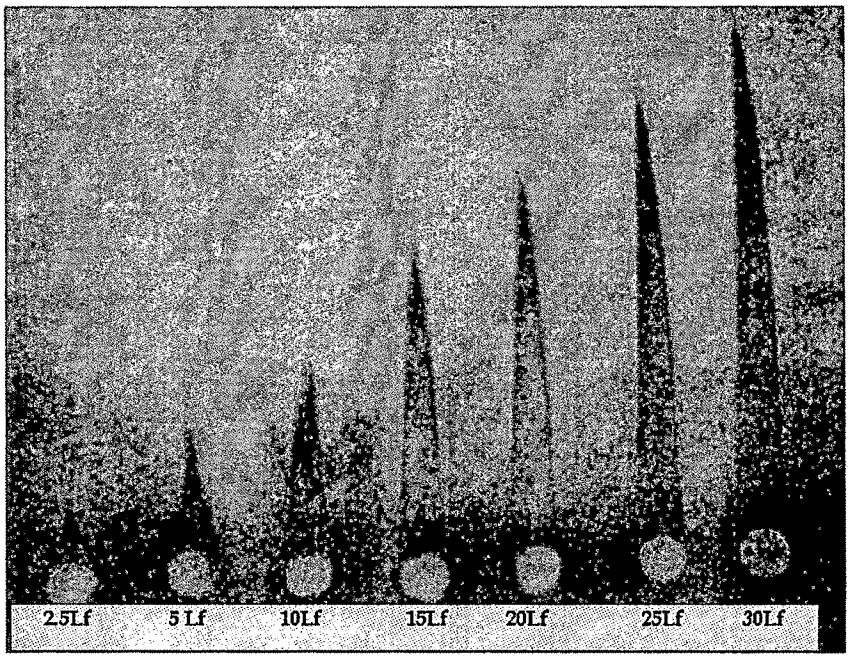


Figure 21: Photograph of Gel showing standard curve for TT



4.4.3. Validation:

4.4.3.1. Accuracy:-

The results of comparison between theoretical and calculated values as shown in **Table 11** indicate that the two values are not significantly different from one another. This establishes the accuracy of the developed RE method.

Table 11: Accuracy of RE for TT

	Theoretical conc. of TT (Lf/ml)	Calculated conc. of TT (Lf/ml)	Student's t-test
1	10	10.16	t = -0.059298
2	15	16.00	p = 0.954648 (Not
3	15	14.04	Significantly Different)
4	25	25.89	

4.4.3.2 Precision:- As the run duration is of 18 hrs, inter-day precision of the method was determined by analyzing the sample of known concentration on consecutive days. The results as shown in **Table 12,** indicate non-significance between theoretical and calculated values, establishing the precision of the mehod.

Table 12: Precision of RE

Day	Theoretical conc. of TT (Lf/ml)	Calculated conc. of TT(Lf/ml)	Student's t-test
1	15	14.25	t = 0.357396
2	15	14.00	p = 0.744459 (Not
3	15	15.04	Significantly Different)
4	15	16.05	

In order to avoid systematic errors, randomization of sample application was also tested. Standard dilutions were loaded randomly, in reverse order and positions of unknown were also changed on the gel. No change in the height of the precipitin rocket was observed, thus eliminating chances of errors due to such occurrences.

4.4.3.3 Repeatability:- The results of repeatability test using different batches of TT **(Table 13)** show that the means were not significantly different (t = -0.017731, p = 0.986145). This established repeatability and reproducibility of the method.

Table 13: Repeatability of RE using Batch I and II of plain TT

Concentration of TT (Lf/ml)	Height of Rocket (mm) (Mean ±S.E.M.; n=4)	
	Batch I	Batch II
2.50	2.45± 0.112	2.83± 0.109
5.00	5.80± 0.115	5.69± 0.271
10.00	12.43± 0.219	11.69± 0.439
15.00	17.08± 0.312	17.43± 0.570
20.00	23.88± 0.271	24.88± 0.214
25.00	29.30± 0.514	30.53± 0.112
30.00	39.92± 0.724	38.69± 0.481

4.4.4. Application of RE in compatibility studies with different additives

RE method was used as an analytical tool in the preformulation studies of the microencapsulation of TT. TT may be prone to denaturation by various conditions during microencapsulation (interaction with polymer and solvents), storage, and rehydration of microspheres. Also, the loss of antigenicity of the antigens is a commonly reported problem especially when they are being formulated into formulations like microspheres, liposomes, nanoparticles etc. This is mainly due to the use of organic solvents, air-liquid interface formed during high-speed homogenization, electrolytes, polymers, cross-linking agents in the preparation of delivery systems and pH conditions. The denaturation of proteins may be due to covalent bond formation, conformation change or other interactions. *(Gupta, RK., 1996; Wilson, K., 2000)*. If any of these changes affect the antigenic epitopes, it will prevent the antigen-antibody reaction and cause loss of antigenicity. As RE also works on the principle of Antigen-Antibody binding, where the immobilized antibody binds with the moving antigen in the applied electrical field, the precipitin complex so formed can be used to quantify the antigen content. This process will not occur if the antigenic epitopes are damaged during manufacturing process or there is an incompatibility with the ingredient in the formulation. In each of such cases either rocket will not be obtained or it will be distorted with lesser height than std. Hence, the developed RE technique can be used as very good and simple tool for verifying the compatibility of various (carrier) polymers, chemicals, reagents, solvents and other formulation excipients proposed to be used in dosage form development of ant antigen. Additives shown in **Table 14** were tested for compatibility with TT.

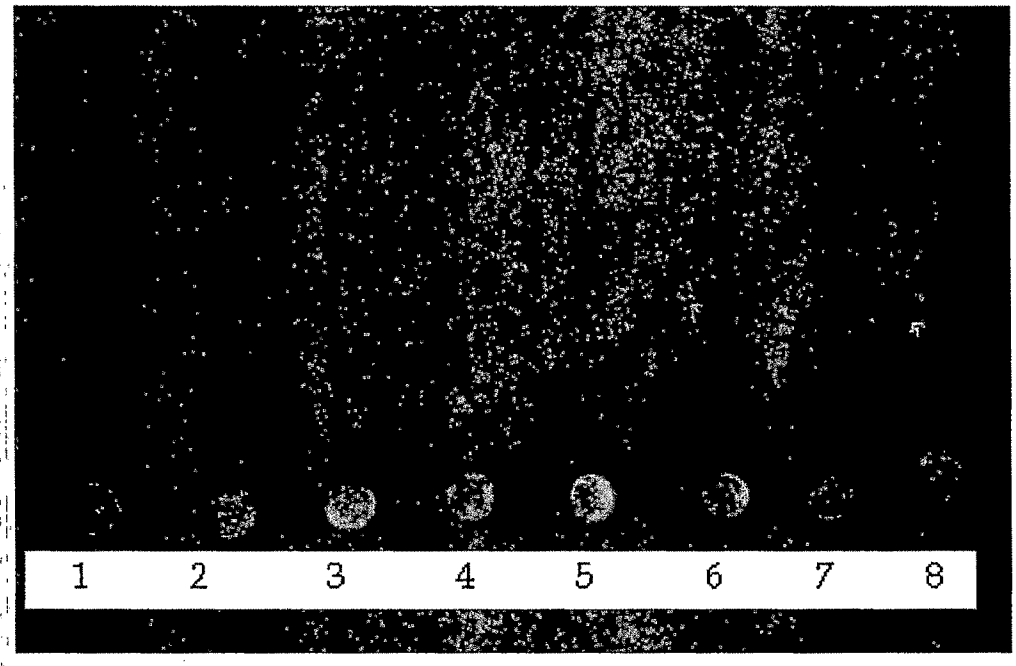
Table 14: Additives tested for compatibility with TT

Additive	Volume	Mixing time	Observation	Conclusion
Chitosan solution, 2% in 1% acetic acid	2ml with 1ml of TT	Vortexed for 5 min	There is no change in height and shape of precipitin rocket	Compatible
Calcium Chloride solution, 2% in MilliQ water *(Vandenberg, G.W., 2001)*	2 ml was added in 1 ml of TT	Stirred for 30min	There is no change in height and shape of precipitin rocket	Compatible
Glycerin	2 ml with 1ml of TT	Stirred for 30min	There is no change in height and shape of precipitin rocket	Compatible
Propylene Glycol	2 ml with 1ml of TT	Stirred for 30min	There is no change in height and shape of precipitin rocket	Compatible
Sodium Tripolyphosphate, 28mg/ml in water *(van der Lubben, I.M., 2003)*	2ml with 1ml of TT	Stirred for 30min	There is no change in height and shape of precipitin rocket	Compatible
Glutaraldehyde, 5% solution *(Zhao, Z., 1996)*	1ml of 5%, added drop-wise in 4ml of TT	Stirred for 30min	There is no formation of precipitin rocket	Incompatible
Isopropyl alcohol	4ml with 1ml of TT	Vortexed for 5 min, centrifuged	The height of the precipitin rocket is reduced as compared to standard	Incompatible
Diethyl Ether	4ml with 1ml of TT	Vortexed for 5 min, centrifuged	The height of the precipitin rocket is reduced and shape is distorted as compared to standard	Incompatible
Sodium Alginate, 2% in MilliQ water *(Vandenberg, G.W., 2001)*	2 ml with 1ml of TT	Vortexed for 5 min	The height of the precipitin rocket is reduced and shape is distorted as compared to standard	Incompatible

In case of Chitosan, Calcium Chloride, Glycerin, Propylene glycol, Ascorbyl Palmitate, and Sodium Tripolyphosphate the shape and height of precipitin rocket was maintained indicating no interaction occurred. **(Table 15, Figure 22).** Student's t-test showed no

significant difference between the standard TT concentration and the TT concentration after exposure to additives (t = -2.137187; p = 0.085622). In case of glutaraldehyde no rocket was observed **(Table 8, Figure 22)**

Figure 22: Gel photographs of RE for additive compatibility study



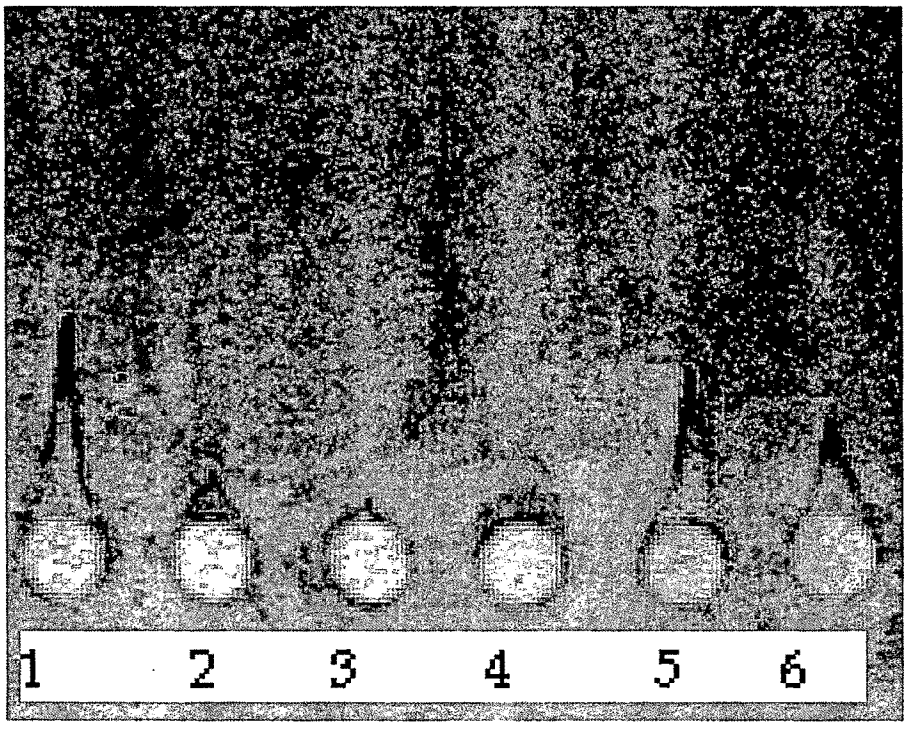
1: Standard TT 15 lf/ml
2: Chitosan
3: Calcium Chloride
4: Glycerin
5: Propylene Glycol
6: Ascorbyl Palmitate
7: Sodium Tripolyphosphate
8: Glutaraldehyde

Table 15: Additive compatibility study: Concentration of TT after exposure to additives

	Additive	Height of Rocket (mm)	Calculated Concentration of TT (Lf/ml)
1	**Std TT 15 Lf/ml**	17.4	15.000
2	**Chitosan**	17.6	15.172
3	**Calcium Chloride**	18.1	15.603
4	**Glycerin**	17.6	15.172
5	**Propylene Glycol**	17.4	15.000
6	**Ascorbyl Palmitate**	17.5	15.086
7	**Sodium Tripolyphosphate**	17.5	15.086
8	**Glutaraldehyde**	nil	0.000

In case of Sodium Alginate, Isopropyl Alcohol, Diethyl ether **(Table 16, Figure 23)** the rocket height was less and shape distorted as compared to standard indicating in compatibility. The difference in height was found to be significant ($p<0.05$).

Figure 23: Gel photographs of RE for additive compatibility study



1: Standard 10Lf

2: Isopropyl Alcohol

3: Diethyl Ether

4: Sodium Alginate

5: Chitosan

6: Standard 5 Lf

Table 16: Additive compatibility study: Concentration of TT after exposure to additives

	Additive	Height of Rocket (mm)	Calculated Concentration of TT (Lf/ml)
1	**Std TT 10Lf/ml**	13.2	10.00
2	**Isopropyl Alcohol**	3.0	2.272
3	**Diethyl Ether**	1.0	0.757
4	**Sodium Alginate**	Distorted	Nil
5	**Chitosan**	11	8.333
6	**Std TT 5 Lf/ml**	6.3	4.778

Sodium alginate has been was reported to show chemical reactivity with TGF-β_1 *(Gombotz, W.R., 1998)*, in which the positively charged protein was found to interact with available carboxylic acid sites on the alginate, resulting in protein inactivation. Same mechanism may be responsible for inactivation of TT.

Organic solvents like IPA, Diethyl ether can disrupt hydrogen bonds contributed by water and eventually alter the protein conformation.

No rocket was observed in case of glutaraldehyde indicating complete loss of epitopes. This dialdehyde reportedly forms covalent bond between side chain amino groups of the amino acid resulting in unstable or stable cross link formation which may have changed the epitope conformation and resulted in loss of antigenicity of TT. Thus, aldehydes like formaldehyde and glutaraldehyde used for cross-linking of the chitosan particles but will also result in cross-linking of TT.

The result of these studies prove that the RE method was a clear indicator of compatibility or incompatibility between additives and TT. Therefore, incompatible additives like Sodium Alginate, Isopropyl Alcohol, Diethyl ether and glutaraldehyde, which would be responsible for any alteration in the antigenic conformation of TT in formulation development were not selected. Formulation experiments for entrapment of TT and DT were performed by using following compatible additives: Chitosan solution in 1% acetic acid, Sodium tripolyphosphate, Propylene glycol and Glycerol.

4.4.5. *In-silico* Study of interactions between Chitosan and toxoids.

In order to study the interaction between Toxoids and CS, amino acid sequence of Tetanus Toxin and Diphtheria toxin was used as the exact sequence of Tetanus and Diphtheria toxoid was not available. Geometrically optimized minimum energy structure of CS, Toxins was achieved using Hyperchem Molecular Modeling software. To study the interaction between CS & Tetanus Toxin and CS & Diphtheria Toxin, geometrically optimized minimum energy structures were kept in contact with each other and both molecules were selected and geometrically optimized minimum energy structure of the complex was achieved. Using this software, we could only compute H-bonds. Hydrogen bonds formed between the TT and Chitosan fragment were recomputed. **Figure 24** shows the number of hydrogen bonds formed between CS and TT and **Figure 25** shows the number of hydrogen bonds formed between CS and DT. There are at least 5 H-bonds between CS-TT and 3 H-bonds for CS-DT system for 20

amino acids of toxins and 20 glucosamine units of CS. This clearly shows the extent of H-bond formation when whole length of TT, DT and CS is considered.

Figure 24: Interaction between Chitosan (CS) and Tetanus Toxin (TT-green backbone) fragments.

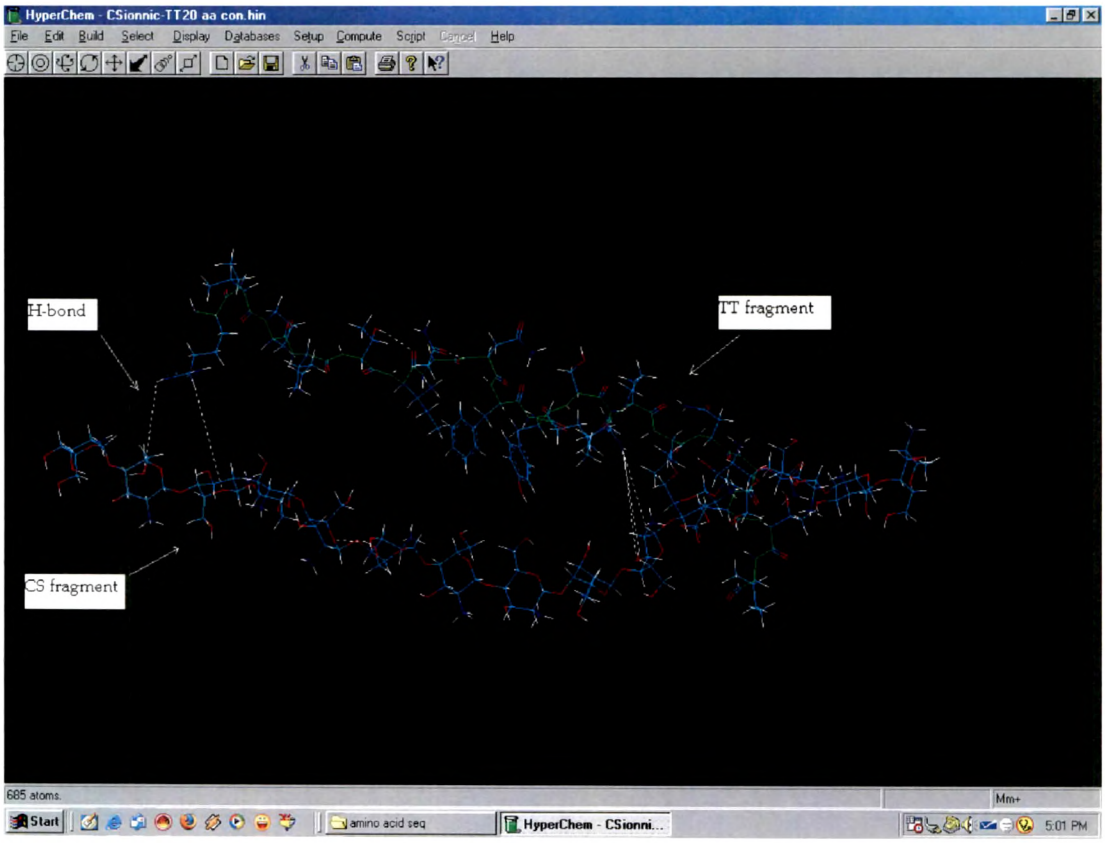
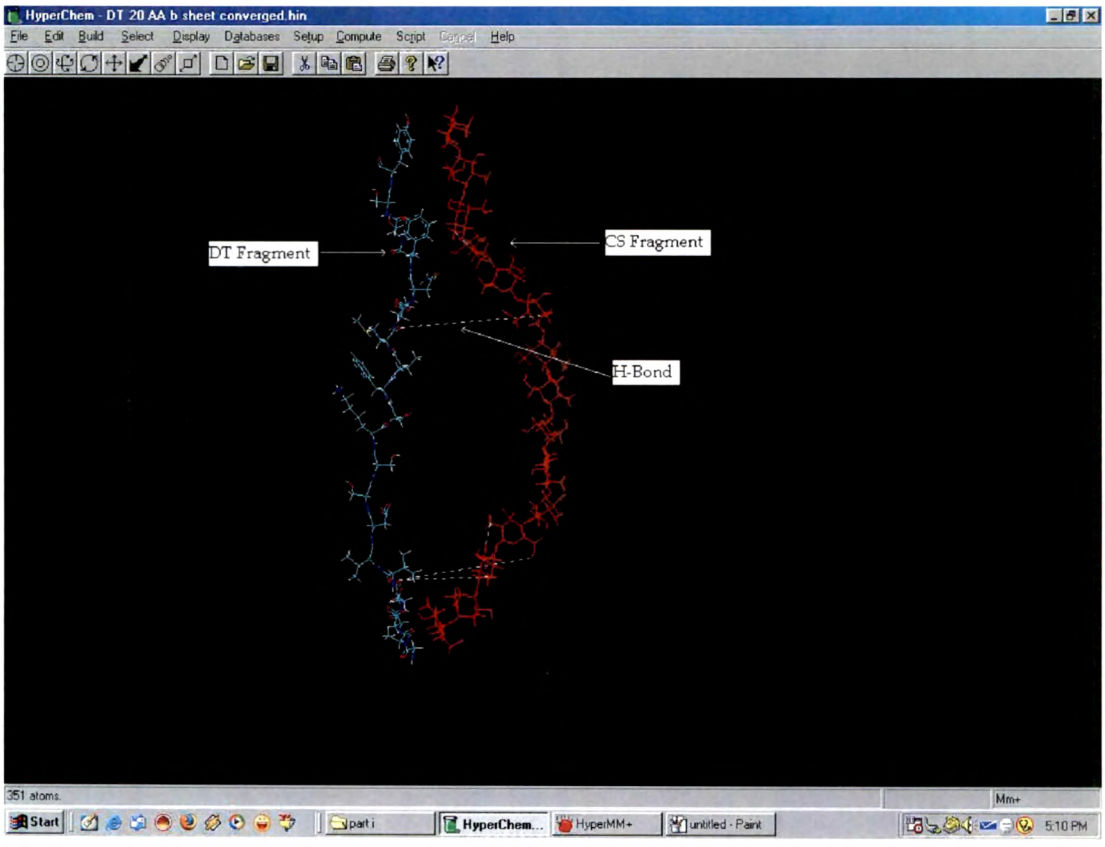


Figure 25: Interaction between Chitosan (CS red color) and Diphtheria Toxin (DT) fragments.



4.5. Conclusions:-

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 developed which was used for quantitative determination of TT as well as for compatibility study in preformulation for the chitosan based microparticle system for TT. RE can 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. Molecular modeling studies showed formation of H-bond between CS and toxoids but

that has not affected the anitigenicity of the toxoids as we could observe the rocket for the CS-TT sample.

4.6 References:

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