

**STUDY OF PERCENTAGE ADSORPTION OF RECOMBINANT PROTEIN ANTIGEN
ON THE REDUCED ALUMINIUM PHOSPHATE ADJUVANT PARTICLES**

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ABSTRACT

The main objective of the present study is estimate the % adsorption of recombinant protein antigen on the reduced aluminium phosphate adjuvant particles. Bovine serum albumin was used as a working standard for estimation of protein by lowry method. Bovine serum albumin was studied using with the concentration from 20, 40, 60,80and 100 µg/mL. Different size containing aluminium phosphate adjuvant particles are prepared by using homogenization, sonication, freezed and thawed methods. Homogenization study is conducted at 10,000 rpm by different time intervals i.e. 15, 30, 60, and 90 minutes. Sonication study was conducted by using elma sonicator at different time intervals i.e. 60 and 180 minutes. During sonication the alumnium phosphate particle size is reduced from 2.20µm to 1.82µm. Freezed at -20C and thawed at room temperature. Different size containing aluminium phosphate particles are estimated by using xylenol orange as an indicator. From the above methods the homogenization at 10,000 rpm at 90 minutes (Batch-8) has less particle size hence the rate of adsorption of recombinant protein antigen is also high. Sonicated aluminium particles size is greater than homogenized particles hence the rate of adsorption is less than homogenized particles. It was conclude that the rate of shearing and time plays a major role for reduced particle size during homogenization and shows better adsorption.

KEYWORDS: Aluminium phosphate adjuvant, homogenization, sonication, freezed and thawed, particle size, xylenol orange, Bovine serum albumin, Model recombinant protein antigen adsorption.

INTRODUCTION

The origin of modern day vaccination began in the 18th century when in 1796 a country doctor named Edward Jenner first noticed that milkmaids infected with cowpox did not become infected with smallpox. Smallpox was a disease that had killed 10% of Europe's population, and the survivors were left with disfiguring scars and blindness.^[1,2] His injection of a young boy with the fluid from a cowpox lesion was the first documented attempt to provide protection from disease through inoculation. More than 200 years later there are 27 preventable diseases that have available vaccines and vaccines were a \$10.6 billion dollar industry.^[3,4] Historically there have been two main branches of vaccine production. One is the development of live, attenuated vaccines. These are vaccines where non-virulent strains of the target microorganism are used or have been made non-pathogenic by modification of their genome. Vaccines for smallpox, measles, mumps, rubella, and cholera fall into this class. The second most recognized branch is the inactivated, whole organism group. These are vaccines where the microorganism is killed by heat or chemical means and its entire structures are used in the production of the vaccine. Influenza, pertusis, anthrax, and hepatitis

A are diseases represented by this class.^[5] Both of these categories are strongly immunogenic and are capable of generating sufficient antibody production to provide protection against disease.

In the early 20th century, a third type of vaccine called the subunit vaccine arose. These vaccines are made from a fragment of the microorganism, such as a protein, polysaccharide, DNA strand or toxin. They can be used separately or in conjugation, such as with the type B influenza vaccine. Diphtheria and tetanus toxoids were the first subunit vaccines to be developed. These types of vaccines are ideal because some of the risks that accompany the live and inactivated vaccines are eliminated but they lack their potency because of reduced immunogenic effect.^[6] They must be aided by a biological or chemical agent commonly referred to as an adjuvant.

Adjuvants can enhance the effectiveness of a vaccine in a number of ways. The most significant is the increased immune response provided by the adjuvant. The second role is Sustained release at a specific site over an extended period of time. And the final role is through selected targeting to specific cell types that are crucial in

invoking immune memory. Currently in the United States the only licensed adjuvants are aluminum based products such as aluminum hydroxide, aluminum phosphate and potassium aluminum sulfate. The efficacy of aluminum based adjuvants varies greatly on the type of antigen delivered.^[6,7] The drawbacks of using aluminum adjuvants are that they can induce severe tissue reactions and hypersensitivity, they cannot induce cell-mediated immunity, and they cannot be processed for long-term storage by means of lyophilization or freezing.

The bridge between innate and acquired immunity is linked by the antigen presenting cells (APCs). Antigen presenting cells are activated dendritic cells (DCs), activated macrophages, and activated B cells.^[7] The role of these cells is to activate T cells via class I and class II Major Histocompatibility complex and a co-stimulatory protein located on the cell surface. As stated in the previous section, macrophages can operate in a phagocytic capacity. However they also have roles in cytokine production and antigen presentation.^[8] After they engulf antigens and present the processed fragments on their surface, they can bind to receptors of the helper T cells (TH). The binding process activates the T cell, which plays a significant role in acquired immunity.

The dendritic cell (DC) is the most potent antigen presenting cell. Therefore many vaccine and adjuvant strategies have directly attempted to target DCs. The cell functions by encapsulating antigens via macro pinocytosis or receptor-mediated endocytosis, processing them and presenting a portion of the antigen on its surface to the T cell through both class I and class II Major Histocompatibility complexes (MHC). The internalization of the microbe stimulates the dendritic cell to transform from a naïve cell into a mature effector cell which possesses surface embedded co-stimulatory molecules. Their most significant role in adaptive immunity is their ability to strongly activate both memory T cells and naïve T cells. T cells function to either directly kill infected cells or to activate stimulate B cells to produce antibodies. The mature cell also works in the innate immune system to activate Natural killer cells (NK) and to secrete IL-12 cytokines. In attempts to directly target DCs biomaterial antigen-delivery vehicles have been that induce endocytosis through DC receptors such as mannose, or surface modified with DC specific antibodies to bind DCs selectively. Additionally studies have shown that in an invitro test polystyrene spheres showed. It was also noted that surface charge effects on particle uptake become more pronounced in particles > 0.5 μm .^[9,10,11]

MATERIALS AND METHODS

Materials

Aluminium phosphate was purchased from Sigma Aldrich. Sodium acetate, sodium carbonate, sodium phosphate tartarate, acetic acid and hydrochloric acid was purchased from Sigma Aldrich.

Methods

Preparation of different particle size containing aluminium phosphate solution by homogenization (1mg/ml) :- (Batch-2 to 5)

Take 4 batches of each 50ml of alphas solution and subjected to homogenization for 15, 30, 60&90 min at 10,000 rpm. After completion of homogenization for from each batch 1.887 ml of homogenized aluminium phosphate containing solution was added to 8.113 ml of normal saline solution by using micropipette. Repeat the procedure for remaining batches now 1mg/ml of different aluminium phosphate particle size containing solution was obtained.

Preparation of different particle size containing aluminium phosphate solution by sonication (1mg/ml) :- (Batch-6&7)

Take 2 batches 50ml of alphas solution and subjected to sonication for 1hr and 3hrs, after completion of sonication for 1 hrs batch 1.887 ml of sonicated aluminium phosphate containing solution was added to 8.113 ml of normal saline solution by using micropipette. Repeat the procedure for remaining batch now 1mg/ml of different aluminium phosphate particle size containing solution was obtained. These two batches are considered as batch-6&7.

Preparation of different particle size containing aluminium phosphate solution by freeze&thawed (1mg/ml) :- (Batch-8)

Take one batch of 50ml of alphas solution and subjected to Freezed at -20°C and thawed at 37°C , after completion of freeze&thawing 1.887 ml of aluminium phosphate containing solution was added to 8.113 ml of normal saline solution by using micropipette. Now 1mg/ml of different aluminium phosphate particle size containing solution was obtained. This is considered as batch-8.

Determination of sedimentation volume ratio of different size particles containing alphas

Numbering the individual batches with neat labeled paper on clean sterile 10ml measuring cylinders. Pipette out 10 ml of aluminium phosphate solution from stock solution and transfer in to a clean sterile 10ml measuring cylinders. Repeat the same procedure for all batches containing aluminium phosphate solution. Set all pipettes uniformly with neat labeled indicating batches on a stand. Set the time in stop watch and switch on the watch for easy identification and observing of the sediment particles. Note down the readings of all batches as 10 ml at 0 times. Mention the time in minutes for easy identification in a note book. Note down the sedimentation volume readings for every 5 minutes time intervals up to first 30 minutes without any time delay. Note down the sedimentation volume readings up to 6th hrs.

Determination of pH for size reduced aluminium phosphate particles

After completion of homogenization, sonication, freeze & thawed the sample solution was determined pH by using mettler Toledo pH and conductivity meter. To get precise value measurements, ensure the calibration status of PH meter. Remove the electrode from PH electrode storage buffer and rinse with WFI and dry it with lint free cloth. Place the electrode in sample and press **Read** key. The measurement ends automatically when the measured value is stable and note down the value in the respective documents. Remove the PH electrode from sample, wash with WFI and blot it dry with lint free cloth. Place the electrode back in electrode storage buffer.

Estimation of Aluminium phosphate by Xylenol orange

Procedure:-Xylenol orange solution:- 1mg/mL (by dissolving 10 mg of xylenol orange in 10 ml of water for injection)

Sodium acetate buffer pH 5.0 was prepared by dissolving 13.6 gm of sodium acetate and 6ml of glacial acetic acid in sufficient water to produce 1000ml and adjust the buffer to pH if necessary.

Prepared aluminium phosphate stock solution:- 1 mg/mL with normal saline.

Standard 1(100µg/mL):- 200µL aluminium phosphate stock solution +1ml of sodium acetate+66µL of xylenol orange+734µL water.

Standard 2(50µg/mL):- 100µL aluminium phosphate stock solution +1ml of sodium acetate+66µL of xylenol orange+834µL water.

Standard 3(25µg/mL):- 50µL aluminium phosphate stock solution +1ml of sodium acetate+66µL of xylenol orange+884µL water.

Standard 4(12.5µg/mL):- 25µL aluminium phosphate stock solution +1ml of sodium acetate+66µL of Xylenol orange+909µL water.

Standard 5(6.25µg/mL):- 12.5µL aluminium phosphate stock solution +1ml of sodium acetate+66µL of Xylenol orange+921.5µL water.

Blank:-1mL sodium acetate buffer +66µL of Xylenol orange + 934 µL water.

Incubate at 37 °C for 4 hours.

After incubation, al-morin complex absorbance was determined at 550nm by UV-Visible spectrometry.

Estimation of recombinant protein

It is conducted by using Lowry's method, by preparation of Lowry's reagent (100; 1; 100) and 1:1 follins reagent. The sample solution should mix with Lowry's reagent and measure at 750nm.

Lowry's method

Determination of protein concentration lies in the reactivity of the peptide nitrogen with the copper ions under alkaline conditions and the subsequent reduction of the Follins CIO plateau phospho molybdic phospho tungsten acid to hetero poly molybdenum blue by the copper-catalyzed oxidation of aromatic acids. It is pH sensitive method hence this method conducted at pH 10-10.5. The absorbance is measured at 750nm.

- Take 1mg/ml of BSA as working standard. Prepare 40, 60, 80, 100, 150, 200µg/ml solution from 1mg/ml working standard.
- The test tube with 1 ml distilled water serves as blank.
- Add 460, 440, 420, 400, 350 and 300µL of Reagent I and incubate for 10 minutes.
- After incubation add 0.5 ml of reagent II and incubate for 10 minutes.
- Measure the absorbance at 750 nm and plot the standard graph.
- Estimate the amount of protein present in the given sample from the Standard graph.

Repeat the same procedure by using recombinant Ag antigen instead of BSA and compare the amount of protein present in the given sample from the standard graph.

Adsorption of recombinant protein on different particle size aluminium adjuvants

Method

1. Calculate the required quantity of recombinant antigen Bulk in normal saline (20µg/mL) and aluminium phosphate gel (0.40mg/ml) for adsorption.
2. Transfer the calculated quantity of aluminium phosphate gel aseptically into a sterile glass bottle.
3. Transfer the calculated quantity of recombinant antigen Bulk to the glass bottle containing aluminium phosphate gel under continuous stirring.
4. Adjust the pH to 5.5 using sterile 0.5M acetic acid following aseptic conditions.
5. After pH adjustment, incubate the adsorbed material at 25±3.0°C and continue stirring at 150rpm for 18-26 hours.
6. After adsorption adjust the pH of adsorbed recombinant antigen to 6 to 7 using sterile 0.5M Sodium hydroxide solution aseptically.
7. Repeat the procedure with different size reduced particles (at different rpm and different time interval of aluminium adjuvants).

RESULTS AND DISCUSSION

Determination of Homogenized aluminium phosphate particle size

Upon homogenization the aluminium phosphate particle size was reduced than control. During homogenization the time also one of the important factors when increase the time of homogenization then the rate of particle size

reduction also increased. Hence the size of aluminium phosphate shows the following order Control>homogenization/15min>homogenization/30min>homogenization/60min>homogenization/90min..Stress the particle size distribution in terms of particle counts were evaluated for all vaccines formulations containing Aluminium phosphate the majority of particles in all formulations are in the 1.0–2.0µm range.

Table 2: Homogenized aluminium phosphate particle size.

S. No.	Batch. No.	Particle size
1	2	1.62±0.012µm
2	3	1.57±0.012µm
3	4	1.50±0.013µm
4	5	1.44±0.013µm

Table 3: Determination of sonicated aluminium phosphate particle size.

S. No.	Batch. No.	Particle size
1	6	2.01±0.012µm
2	7	1.82±0.012µm

During sonication also the aluminium phosphate particle size reduced. Here also the time plays a major role.

Control>sonication/1hr>sonication/3hrs.

Freezed and thawed aluminium phosphate particles are increased than control. Stress the particle size distribution in terms of particle counts were evaluated for all vaccines formulations containing Aluminium phosphate the majority of particles in all formulations are in the 1.0–2.0µm range.

Determination of sedimentation volume ratio of aluminium phosphate solution

Control (batch-1) particles are easily settled than homogenized particles. The order of sedimentation rate time as follows;

Control<homogenization/15min<homogenization/30min<homogenization/60min<homogenization/90min.

During homogenization, sonication, Freezed and thawed conditions the PH was not changed. Hence during the above all conditions the aluminium phosphate pH was maintained.

Table no 4: Determination of sedimentation volume ratio of aluminium phosphate solution.

S. No.	Time	Sedimentation volume ratio of aluminium phosphate adjuvant $\bar{X} \pm S.D$					
		Batch-1	Batch-2	Batch-3	Batch-4	Batch-5	Batch-8
1	0	10±0.012	10±0.011	10±0.011	10±0.011	10±0.011	10±0.011
2	5	10±0.012	10±0.011	10±0.011	10±0.011	10±0.011	10±0.011
3	10	10±0.012	10±0.011	10±0.011	10±0.011	10±0.011	9.9±0.02
4	15	10±0.012	10±0.011	10±0.011	10±0.011	10±0.011	9.9±0.02
5	20	10±0.012	10±0.011	10±0.011	10±0.011	10±0.011	9.8±0.03
6	25	10±0.012	10±0.011	10±0.011	9.9±0.02	9.9±0.02	9.8±0.03
7	30	10±0.012	9.9±0.012	9.9±0.02	9.9±0.02	9.9±0.03	9.7±0.02
8	40	9.9±0.03	9.8±0.013	9.9±0.012	9.8±0.013	9.9±0.012	9.6±0.02
9	50	9.9±0.012	9.8±0.013	9.8±0.013	9.8±0.013	9.8±0.012	9.5±0.012
10	60	9.8±0.013	9.7±0.012	9.8±0.013	9.7±0.012	9.8±0.011	9.4±0.013
11	75	9.7±0.012	9.7±0.012	9.7±0.012	9.7±0.012	9.8±0.011	9.3±0.012
12	90	9.6±0.013	9.6±0.012	9.6±0.012	9.6±0.012	9.7±0.013	9.2±0.012
13	105	9.5±0.012	9.6±0.013	9.6±0.013	9.5±0.012	9.7±0.012	9.0±0.012
14	120	9.4±0.013	9.5±0.012	9.5±0.012	9.5±0.011	9.7±0.012	8.8±0.012
15	150	9.3±0.012	9.4±0.01	9.5±0.013	9.4±0.012	9.6±0.012	8.5±0.012
16	180	9.2±0.011	9.3±0.012	9.4±0.012	9.4±0.012	9.6±0.012	8.2±0.012
17	210	9.1±0.012	9.2±0.012	9.3±0.013	9.3±0.012	9.6±0.012	7.8±0.012
18	240	9.1±0.012	9.2±0.013	9.2±0.012	9.3±0.013	9.5±0.012	7.0±0.013
19	270	9.0±0.012	9.1±0.012	9.2±0.013	9.3±0.012	9.5±0.013	6.2±0.012
20	300	9.0±0.013	9.1±0.012	9.2±0.012	9.2±0.013	9.5±0.012	5.6±0.012
21	330	8.9±0.012	9.0±0.013	9.1±0.012	9.2±0.012	9.4±0.012	5.4±0.013
22	360	8.9±0.012	9.0±0.012	9.1±0.012	9.2±0.013	9.4±0.012	5.2±0.012

Determination of pH for size reduced aluminium phosphate particles solution

S. No.	Batch. No.	pH
1	1	6.48±0.012
2	2	6.46±0.012
3	3	6.46±0.011
4	4	6.46±0.012

5	5	6.46±0.012
6	6	6.48±0.012
7	7	6.46±0.012
8	8	6.41±0.012

Table 5: Estimation of aluminium phosphate (Control) by Xylenol orange.

S. No.	Concentration (µg/ml)	Absorbance	Practical Concentration
1	6.25	0.022±0.012	5.205±0.012
2	12.5	0.047±0.013	12.558±0.012
3	25	0.091±0.012	25.5±0.013
4	50	0.174±0.012	49.911±0.012
5	100	0.340±0.013	98.735±0.012

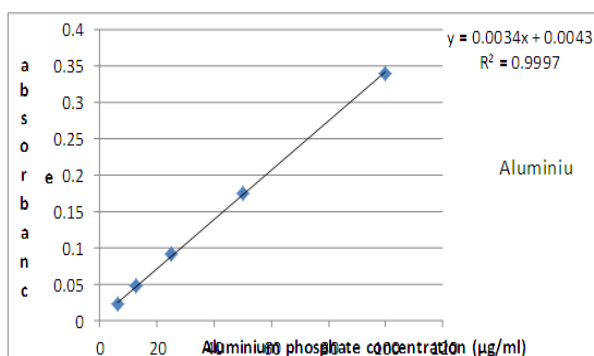


Fig 1: Estimation of aluminium phosphate by Xylenol orange.

Estimation of homogenized (10,000rpm) aluminium phosphate by Xylenol orange

Homogenized samples are having less absorbance value than control, sonicated, Freezed and thawed samples. Hence I observed that the when the rate of shearing is increased then the rate of concentration will be decreased. The absorbance values of homogenized samples were slightly increased than control.

Table 6: Estimation of homogenized (10,000rpm) aluminium phosphate by Xylenol orange.

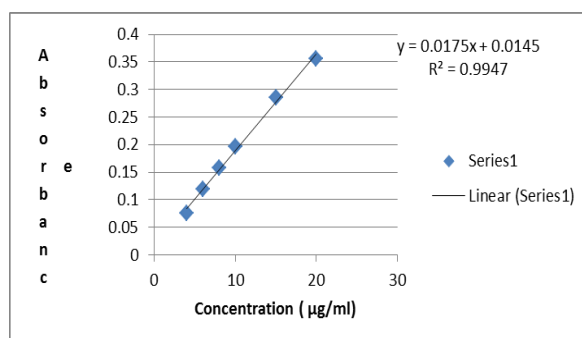
S. No.	Batch. No.	Theoretical concentration(µg/mL)	Absorbance value	Practical concentration
1	1	25	0.087±0.012	24.323±0.012
2	2	25	0.082±0.012	22.852±0.012
3	3	25	0.086±0.012	24.029±0.012
4	4	25	0.087±0.013	24.323±0.013
5	5	25	0.085±0.012	23.735±0.012
6	6	25	0.531±0.013	34.11±0.013
7	7	25	0.524±0.012	30.11±0.012
8	8	25	0.237±0.012	68.73±0.012

Table 7: Estimation of known (recombinant Ag) protein by Lowry method.

Recombinant Ag sample measure at 750 nm.

S. no.	Concentration (µg/ml)	Absorbance value	Practical Concentration (µg/ml)
1	4	0.076±0.012	3.514±0.012
2	6	0.119±0.012	5.971±0.012
3	8	0.157±0.013	8.142±0.013
4	10	0.196±0.014	10.371±0.012
5	15	0.286±0.012	15.514±0.013
6	20	0.355±0.13	19.457±0.012

Estimation of recombinant protein by Lowry method



Adjustment of pH for Adsorption at PH 5

Batch 1: Recombinant Ag batch size:-100ml.

S. No.	Composition	Concentration	Required volume (mL)	Round off volume (mL)	0.5M acetic acid for PH adjustment	0.5M NaoH for PH adjustment	Final volume (mL)
1	Antigen bulk	120µg/mL	16.66	16.7	200µl	160µl	24.66
2	Aluminium phosphate	5.3mg/mL	7.54	7.6	-----	-----	-----

Batch 2: Recombinant Ag batch size:-100ml.

S. No.	Composition	Concentration	Required volume (mL)	Round off volume (mL)	0.5M acetic acid for PH adjustment	0.5M NaoH for PH adjustment	Final volume (mL)
1	Antigen bulk	120µg/ml	16.66	16.7	200µl	2000µl	25.3
2	Aluminium phosphate	5.3mg/ml	8.16	8.2	-----	-----	-----

Batch 3: Recombinant Ag batch size:-100ml.

S. No.	Composition	Concentration	Required volume (mL)	Round off volume (mL)	0.5M acetic acid for PH adjustment	0.5M NaoH for PH adjustment	Final volume (mL)
1	Antigen bulk	120µg/mL	16.66	16.7	200µl	140µl	24.24
2	Aluminium phosphate	5.3mg/mL	7.18	7.2	-----	-----	-----

Batch 4: Recombinant Ag batch size:-100ml.

S. No.	Composition	Concentration	Required volume (ml)	Round off volume (ml)	0.5M acetic acid for PH adjustment	0.5M NaoH for PH adjustment	Final volume (ml)
1	Antigen bulk	120µg/ml	16.66	16.7	200µl	150µl	24.65
2	Aluminium phosphate	5.3mg/ml	7.54	7.6mg/ml	-----	-----	-----

Batch 5 Recombinant Ag batch size:-100ml.

S. No.	Composition	Concentration	Required volume (ml)	Round off volume (ml)	0.5M acetic acid for PH adjustment	0.5M NaoH for PH adjustment	Final volume (ml)
1	Antigen bulk	120µg/ml	16.66	16.7	200µl	140µl	24.84
2	Aluminium phosphate	5.3mg/ml	7.73	7.8	-----	-----	-----

Batch 6: Recombinant Ag batch size:-100ml.

S. No.	Composition	Concentration	Required volume (ml)	Round off volume (ml)	0.5M acetic acid for PH adjustment	0.5M NaoH for PH adjustment	Final volume (ml)
1	Recombinant Ag bulk	120µg/ml	10.30	10.3	500µl	300µl	18.7
2	Aluminium phosphate	5.3mg/ml	7.54	7.6	-----	-----	-----

Batch 7: Recombinant Ag batch size:-100ml.

S. No.	Composition	Concentration	Required volume (ml)	Round off volume (ml)	0.5M acetic acid for PH adjustment	0.5M NaoH for PH adjustment	Final volume (ml)
1	Recombinant Ag bulk	120µg/ml	10.3	10.3	540µl	310µl	18.75
2	Aluminium phosphate	5.3mg/ml	7.54	7.6mg/ml	-----	-----	-----

Batch. 8: Recombinant Ag batch size:-100ml.

S. No.	Composition	Concentration	Required volume (ml)	Round off volume (ml)	0.5M acetic acid for PH adjustment	0.5M NaoH for PH adjustment	Final volume (ml)
1	Recombinant Ag bulk	120µg/ml	10.30	10.3	4100µl	220µl	13.73
2	Aluminium phosphate	5.3mg/ml	2.80	2.80	-----	-----	-----

Recombinant antigen sample measure at 750 nm**Estimation of unknown protein (recombinant Ag) by Lowry method**

S. no.	Batch.no	Volume of protein (µl)	Absorbance	Practical Concentration (µg/ml)
1	1	500	0.135±0.012	5.70±0.012
2	2	500	0.109±0.012	4.56±0.012
3	3	500	0.129±0.013	5.43±0.013
4	4	500	0.136±0.012	5.74±0.012
5	5	500	0.132±0.012	5.57±0.012
6	6	500	0.515±0.013	28.6±0.013
7	7	500	0.473±0.012	26.2±0.012
8	8	500	0.237±0.012	68.73±0.012

Table 10: % adsorption of recombinant antigen.

S. No	Batch. No	% of Adsorption
1	1	85.971±0.012%
2	2	88.487±0.012%
3	3	86.937±0.013%
4	4	85.867±0.012%
5	5	86.192±0.012%
6	6	66.08±0.013%
7	7	65.750.012
8	8	61.36±0.012%

%Adsorption of recombinant Ag protein antigen on homogenized aluminium phosphate is increased than control, % adsorption of recombinant Ag protein on aluminium phosphate Control<homogenized/15min < homogenized/30min<homogenized/60min<

homogenized /90min and control> sonicated /1hr> sonicated /3hrs> Freezed and thawed.

CONCLUSION

By using aluminium phosphate and recombinant protein antigen are prepared in scheme. All the 8 batches of formulations were evaluated with physical, analytical characterization such as particle size determination, pH, and osmolality. From the above result it is evident that prepared vaccine formulations 5th batch showed 1.49µm When compared with standard. In particulars, vaccine formulation containing the less particle size of adjuvant shows the better adsorption compared with other batches formulations. A future study aspect of the size reduced aluminium phosphate containing vaccine formulation is carryout in-vivo studies to bring potential effects.

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