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Development of Rocket Electrophoresis Technique as an Analytical Tool in Preformulation Study of Tetanus Vaccine Formulation

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ABSTRACT Rocket Electrophoresis (RE) technique relies on the difference in charges of the antigen and antibodies at the selected pH. The present study involves optimization of RE run conditions for Tetanus Toxoid (TT). Agarose gel (1% w/v, 20 ml, pH 8.6), anti-TT IgG – 1 IU/ml, temperature 4–8°C and run duration of 18 h was found to be optimum. Height of the rocket-shaped precipitate was proportional to TT concentration. The RE method was found to be linear in the concentration range of 2.5 to 30 Lf/mL. The method was validated and found to be accurate, precise, and reproducible when analyzed statistically using student's t-test. RE was used as an analytical method for analyzing TT content in plain and marketed formulations as well as for the preformulation study of vaccine formulation where formulation additives were tested for compatibility with TT. The optimized RE method has several advantages: it uses safe materials, is inexpensive, and easy to perform. RE results are less prone to operator's bias as compared to flocculation test and can be documented by taking photographs and scanned by densitometer; RE can be easily standardized for the required antigen concentration by changing antitoxin concentration. It can be used as a very effective tool for qualitative and quantitative analysis and in preformulation studies of antigens.

KEYWORDS Tetanus toxoid, Rocket Electrophoresis, Preformulation, Additive Compatibility

INTRODUCTION

Many indirect and direct techniques have been developed for the analysis of protein. There are many immunochemical methods: Chemical methods like peptide mapping, mass spectroscopy, colorimetric assays, chromatography, electrophoresis; Spectroscopic methods like circular dichroism, fluorescence, infrared, nuclear magnetic resonance, electron microscopy, dynamic light scattering for in vitro analysis and characterization of antigens and vaccines. All these techniques have their own pros and cons. However, they can

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be used collectively for fingerprinting any antigen or biotech products (Metz, 2002).

Most of the techniques are either expensive due to high initial cost of setup and recurring cost of chemicals or require of special expertise. However, the non-labeled immunoassays (flocculation, immunodiffusion, immunoelectrophoresis) are quite easy use, economic, and also provide sufficient information about the antigen.

The trend toward the use of peptides and subunit proteins in modern vaccine design has necessitated the use of immunological adjuvants to achieve effective immunity (Dunn, 1990). However, due to the interference of adjuvants, the in vitro determination of antigen has proved to be difficult. Most of the in vitro tests do not give any information of the antigenicity of the vaccine/antigen making in vivo test mandatory. Hence, in vivo assays are used to determine the antigenic potency of adjuvanted vaccines (Katz, 1989) such as vaccines adsorbed on aluminium hydroxide or phosphate, developmental vesicular adjuvants such as liposomes (Weiner, 1990), niosomes (Brewer, 1992), particulate adjuvants such as poly (DL-lactic-co-glycolic acid) (PLGA) microparticles with stabilizers (Gupta, 1996), and chitosan microparticles. The disadvantages of in vivo tests are numerous: they are expensive, inaccurate, slow, show large variations in results between laboratories, and are ethically questionable (Festing, 1994). If correlation between a range of chemical and biological tests of the antigen/vaccine/biological product of interest is demonstrated, potency testing in animals could be replaced by a corresponding in vitro method. Hence, a test which can be used for in vitro estimation of the antigen/vaccine in the presence of adjuvant and which will also give information regarding the antigenicity of the antigen/vaccine is required.

Antigens can be quantified by electrophoresing them into antibody containing gel in the technique termed as 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; the height of the rocket being proportional to antigen concentration (Roitt, 1994). The present work involves development of a modified RE method for Tetanus Toxoid (TT) and its application:

In qualitative and quantitative estimation of TT

In estimation of TT from marketed Tetanus Vaccine

As an analytical tool in Preformulation studies for the development of chitosan-based vaccine delivery system for TT

Chitosan is a natural, biocompatible, and biodegradable polymer. Chitosan microparticles have been successfully used for the delivery of protein-peptides, vaccines, and DNA (Genta, 1998). TT is a large protein with a molecular weight of 150,000, prepared by detoxification of tetanus toxin using formaldehyde. TT may be prone to denaturation by various conditions such as microencapsulation (interaction with polymer, crosslinking agents and solvents), storage, and rehydration of microspheres (Gupta, 1996).

MATERIALS AND METHODS

Purified Tetanus toxoid (Batch I ~3000 and Batch II ~3840 Lf/mL) and Equine tetanus antitoxin (500 IU/mL) were 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 (medium EEO for electrophoresis), 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, tri-sodium citrate 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 analysed using Quantity One™ software. All the chemicals were of analytical reagent grade unless otherwise specified. MilliQ water was used wherever required.

Tris-glycine gel and tank buffer (pH 8.6) were prepared by dissolving tris (3 g) and glycine (14.2 g) in MilliQ water and volume was made up to 1000 mL after adjusting pH to 8.6.

Staining solution was prepared by dissolving coomasie brilliant blue R-250 (300 mg) in methanol (45 mL), and adding acetic acid (10 mL) and MilliQ water (45 mL). Destaining solution was prepared by

mixing methanol (45 mL) with acetic acid (10 mL) and MilliQ water (45 mL).

Chitosan Solution was prepared by adding chitosan (2 g) to 100 mL MilliQ water containing 1 mL glacial acetic acid under continuous stirring. After overnight stirring, the chitosan solution obtained was filtered through sterile dacron cloth to get 20 mg/mL solution of chitosan.

In evaluating the results of RE, 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 concentration and was used for the optimization, linearity, validation, and compatibility studies.

Optimization of RE Run Conditions

Rocket electrophoresis (RE) run was optimized by studying the effect of variables like agarose gel amount, equine tetanus antitoxin (ATS) amount, run environment temperature, and run duration on the precipitin rocket height.

The final optimized conditions for the RE run were as follows: Rocket electrophoresis was performed using 1% w/v agarose gel prepared in tris-glycine buffer (pH 8.6). Required amount of agarose was dissolved in 20 mL buffer. Required amount of ATS was added at 50°C with continuous stirring, to give concentration of 1 IU/mL in agarose gel. The agarose solution containing ATS was poured evenly on 10×7 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 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. TT was found to travel toward anode when current was allowed to pass through the gel. The gel was electrophoresed at 4–8°C for 18 h 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 solution and destained with destaining solution. The heights of the rocket shaped precipitate were measured from the top of the well to the top of the precipitate using digital caliper.

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 six times and gel 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.

Validation of RE

Accuracy

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 the linear relationship between height of rocket and concentration of TT. Student's t-test was applied to the set of values.

Precision

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.

Reproducibility

Reproducibility of the method was studied by using Batch I and II of TT which were diluted to get the standard concentration in the range of 2.5 to 30 Lf/mL.

Applications of RE

Estimation of TT from different batches of plain TT

Plain TT, i.e., non-adsorbed TT, was taken from different batches and loaded in agarose gel sample well, along with standards, after suitable dilution. RE was run as per the optimized procedure. The relationship between the height and concentration of TT was used to calculate the unknown concentration.

Estimation of TT from Marketed Product

A vial containing 10 Lf/mL of TT was taken. To this, sufficient sodium citrate powder was added to give 10% w/v concentration and incubated at 37°C for 16 h. It was centrifuged, the supernatant loaded in agarose gel sample well (IP, 1996), and RE was carried out in accordance with the earlier described procedure.

Compatibility Studies with Different Additives

Depending upon the additive used for the preparation of TT encapsulated chitosan microspheres, it was brought in contact with the TT for the same time as used in the process of manufacturing of microspheres. 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. Chitosan, calcium chloride, ascorbyl palmitate, glycerin, sodium alginate, isopropyl alcohol, diethyl ether, propylene glycol, and sodium tripolyphosphate were tested for compatibility with TT.

RESULTS AND DISCUSSION

Optimization of Run Conditions

The standard dilutions of TT were prepared in tris-glycine buffer and loaded in sample wells. The pH of agarose gel and tank buffer was kept at 8.6 as at this pH, TT molecule is negatively charged and IgG in ATS is positively charged. Rocket electrophoresis run conditions were optimized by studying the effect of variables as shown in Table 1.

Agarose gel, 20 mL of 1% w/v, 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. The movement of TT may be impeded at a higher quantity and thickness of gel. Run environment temperature of 4–8°C was found to give best results whereas the gel was often found to be distorted at 20–25°C, probably due to the heat generated during the run. Run duration of 18 h at 4–8°C was found to give reproducible rocket height. At lower run duration, the rocket formation is not complete as there is no proportionate difference in height of rocket formed for different loaded concentrations.

The present optimized RE method has many advantages: It uses less amount of agarose and safe materials like tris-glycine instead of controlled substances like sodium barbital buffer as reported by Ljungqvist (1987), with sample volume of only 8 µl. The RE method is easy to perform and the cost of run is less 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. This indicates 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 documentation system making the method beneficial in validation procedures and quality control audits.

Determination of Linearity for TT in RE

The relationship between concentration of TT in the sample well and height of the precipitin rocket was found to be linear in the range of 2.5 to 30 Lf/ml. The standard calibration curve Table 2 is represented

TABLE 1 **Optimization of RE Run Conditions**

Parameter	Levels				Remarks
Agarose gel amount (mL)	30		20		20 mL of 1% agarose gel was found to give reproducible and clear rockets were obtained as compared to 30 mL.
ATS amount (IgG IU/mL)	1.5	1.5	0.5		1 mL was found to give rocket height in between 2 to 40 mm for standard curve of TT.
Run Environment Temperature (°C)	20–25		4–8		4–8°C was found to give best results.
Run duration (hrs)	6	12	16	18	18 hrs at 4–8°C was found to give reproducible rocket height.

TABLE 2 **Calibration Curve for Tetanus Toxoid**

Std concentration of TT (Lf/mL)	Height of precipitin rocket (mm) Mean ± S.E.M. (n = 6)
02.50	02.82 ± 0.129
05.00	06.23 ± 0.117
10.00	13.81 ± 0.312
15.00	18.58 ± 0.447
20.00	24.49 ± 0.280
25.00	30.72 ± 0.604
30.00	40.50 ± 0.864

graphically in Fig. 1. Gel photograph taken using Bio-Rad gel documentation system, USA. Fig. 2 also clearly indicates the sensitivity of the developed method.

Equation of the regression line is

$$y = 1.3034x - 0.4246,\ R^2 = 0.9921 \quad (1)$$

Validation of RE

Accuracy

The results of comparison between theoretical and calculated values as shown in Table 3 indicate that the two values are not significantly different from one another. This establishes the accuracy of the developed RE method.

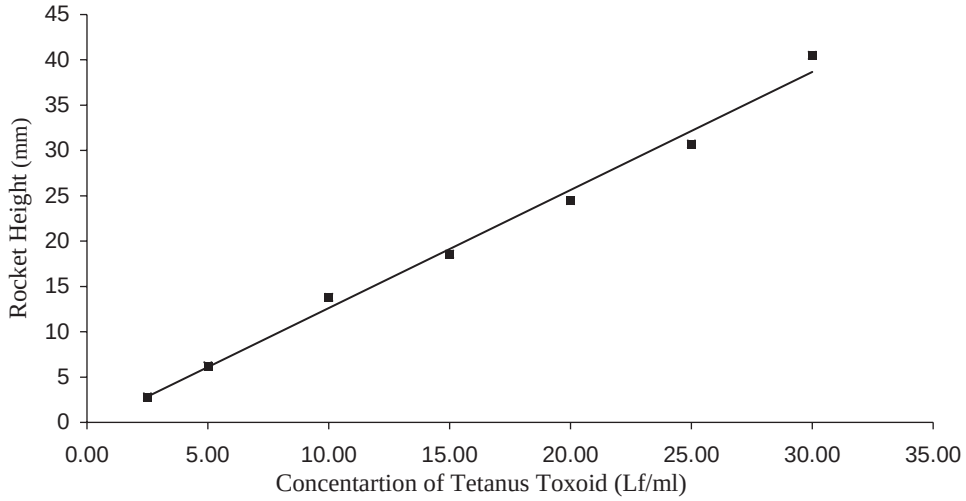

FIGURE 1 **Calibration Curve for Tetanus Toxoid.**

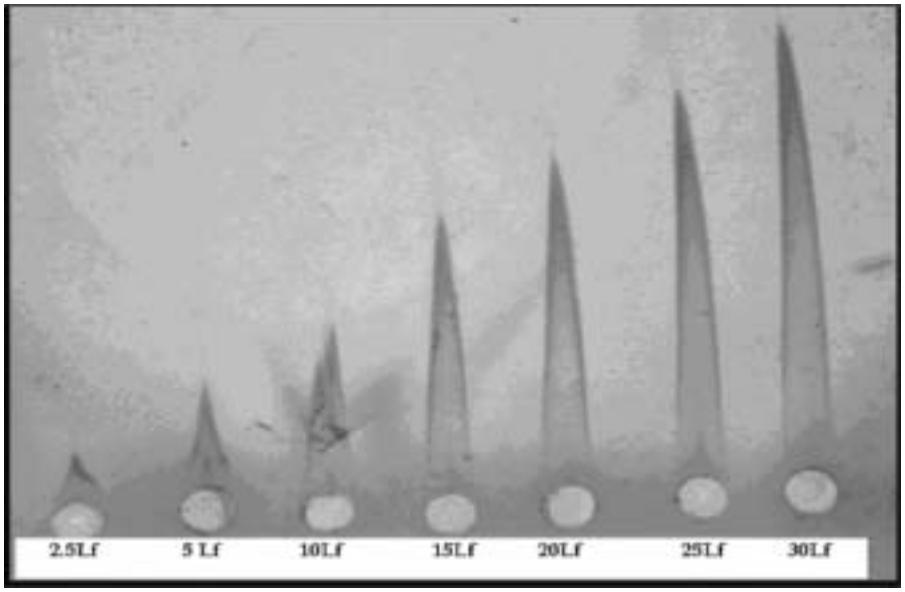

FIGURE 2 **Photograph of Gel Showing Calibration Curve for TT.**

TABLE 3 Accuracy of RE

Sr.No.	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
3	15	14.04	(Not significantly
4	25	25.89	different)

TABLE 4 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
3	15	15.04	(Not significantly
4	15	16.05	different)

Precision

As the run duration is of 18 h, inter-day precision of the method was determined by analyzing the sample of known concentration on consecutive days. The data, analyzed statistically by Student's t-test (Table 4), indicated non-significance between theoretical and calculated values, establishing the precision of the method.

In order to avoid systematic errors, due to minor differences in gel thickness or in readings which might influence the outcome of the test, randomization of sample application was done. Standard dilutions were loaded randomly and positions of test samples 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.

Reproducibility

The results of reproducibility test (Table 5) showed that the means of the two different batches were not significantly different ($t = -0.017731$, $p = 0.986145$). This established reproducibility of the method.

APPLICATIONS

Estimation of TT from Different Batches of Plain TT

Flocculation method is usually used for estimation of TT from production batches. As developed by Ramon (Lyng, 1987), the concentration in Lf units of an unknown tetanus toxoid preparation is estimated relative to reference preparation of tetanus antitoxin. Immunoprecipitation tests in gels are preferred to flocculation tests in some control laboratories because they give more information about the quality and concentration of the vaccine antigens (Ljungqvist, 1987; Winsens, 1985). By replacing the antitoxin reference preparation with toxoid reference preparation, it will now be possible to use RE method instead of flocculation method for the quantitative estimation of toxoids. Tetanus or any other toxoid can be standardized against the International Antitoxin Standard by RE. The appropriate equation can be obtained between the antigen and antitoxin concentration. This relationship between the toxoid and antitoxin can be used to measure the unknown concentration of antigen. The rocket height is directly proportional to the concentration of the TT in the well and inversely proportional to the amount of antitoxin (ATS) mixed into the agarose gel. In this case, ATS of 1 IU/mL of agarose gel was taken to get the rocket height in the range of 2 to 40 mm.

TABLE 5 Reproducibility of RE Using Batch I and II of Plain TT

Conc 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

Estimation of TT in Marketed Product

Three marketed products of adsorbed TT vaccines were studied after treating with tri-sodium citrate for complete desorption of the TT. Concentration of TT was calculated using the linear equation between TT concentration and height of the rocket. The results given in Table 6 shows that the labeled and calculated means were not significantly different. This establishes the applicability of the RE method in quantitative estimation of TT from formulation containing adjuvant.

TABLE 6 Application of RE in Analysis of Marketed Products

Product	Label claim of TT (Lf/mL)	Calculated conc. of TT (Lf/mL)	Student's t-test
Product A	10	9.87	t = 0.005739
Product B	10	11.06	p = 0.995942
Product C	10	9.06	(Not significantly different)

Compatibility Studies with Different Additives

RE method was used as an analytical tool in the preformulation studies of the microencapsulation of TT using chitosan, a biodegradable polymer. TT is a large protein with a molecular weight of 150,000 and has been found to be stable at 37°C for several weeks. However, TT may be prone to denaturation by various conditions such as microencapsulation (interaction with polymer, solvents and crosslinking agents), storage and rehydration of microspheres. The loss of antigenicity of the antigen is a commonly reported problem especially when they are being formulated into formulations like microspheres, liposomes, and nanoparticles for the controlled delivery of the antigen. This is mainly due the use of organic solvents (air-liquid interface formed due to high-speed homogenization), electrolytes, polymers, pH conditions, and cross-linking agents during the preparation of the delivery systems. The denaturation of proteins may be due to covalent bond formation, conformation change, or other interactions (Gupta, 1996; Wilson, 2000).

Antigenic epitopes are recognized by the immune system of the body, and specific antibodies are produced. The antigenicity of the antigens is lost if its antigenic epitopes are damaged during the process. The same mechanism of antigen-antibody binding is used in RE, where the immobilized antibody binds with the moving antigen in the applied electrical field. This precipitin complex can be visualized after staining and destaining in the form of rocket. 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 the cases either rocket will not be obtained or will be distorted with lesser height than standard. Thus formation and integrity of the rocket will indicate retention of antigenicity of the toxins. Hence, RE was used to test the compatibility of TT with various additives as shown in Table 7.

In case of chitosan, calcium chloride, sodium tripolyphosphate, ascorbyl palmitate, glycerin, and propylene glycol, no interaction was observed as the shape and height of precipitin rocket was maintained (Table 8, Fig. 3). Student's t-test showed no significant difference between the standard TT concentration and the TT concentration after exposure to additives ($t = -2.137187$; $p = 0.085622$), indicating their compatibility with TT. In case of sodium alginate, isopropyl alcohol, diethyl ether, and glutaraldehyde, the rocket height was significantly reduced ($p < 0.05$) and shape distorted as compared to the standard, indicating incompatibility with TT (Table 9, Fig. 4).

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

Organic solvents like isopropyl alcohol and diethyl ether can disrupt hydrogen bonds contributed by water and eventually alter the protein conformation.

No rocket was observed in the 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 total loss of antigenicity of TT. This implies that aldehydes like formaldehyde and glutaraldehyde should not be used for cross-linking of the chitosan particles as they will also cross-link TT.

Thus, RE method proves to be a clear indicator of compatibility or incompatibility between additives and TT. This will be useful tool to screen out incompatible additives which are responsible for any alteration in the antigenic conformation of TT, and final formulation can be prepared by using compatible additives like Chitosan, sodium tripolyphosphate, propylene glycol, and glycerol.

CONCLUSIONS

Modified Rocket Electrophoresis method was developed, optimized, and validated for accuracy,

TABLE 7 **Additives Tested for Compatibility with TT**

Additive	Use	Volume	Mixing time	Observation
Chitosan solution, 2% in 1% acetic acid	Carrier biodegradable polymer	2 mL with 1 mL of TT	Vortexed for 5 min	Compatible as there is no change in height and shape of precipitin rocket
Calcium chloride solution, 2% in MilliQ water	Cross-linking agent for alginate and for chitosan- alginate particles	2 mL was added in l mL of TT	Stirred for 30 min	Compatible as there is no change in height and shape of precipitin rocket
Glycerin	Solvent for stabilization of formulation	2 mL with 1 mL of TT	Stirred for 30 min	Compatible as there is no change in height and shape of precipitin rocket
Propylene glycol	Solvent for stabilization of formulation	2 mL with 1 mL of TT	Stirred for 30 min	Compatible as there is no change in height and shape of precipitin rocket
Sodium tripolyphosphate, 28 mg/mL in water	Cross-linking agent for chitosan	2 mL with 1 mL of TT	Stirred for 30min	Compatible as there is no change in height and shape of precipitin rocket
Glutaraldehyde, 5% solution	Cross-linking agent for chitosan	1 mL of 5%, added drop-wise in 4 mL of TT	Stirred for 30 min	Incompatible as there is no formation of precipitin rocket
Isopropyl alcohol	Used for washing and hardening of chitosan microparticles	4 mL with 1 mL of TT	Vortexed for 5 min, centrifuged	Incompatible as the height of the precipitin rocket is reduced as compared to standard
Diethyl ether	Used for washing of chitosan microparticles	4 mL with 1 mL of TT	Vortexed for 5 min, centrifuged	Incompatible as the height of the precipitin rocket is reduced and shape is distorted as compared to standard
Sodium alginate, 2% in MilliQ water	Carrier polymer can be used alone and also cross-linking agent for chitosan	2 mL with 1 mL of TT	Vortexed for 5 min	Incompatible as the height of the precipitin rocket is reduced and shape is distorted as compared to standard

TABLE 8 **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

TABLE 9 **Additive Compatibility Study: Concentration of TT After Exposure to Additives**

	Additive	Height of rocket (mm)	Calculated concentration of TT (Lf/mL)
1	Std TT 10 Lf/mL	13.2	10.000
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

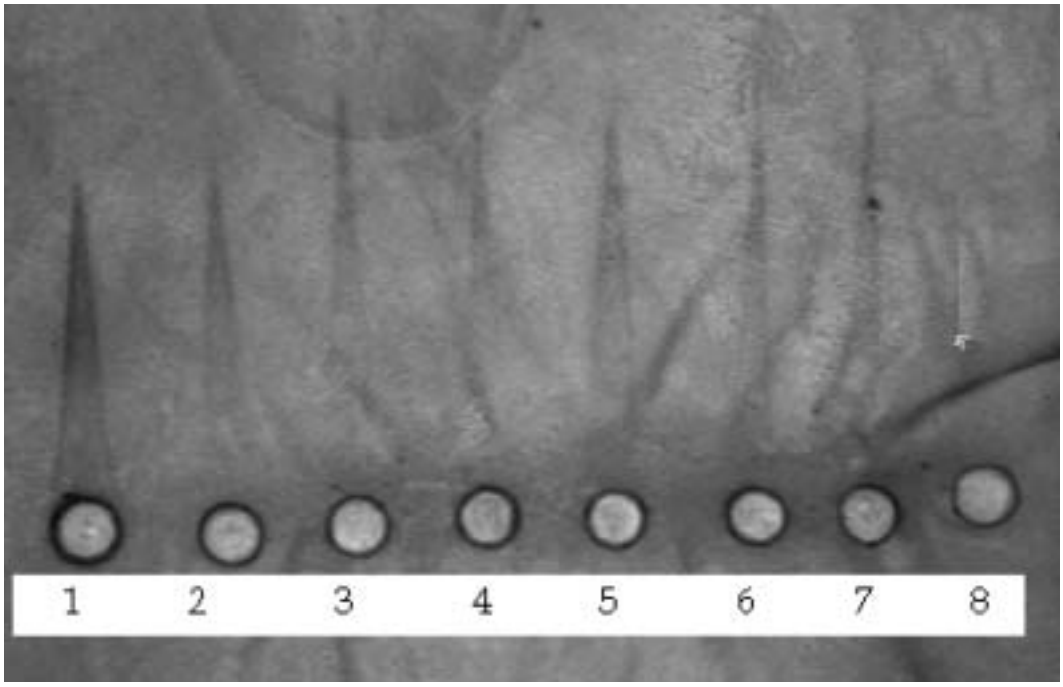

FIGURE 3 **Gel Photographs of RE for Additive Compatibility Study 1: Standard TT 15 Lf/mL; 2: Chitosan; 3: Calcium Chloride; 4: Glycerin; 5: Propylene Glycol; 6: Ascorbyl Palmitate; 7: Sodium Tripolyphosphate; 8: Glutaraldehyde.**

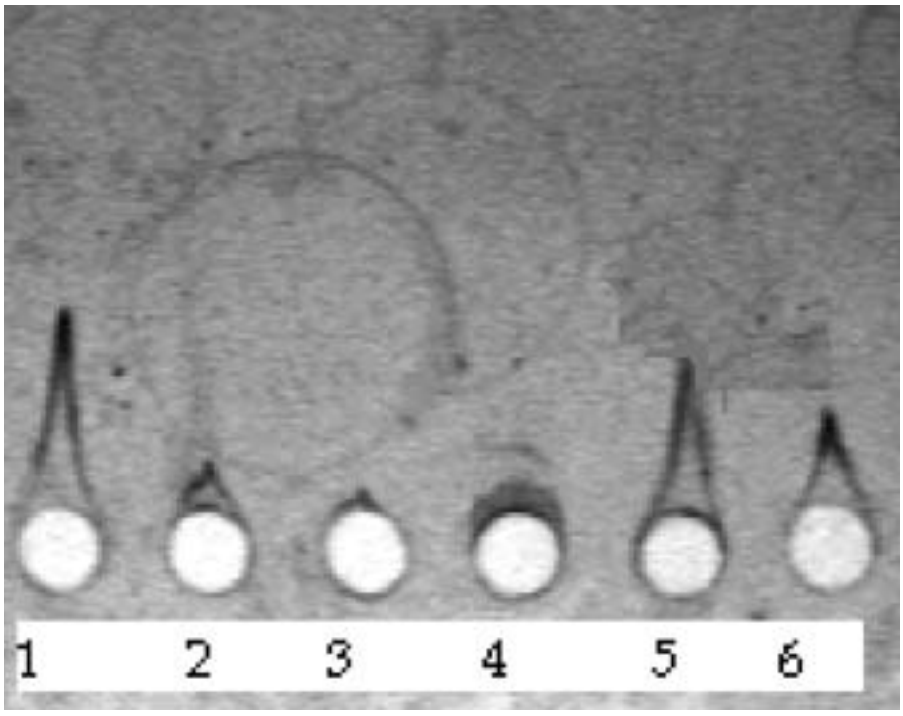

FIGURE 4 **Gel Photographs of RE for Additive Compatibility Study 1: Standard 10 Lf/mL; 2: Isopropyl Alcohol; 3: Diethyl Ether; 4: Sodium Alginate; 5: Chitosan; 6: Standard 5 Lf/mL.**

precision, and reproducibility. Linear relationship in a range of 2.5 to 30 Lf/mL between concentration of standard TT and height of precipitin rocket in agarose gel was developed, which was used for determination of TT concentration in batches of plain TT, marketed product, and for compatibility study in preformulation for the chitosan-based microparticle system for TT. 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. 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 photograph or scanned by densitometer.

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