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Formulation development, systematic optimization of PLGA-CS-Tween 80 nanoparticles of Rivastigmine Tartrate for treatment of Alzheimer's disease

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ABSTRACT

Aimed to formulate, optimize (using CCD design) and characterize PLGA-CS-Tween 80 nanoparticles, of the drug Rivastigmine Tartrate (RT), for the treatment of Alzheimer's disease (AD). The pharmacodynamics performance of the nanoparticles (NPs) were evaluated for brain targeting and memory improvement in Aluminium chloride treated model using Morris water maze test and AchE activity analysis. PLGA-CS-Tween 80 nanoparticles prepared with emulsification-solvent evaporation method. Effect of important factors on the particle size, polydispersity, entrapment efficiency and in vitro drug release was studied using CCD design. Prepared nanoparticles showed particle size 143.0nm, polydispersity 0.164, entrapment efficiency 79.649% and in vitro drug release 69.30±0.262% (60h). FTIR studies showed there was no interaction between drug and polymers. DSC studies indicated that RT was evenly dispersed as amorphous form into NPs. SEM studies indicated that the NPs were spherical in shape and rough at surface. The stability study for six months demonstrated that the formulations were stable at refrigerator (3-5°C) condition is most suitable for storage of nanoparticles. In vivo behavioral study and AchE activity analysis demonstrated that, the rats treated with NPs showed markedly better memory retention compare to pure drug treatment. The study demonstrated that successful targeting of RT, to the brain by chitosan (CS) and Tween 80 coated NPs, have significant therapeutic potential to treat AD and potential carrier for providing sustained brain delivery of RT.

Key words: PLGA-CS-Tween 80 nanoparticles, Alzheimer's disease, Brain targeting, Rivastigmine Tartrate, Morris water maze test

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INTRODUCTION

Alzheimer's disease (AD) is one of the most frequent causes of dementia, representing 50 to 60% of all dementia cases and affecting 10 to 20% of people older than 65 years[1]. This progressive neurodegenerative disease, characterized by a global cognitive decline, behavioral and functional changes has a great impact on the ability of individuals to perform basic activities of daily living[2, 3]. Although many intellectual functions are impaired (attention, orientation, language, judgmental) the most prominent symptom of AD is represented by a progressive memory loss[4]. AD is 1.5 times more common than stroke or epilepsy and is as common as congestive heart failure[5]. It affects 15 million people worldwide. Moreover, Alzheimer's disease has a tremendous negative economic impact amounting to over $ 100 billion a year[6].

Rivastigmine Tartrate (RT) was approved by the US Food and Drug Administration for the treatment of AD. But its current therapy has many disadvantages, because of its hydrophilicity it could not enter into brain, so necessitating

frequent dosing and cholinergic side effects. The BBB represents an effective obstacle for the delivery of neuroactive agents to the central nervous system (CNS) and it makes the treatment of many CNS diseases difficult to achieve[7].

RT is a reversible cholinesterase inhibitor used for the treatment of AD. RT has been shown to improve or maintain patient's performance in three major domains: cognitive function, global function and behavior. However, limitations with its oral therapy include restricted entry into brain due to its hydrophilicity, necessitating frequent dosing and cholinergic side effects like sever bradycardia, nausea, dyspepsia, vomiting and anorexia[8].

Targeting of drugs to the brain is one of the most challenging issues for pharmaceutical research, as many hydrophilic drugs and neuropeptides are unable to cross the blood brain barrier (BBB)[9]. Many strategies have been developed to overcome this problem which includes chemical delivery systems, magnetic drug targeting or drug carrier system such as antibodies, liposomes or nanoparticles[10].

Among these, polymeric nanoparticles have recently attracted great attention as potential drug delivery systems. Due to their small size, NPs penetrate into even small capillaries and are taken up within cells, allowing an efficient drug accumulation at the targeted site over a period of days or even weeks after injection[11]. Biodegradable NPs can be successfully used for transferring the drug release profile by controlling the polymer degradation. Poly lactide–co-glycolide (PLGA) is one of the well known biodegradable carrier[12]. PLGA microparticles and nanoparticles have been extensively studied as drug carriers based upon the properties of degradability and biocompatibility[13, 14]. Moreover, PLGA matrix can be successfully encapsulated both hydrophilic and lipophilic drugs[15]. PLGA has been used for oral[16, 17] and parenteral[18, 19] delivery of drugs. However, the lack of functional groups on the surface of PLGA NPs for covalent modification has been limited the potential for surface tethering bioactive molecules including DNA , ligands[20] or vaccines[21].

Thus, various attempts for physical surface modification of PLGA NPs have been made by coating PLGA with surfactants or polymers. As such cationic surface modification based upon the electrostatic interaction with the negatively charged surface of PLGA has been suggested as a potential method to modify the surface of PLGA NPs[22]. Since the cell membrane is negatively charged, cationic particles can easily interact with the cell membrane and promote subsequent bioactivity[23].

CS has the ability to adsorb on PLGA nanoparticles, because of its cationic nature, high surface energy and microporous non-uniform surface of PLGA nanoparticles[24]. It has been established that CS is capable of opening the tight junctions of epithelial cells and it can improve the uptake of hydrophilic peptide[25]. Moreover, Tween 80 coating of CS nanoparticles was demonstrated to maximize the translocation of these nanosystems from blood to brain[26].

Therefore, we aimed to prepare PLGA NPs with double coating with CS and Tween 80, to investigate the potential of RT loaded PLGA-CS-Tween 80 nanoparticles to overcome the BBB and deliver RT to the brain. We hypothesized; indeed that the positive surface charges of these PLGA-CS-Tween 80 NPs would be favorable for their transport across the BBB by adsorptive-mediated transcytosis (AMT). AMT is a vesicular transport route of cationic substances through the BBB and, unlike receptor-mediated transcytosis (RMT), it does not require specific binding sites on cell surfaces but involves electrostatic interactions between polycationic substance and negative charges on the endothelial surfaces[27]. Hence, the present investigation was aimed at formulating nanoparticulate systems of RT that can improve brain targeting, provide sustained release, reduce dosing frequency and minimize side effects.

MATERIALS AND METHODS

Materials

Rivastigmine Tartrate (RT) was received as a gift sample from Sparc (Vadodara, India). Poly (D,L-Lactide-co Glycolide) (PLGA) (50:50) was purchased from Durate Corporation (Birmingham AL, USA) and was used without further purification. Polyvinyl alcohol (PVA), Tween 80, and chitosan (CS) were purchased from S D Fine Chemicals (Mumbai, India). All other chemicals and reagents used in this study were of analytical grade and used as received.

Methodology

Drug polymer compatibility study

Compatibility studies were carried out to know the possible interactions between RT and polymers used in the formulation. Physical mixtures of drug and polymers were prepared to study the compatibility. Drug polymer compatibility studies were carried out using FTIR spectroscopy (ATR technique). IR spectra of drug and along with polymers were seen in between 600-4000 cm^{-1}.

PLGA-CS-Tween 80 NPs preparation method

PLGA-CS-Tween 80 NPs have been prepared by nanoprecipitation method, which was an emulsification - solvent evaporation method. The polymer PLGA (85mg) has been added to 3ml of acetone and was dissolved with magnetic stirrer (By Remi Equipment Pvt Ltd, Bangalore, India). In above organic solution, RT (4.3mg) was added and allowed to dissolve. This solution has been added with 23G needle to an aqueous phase of PVA (1% w/v) to form O/W emulsion. Once all the drug/polymer mixture has been added to PVA solution, the contents were allowed to mix for 5 mins with homogenizer (T25 digital Ultra turax by IKA, Germany) at 18000 RPM. The resulting suspension was sonicated for 10 mins at 45% amplitude with a ultrasonic probe (By Dakshin, Bombay), immediately after sonication the emulsion was poured into excess of aqueous phase of PVA (1%), CS (0.25 %w/v) and Tween 80 (0.5 %v/v) for solvent evaporation under rapid stirring and coating of chitosan on PLGA-CS-Tween 80 NPs with a magnetic stirrer for 24h. Then the nanoparticles were collected by centrifugation and washed 3 times with distilled water. Finally, they were resuspended into 2 ml of cryoprotectant solution (Sucrose (2% w/w), dried with lyophilizer (Eqsquire Biotech, Germany) and stored at 4°C[28].

Experimental design for optimization of formulation

Preliminary experiments indicated that variables such as an amount of polymer PLGA, CS concentration and volume of acetone were the main factors that affected the particle size, polydispersity, and percentage drug release and encapsulation efficiency of the PLGA-CS-Tween 80 NPs. A CCD model has been used to statistically optimize the formulation parameters and evaluate the main effects, interaction effects and quadratic effects of the formulation factors on the particle size (Y_1), polydispersity (Y_2), encapsulation efficiency (Y_3) and percentage drug release (Y_4) of NPs. Details of the design are listed in Table 1. For each factor, the experimental range has been selected on the basis of the results of preliminary experiments and the feasibility of preparing the PLGA-CS-Tween 80 NPs at the extreme values. The value range of the variables was: amount of PLGA (X1): 50–120 mg, CS concentration (X2): 0.2–0.3%, and volume of acetone (X3): 2.0–5.0 ml. The design consists of 15 runs (8 factorial points, 6 star points and 1 center point) and 5 replicated runs (center points) yielding 20 experiments in total (Table 2). The purpose of replication was to estimate experimental error and increase the precision. Each experimental run has been repeated thrice. The star points represent extreme values (low and high) for each factor in the design and allow for estimation of second-order effects. The star points are at some distance, alpha, from the center based on the properties desired for the design and the number of factors in the design. Alpha in coded units is the axial distance from the center points and makes the design rotatable. A rotatable design provides equally good predictions at points equally distant from the center, a very desirable property for Response Surface Methodology[29].

Table 1: Relationship between factors and responses used for PLGA-CS-Tween 80 formulation

Factors (Independent Variables)	Levels of variables					Responses (Dependent Variables)
	-α	**-1**	**0**	**1**	**+ α**	Y_1= Particle Size (nm)
X_1= Amount of PLGA (mg)	26.14	50	85	120	143.86	Y_2= Polydispersity
X_2=Amount of coating agent (% w/v)	0.17	0.2	0.25	0.3	0.33	Y_3=Encapsulation Efficiency (%)
X_3= Acetone (ml)	0.98	2	3.5	5.0	6.02	Y_4= Drug release (%)

Determination of encapsulation efficiency and drug loading

The amount of 10 ml of RT loaded PLGA-CS-Tween 80 NPs suspension was carefully transferred to centrifugation tube. The nanoparticles in the form of sediment were separated from the solution by ultracentrifugation at 15,000 rpm at 4°C for 40 mins. The supernatant was carefully decanted and analyzed by UV spectrophotometer for RT.

Table 2: Composition of various PLGA-CS-Tween 80 nanoparticle formulations prepared as per the experimental design

Formulation Batch	Coating agent concentration (% w/v)	Polymer concentration (mg)	Volume of internal phase (Organic phase) (ml)
1	0.2	50	2
2	0.2	120	2
3	0.3	50	2
4	0.3	120	2
5	0.2	50	5
6	0.2	120	5
7	0.3	50	5
8	0.3	120	5
9	0.25	26.14	3.5
10	0.25	143.86	3.5
11	0.17	85	3.5
12	0.33	85	3.5
13	0.25	85	0.98
14	0.25	85	6.02
15	0.25	85	3.5
16	0.25	85	3.5
17	0.25	85	3.5
18	0.25	85	3.5
19	0.25	85	3.5
20	0.25	85	3.5

The % entrapment efficiency and % drug loading were calculated using equation as given below[30]:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} * 100 \quad (1)$$

$$\text{Drug loading} = \frac{\text{Total drug} - \text{Free drug}}{\text{Nanoparticles weight}} \quad (2)$$

Particle size analysis and polydispersity

Particle size analysis and polydispersity nanoparticles were determined by particle size analyzer (Brookhaven Instrument Corporation, NY). Lyophilized nanoparticles were dispersed in double distilled water and analyzed in three readings per nanoparticles sample. The polydispersity was also calculated based on the volumetric distribution of particles[29].

Zeta Potential determination

Zeta Potential was measured by using zeta size analyzer (Brookhaven instrument Corporation, NY). About 1.5 ml of the sample was placed in the cuvette and electrode was inserted carefully and click “track” button and the Zeta PALS meter instantly calculates and displays the colloidal’s Zeta potential (or electrophoretic mobility)[31].

***In vitro* drug release**

The *in vitro* drug release profile of PLGA-CS-Tween 80 NPs formulation has been studied using a dialysis bag. Approximately 1 ml of nanoparticle suspension (Corresponding 6 mg of RT) were taken into a dialysis bag (molecular weight cut-off, 12 KDa, Himedia, India) and placed in a beaker containing 150 ml of phosphate buffer saline (PBS) (pH 7.4). Then the beaker was placed over a magnetic stirrer and the temperature of the assembly was maintained at 37±1°C throughout the study. Samples (5ml) were withdrawn at definite time intervals (1, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48 and 60h) and replaced with equal amounts of fresh buffer. The samples were analyzed for drug concentration by UV-VIS spectrophotometer at 264 nm[32].

Stability studies

A study was carried out to assess the stability of PLGA-CS-Tween 80 nanoparticles of drug RT. The samples were stored in room temperature (15-20°C), refrigerator (3-5°C) and 37°C (RH=75%) over a period of 6 months. Samples were periodically withdrawn at monthly intervals for six months and examined for their drug release as well as any changes in physical appearance[33].

Scanning electron microscopy (SEM)
The surface morphology of the formulated nanoparticles was measured by scanning electron microscopy (SEM) (EM-LEO 435VP, Carl Zeiss SMT Inc., NY) equipped with 15 kv, SE detector with a collector bias of 300 V. The lyophilized samples were carefully mounted on an aluminum stub using a double stick carbon tape. Samples were then introduced into an automated sputter coated and coated with a very thin film of gold before scanning the sample under SEM[29].

Differential Scanning calorimeter (DSC) study:
The physical state of RT entrapped in the NPs was characterized by Differential Scanning Calorimetry (DSC - 60, Shimadzu, Japan). Each sample was sealed in standard aluminium pans with lids and purged with air at a flow rate of 40 ml/min. A temperature ramp speed was set at 20 °C/min, and the heat flow was recorded in the range 30-300°C under inert nitrogen atmosphere. Thermograms were taken for RT and PLGA-CS-Tween 80 NPs[34].

Experimental animals
The subjects used in this research work were 40 male and female adult Wistar rats. Wister rats, 200-220 g, procured from, Bioneeds, Bangalore, were used for investigation. The Institutional Animal Ethical Committee approved the protocol. They were kept in the animal house of Department of Pharmacology, Acharya & B M Reddy college of pharmacy (Bangalore, India) for seven weeks (normal) standard environmental condition (relative humidity of 60%, 12h-12h light-dark cycle) with sufficient food, water and under a good ventilation in order for the animals (Wistar rats) to acclimatized. (Registration number for Institutional Animal Ethics Committee (IAEC)-997/c/06/CPCSEA)

Experimental design for animal study
Drug and treatment schedule
Aluminium chloride solution and the optimized formulation (PLGA-CS-Tween 80 Nps) were freshly prepared at the beginning of each experiment. For oral administration, Aluminium chloride was dissolved in distilled water and for intraperitoneal administration, prepared nanoparticles were dispersed in normal saline solution (0.9 %w/v). Dose calculated equivalent to 1.5 mg/kg of RT for standard as well as for nanoparticles formulations. Animals were divided into four groups:
Group 1: Normal control
Group 2: Positive control (Aluminum chloride 100 mg/kg/day p.o)
Group 3: Standard (RT 1.5 mg/kg IP in saline + Aluminum chloride 100 mg/kg/day p.o)
Group 4: PLGA-CS-Tween 80 NPs treated (NPs IP in saline + Aluminum chloride 100 mg/kg/day p.o)

Spatial navigation task
The acquisition and retention of a spatial navigation task was evaluated using Morris water maze[28]. Rats were trained to swim to a visible platform in a circular water pool (100cm in diameter and 45cm deep) located in test room. A hidden circular platform (20cm height, 12cm in diameter and 2cm below the water surface, fixed position). The pool was conceptually divided into four equal quadrants. The rats received a training session considering of two trials per day at 1 min interval for 5 days prior to starting dose regimen. In the first test, rats were placed on the platform for 20s, then the rats were placed in the water facing the pool wall at one of the 4 quadrants at a different place every day, and allowed to swim for a maximum of 90s to find the hidden platform were it was allowed to stay for 10s. If rat did not find the platform in 90s, it was placed on the platform by hand and remained there for 10s. The time to reach the platform (escape latency) was measured with a stopwatch. The escape latency was studied on the day 0, 7, 14, 21, 28, 35 and 42th[35].

Activity of AchE
After the last Morris water maze test, rats were killed by decapitation, the frontal cortex and hippocampus were removed and homogenized in 5% of sodium phosphate buffer (75 mM, pH 7.4, 4°C), respectively. For the assay of AchE activity, a 4ml reaction mixture that contained acetylthiocholine iodide (0.3 mM), sodium phosphate buffer (0.1 mM Ph 7.4) 1 ml and homogenate 0.1-0.2ml was incubated at 37°C for 8 min. The reaction was terminated by adding 1 ml of 3% sodium lauryl sulfate, then 1 ml of 0.2% 5, 5'-dithiobis (2-nitrobenzoic acid) to produce the yellow anion of 5-thio-2 nitrobenzoic acid. The color intensity was measured spectrophotometrically at 440 nm. All samples were assayed in duplicate. AchE activity was calculated as optical density (OD) value/mg protein for AchE[35]. Protein concentrations were determined with the Coomassie blue protein-binding method using bovine serum albumin as standard[36].

RESULTS AND DISCUSSION

Drug polymer compatibility study

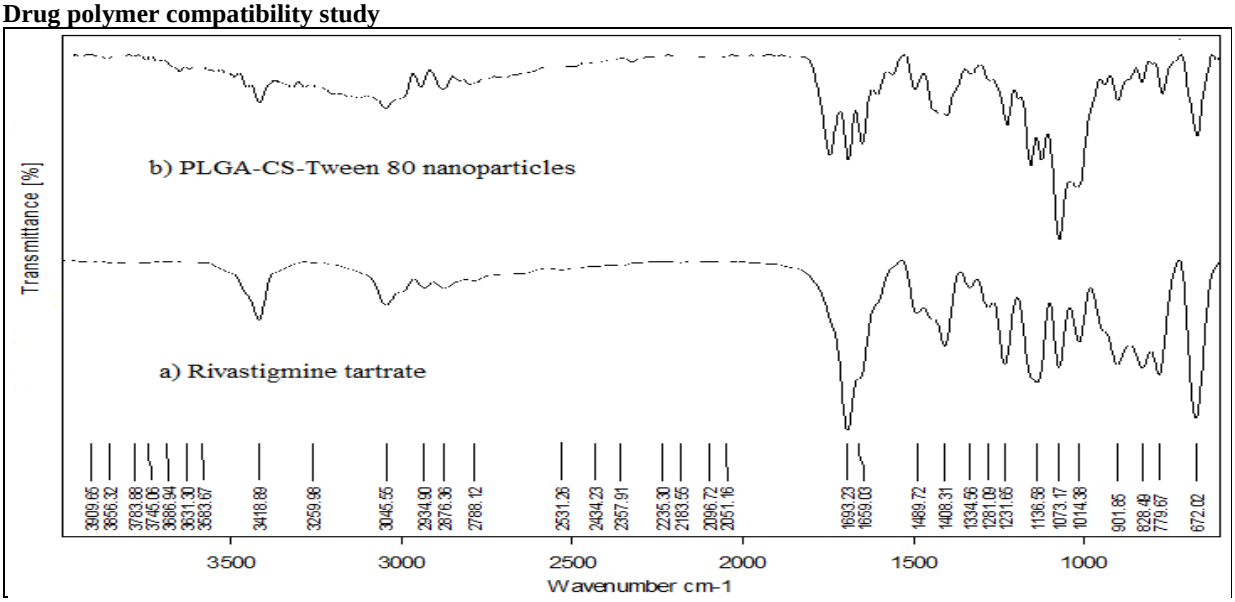

Fig. 1: FTIR spectra of a) Rivastigmine Tartrate and b) PLGA-CS-Tween 80 NPs

The Fourier Transform Infrared (FTIR) spectra of the RT and PLGA-CS-Tween 80 NPs are shown in Fig. 1a) and 1b), respectively. Fig. 1a) shows C-H (str) at 2934 cm^{-1}, C-N (str) at 2876 cm^{-1}, C=O (str) at 1654 cm^{-1} and C=C (str) at 3045 cm^{-1}. The Fig. 1b) shows the FTIR spectra of PLGA-CS-Tween 80 NPs and it shows all the characteristic peaks C-H (str) at 2945 cm^{-1}, C-N (str) at 2880 cm^{-1}, C=O (str) at 1653 cm^{-1} and C=C (str) at 3049 cm^{-1}, which indicates there was no interaction between drug and polymers and they are compatible with each other.

Table 3: Results of particle size, polydispersity, % entrapment efficiency and *in vitro* drug release of PLGA-CS-Tween 80 NPs formulations prepared as per the experimental design

Formulation	Entrapment efficiency (%)	Drug Loading (mg)	Particle Size (nm)	Polydispersity	Drug release (%)
F1	54.675	2.35	127.4	0.162	43.856±0.391
F2	47.159	2.03	43.07	0.199	36.889±0.386
F3	56.749	2.44	109.43	0.155	44.322±0.262
F4	48.648	2.09	146.8	0.175	37.05±0.825
F5	62.235	2.68	89.03	0.138	53.169±0.533
F6	63.147	2.72	136.7	0.149	53.819±0.533
F7	62.895	2.70	101.79	0.144	53.339±0.384
F8	61.483	2.64	141.51	0.163	52.967±0.397
F9	46.988	2.02	135.4	0.169	36.55±0.241
F10	45.716	1.97	200.88	0.26	34.133±0.152
F11	69.748	2.99	160.8	0.242	56.186±0.254
F12	67.423	2.89	151.9	0.229	55.844±0.650
F13	44.856	1.93	178.9	0.127	33.614±0.521
F14	49.586	2.13	89.03	0.124	36.515±0.254
F15	64.019	2.75	114.2	0.166	54.292±0.152
F16	71.640	3.08	123.4	0.189	57.906±0.385
F17	75.428	3.24	122.1	0.165	60.142±0.263
F18	77.815	3.34	120.7	0.186	62.117±0.390
F19	70.183	3.02	131.4	0.161	57.719±0.296
F20	65.836	2.83	121.2	0.257	55.536±0.406

In vitro drug release

The *in vitro* drug release studies were carried out for 60h. Drug release studies of drug loaded NPs showed biphasic release profile. The initial fast release rate may be due to smaller particle size of NPs, which is associated with smaller diffusion path, so drug accessible to the solid/dissolution medium inter phase can diffuse easily to the

surface. Thereafter the release rate decreased, which reflects the release of drug entrapped in the polymer. The release rate in the second phase is assumed to be controlled by diffusion rate of drug across the polymer matrix. PLGA-CS-Tween 80 NPs release rate is dependent upon the molecular weight and lactide content of the polymer. The release rate reduces as the molecular rate and the lactide content of the polymer increases. An increase in the concentration of CS (0.2-0.3% w/v) initially showed no effect on the drug release. After optimal concentration, drug release started decreasing. A trend of decrease in drug release is due to the coating of polymer on the surface of nanoparticles. When the data obtained from drug release have been studied with zero order, first order, Higuchi model and Korsmeyer Peppas model, they were found to be following Higuchi model. Korsmeyer Peppas model showed *n* (diffusion exponent) < 0.5, which suggests that the release pattern of drug is following first order, fickian diffusion kinetics/anomalous transport for PLGA-CS-Tween 80 NPs.

Exploration of key factors effects using response surface methodology (RSM)

In all, nine coefficients (β_0-β_8) were calculated, with β_0 representing the intercept, and β_1-β_8 representing coefficient of various quadratic and interaction terms:

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_1X_2+\beta_5X_1X_3+\beta_6X_2X_3+\beta_7X_1^2+\beta_8X_2^2+ \beta_9X_3^2 \quad (3)$$

Influence of variables on particle size

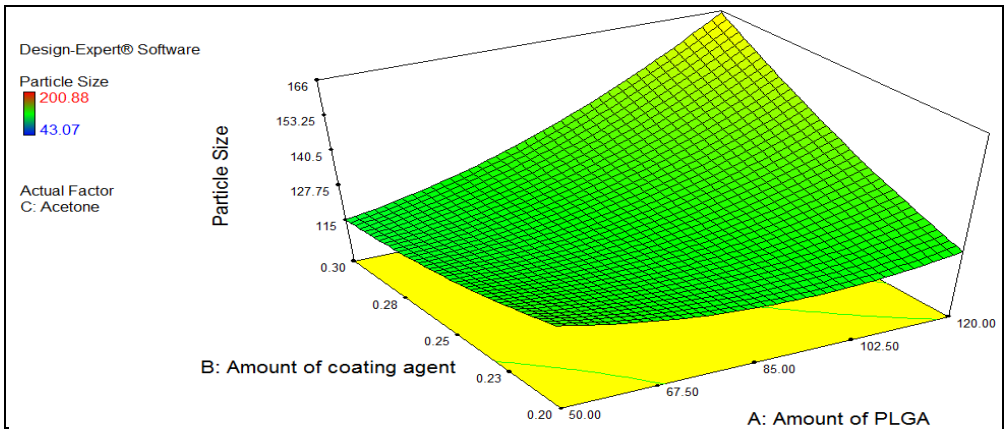

Fig. 2: Response surface plot showing the influence of amount of PLGA (X_1), amount of coating agent (X_2) and acetone concentration (X_3) on particle size of nanoparticles Y_1 (nm)

$$Y_1 = +123.55 +11.02\ X_1 +6.47X_2 -7.97\ X_3 +14.22\ X_1\ X_2 +16.79X_1\ X_3 -8.52X_2\ X_3 +7.24\ X_1^2 +3.07\ X_2^2 -4.\ 84X_3^2 \quad (4)$$

A positive value in regression equation for a response represent an effect that favors the optimization (synergistic effect), while a negative value indicates an inverse relationship (antagonist effect) between the factors and responses.

Particle size is a critical factor for nanoparticles based drug delivery system. It is the one of the factors, which controls the kinetics of drug release. The particle size values for 20 batches show a wide variation in response i.e., the response range from a minimum 43.07 to a maximum 200.88nm (Values are given into Table 3). The Fig. 2 shows the response surface plot obtained for the interaction between the PLGA concentration and amount of coating agent at constant acetone value. The positive sign for the coefficient of PLGA concentration and coating agent concentration showed that particle size increases with increase in the concentration of PLGA and coating agent concentration. The negative sign of coefficient of acetone amount showed that particles size decreased with increase in concentration of amount of acetone. An increase of mean particle size has been observed, when increase in PLGA concentration with 1% amount of PVA. The particle size does not change much initially with increase in solvent volume, but started decreasing with further increase in volume of acetone. An increase in the particle size has been observed with increase in the coating agent concentration.

Influence of variables on polydispersity

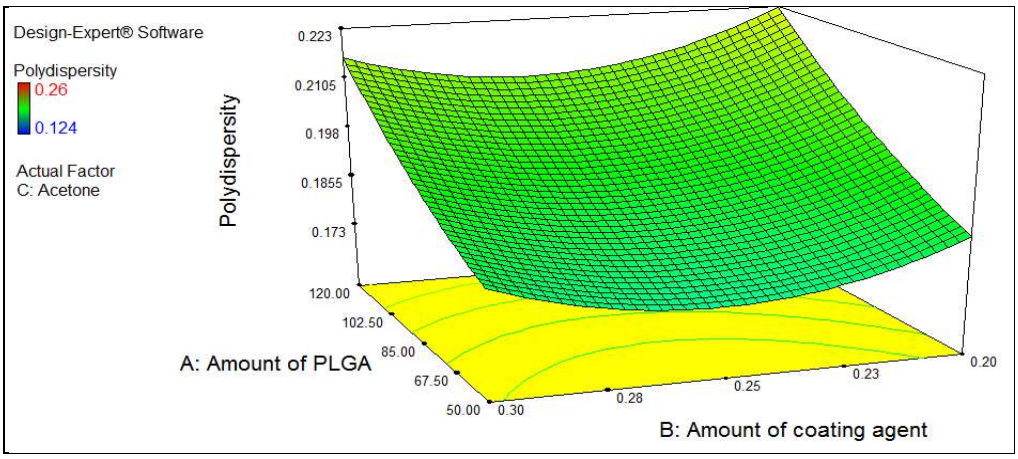

Fig. 3: Response surface plot showing the influence of amount of PLGA (X_1), amount of coating agent (X_2) and acetone concentration (X_3) on polydispersity of nanoparticles Y_2

$$Y_2=+0.32+1.14X_1+1.39X_2-8.32X_3+6.428X_1X_2–6.42X_1X_3-0.085X_2X_3-1.54X_1^2+2.21X_2^2+4.61X_3^2 \quad (5)$$

After nanoparticle formation, the size population of nanoparticle frequently follows a multimodal distribution. The poly dispersity index is very important parameter, which is used to describe variation in particle size in a sample of particle. When polydispersity index is close to 1, the size range becomes wide. A desire optimal value of polydispersity index is closer to zero. The polydispersity values for 20 batches show a wide variation in response i.e., the response range from a minimum 0.124 to a maximum 0.260 (Values are given into Table 3). The Fig. 3 shows the response surface plot obtained from the interaction between the PLGA concentration and amount of coating agent at constant acetone value. The positive sign for the coefficient of PLGA concentration and coating agent concentration showed that polydispersity increases with increase in the concentration of PLGA and coating agent concentration. The negative sign of coefficient of acetone amount showed that polydispersity decreased with increase in concentration of amount of acetone. It has been observed that the polydispersity index increases with increase in the amount of polymer increases and decrease with the amount of organic solvent acetone. The polydispersity did not increase much initially with increase in coating agent but it started increasing with further increase in the concentration of coating agent.

Influence of variables on % Entrapment Efficiency

To transport the drug to the specific site and increase its resident time, there is a need of high entrapment efficiency. PLGA molecules have ability to entrap both hydrophilic and lipophilic drug and target them to specific targeted site. The % Entrapment efficiency values for 20 batches show a wide variation in response i.e., the response range from a minimum 44.856 to a maximum 77.815 % (Values are given into Table 3). The Fig. 4 shows the response surface plot obtained from the interaction between the PLGA concentration and amount of coating agent at constant acetone value. The positive sign for the coefficient of PLGA concentration and coating agent concentration showed that % entrapment efficiency increases with increase in the concentration of PLGA and coating agent concentration. The positive sign of coefficient of acetone amount showed that % entrapment efficiency increased with increase in concentration of amount of acetone. As per the results showed in Table 3, drug loading increases with increase in the concentration of PLGA and decreases after optimum polymer concentration for maximum loading.

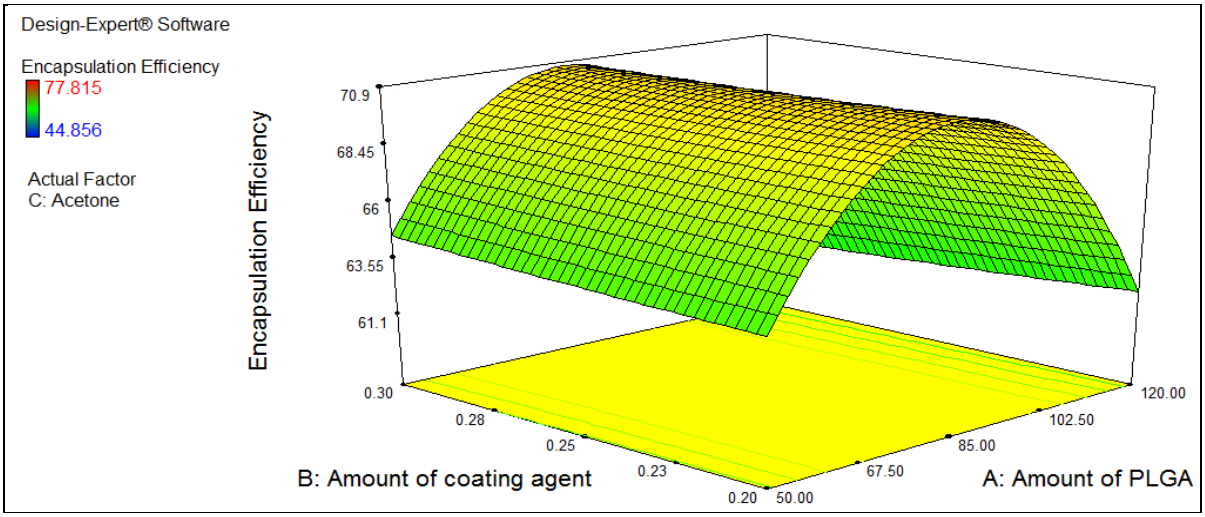

Fig. 4: Response surface plot showing the influence of amount of PLGA (X_1), amount of coating agent (X_2) and acetone concentration (X_3) on % entrapment efficiency of nanoparticles Y_3 (%)

$$Y_3 = +70.69 +1.35X_1 +0.11X_2 +3.71X_3 +0.39X_1X_2 +1.91X_1X_3 +0.55X_2X_3 +7.79X_1^2 +0.068X_2^2 +7.49X_3^2 \quad (6)$$

Influence of variables on *in vitro* drug release

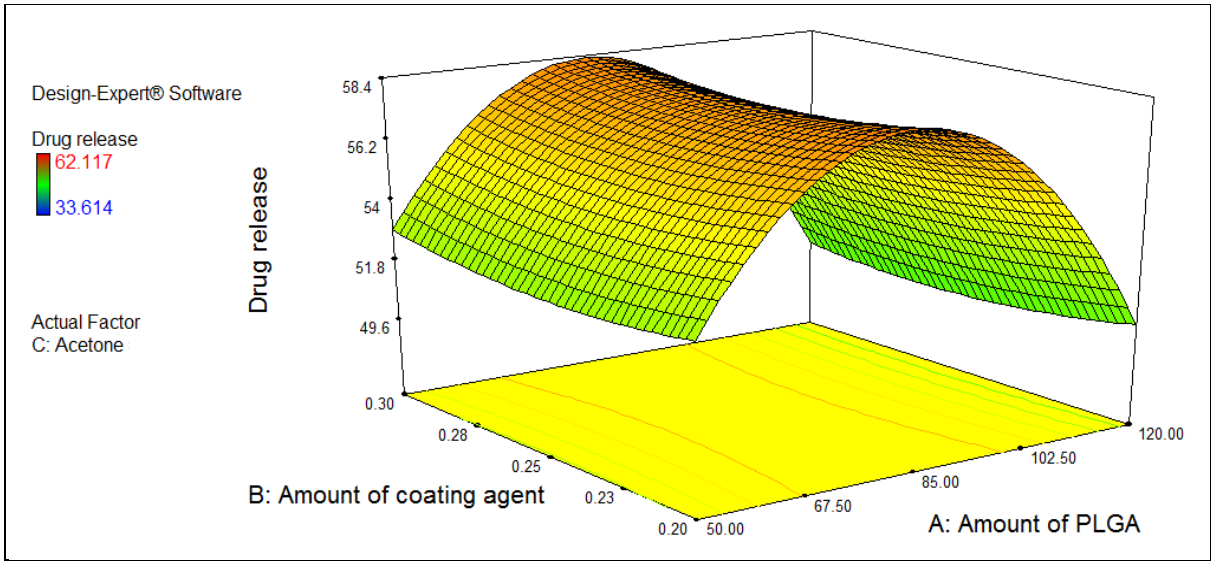

Fig. 5: Response surface plot showing the influence of amount of PLGA (X_1), amount of coating agent (X_2) and acetone concentration (X_3) on % *in vitro* drug release of nanoparticles Y_4 (%)

$$Y_4 = +57.77 +1.32X_1 -0.046X_2 +4.10X_3 -0.17X_1X_2 +1.81X_1X_3 -0.16X_2X_3 +6.78X_1^2 +0.53X_2^2 +6.88X_3^2 \quad (7)$$

The *in vitro* drug release values for 20 batches show a wide variation in response i.e., the response range from a minimum 33.614 to a maximum 62.117 % (Values are given into Table 3). The Fig. 5 shows the response surface plot obtained for the interaction between the PLGA concentration and amount of coating agent at constant acetone value. The positive sign for the coefficient of PLGA concentration and acetone concentration showed that % cumulative drug release increases with increase in the concentration of PLGA and acetone concentration. The negative of coefficient of coating agent amount showed that % cumulative drug release decreased with increase in concentration of coating agent amount.

Optimized formulation was selected based on following criteria:

Particle size < 200, Polydispersity- minimum, % entrapment efficiency- maximum and *in vitro* drug release- maximum

Based on this research, the formulation containing 85 mg of PLGA, 0.25% of CS and 3 ml of acetone was selected as optimized formulation. The selection of the optimized formulation was based on minimization of particle size below 200 nm to facilitate brain targeting[37], minimization of polydispersity, maximization of entrapment efficiency and maximization of *in vitro* drug release. The optimized formulation exhibited particle size 145.07nm, polydispersity 0.164, entrapment efficiency 79.649 % and *in vitro* drug release 69.302±0.262 % (60h).

Stability study

The optimized formulations was kept for stability studies at room temperature (15-20°C), refrigerator (3-5°C) and 37°C (RH=75%) over a period of 6 months. Samples have been evaluated at 0 to 6 each month for their drug release as well as any changes in physical appearance. The results of the stability study showed that there was no significant change in the drug release study and appearance of the optimized formulation, stored at refrigerator (3-5°C). While room temperature (15-20°C) and 37°C (RH=75%) showed that there was agglomeration of particles present and drug release decreased significantly. Thus, it can be concluded that refrigerator (3-5°C) condition is the most suitable for storage of optimized PLGA-CS-Tweeen 80 NPs.

Scanning electron microscopy (SEM)

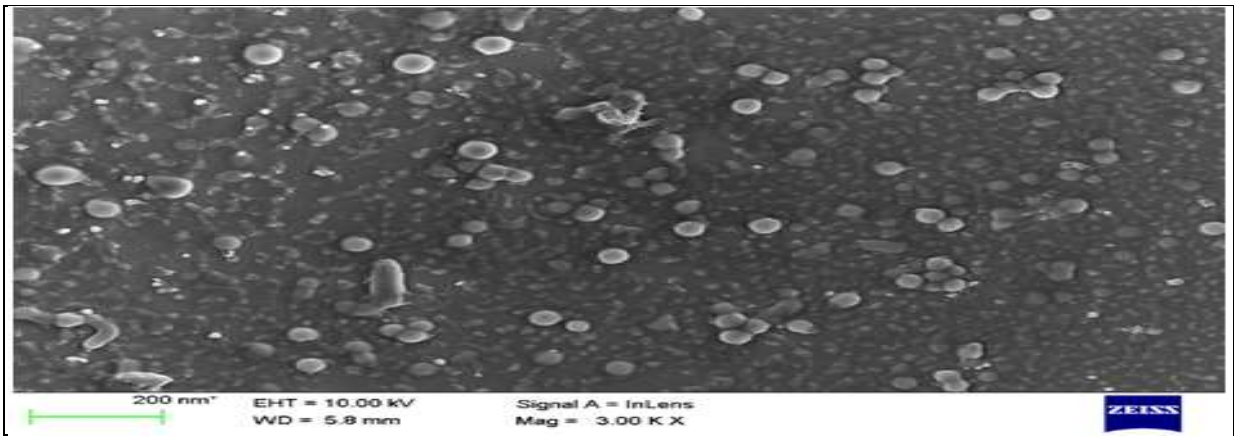

Fig. 6: Nanoparticle morphology of freeze dried PLGA-CS-Tween 80 NPs as studied by SEM

Surface morphology of the specimens has been determined by using the SEM (EM-LEO 435VP, Carl Zeiss SMT Inc., NY). The SEM photograph of optimized formulation has been shown in Fig. 6**.** It has been observed that the optimized PLGA-CS-Tween 80 NPs have rough surface and spherical shape.

Differential scanning calorimetry

Differential scanning calorimetry study gives information regarding the physical properties like crystalline or amorphous nature of the sample. The DSC thermogram of RT (Fig. 7a) shows an exothermic peak at 127.16°C corresponding to its melting temperature. However, no sharp endotherm was seen at 127.16°C (Fig. 7b) (Optimized formulation). This shows that crystallinity of the drug has been reduced significantly in nanoparticles. Hence it could be concluded that the drug was present in the optimized formulation, as amorphous phase and may have been homogeneously dispersed in the PLGA matrix.

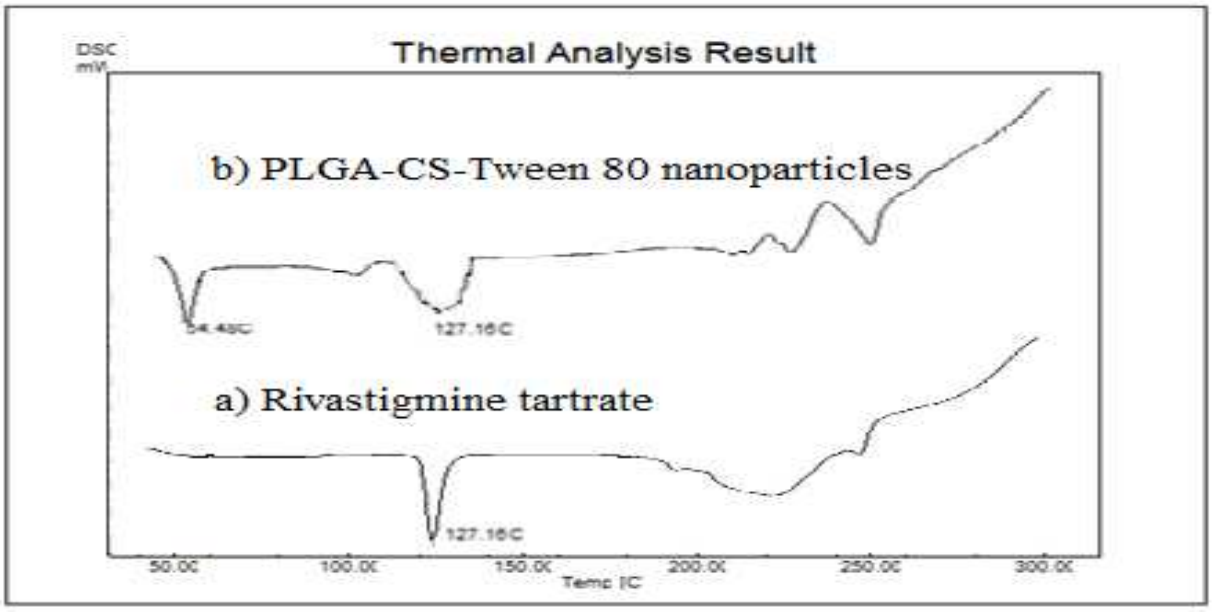

Fig.7: DSC thermogram of a) Rivastigmine Tartrate and b) PLGA-CS-Tween 80 NPs

Spatial navigation task (Morris water maze)

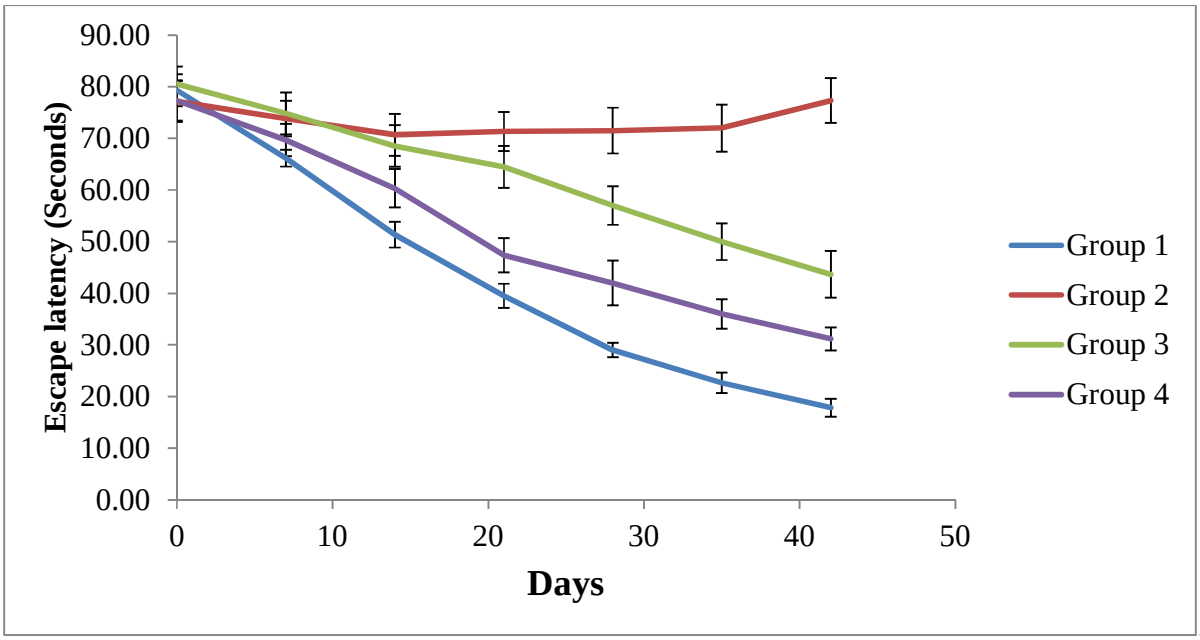

Fig. 8: Comparison of escape latency in various groups of rats using spatial navigation task. (Morris water maze) The values are depicted as mean ± SD (*n* = 6)

In spatial navigation task, the normal, standard and formulations treated groups of rat quickly learned to swim directly to the platform in the Morris water maze. Aluminum chloride treated rats showed an initial increase in escape latency, which declined during following weeks of Morris water maze test. The rats that received pure drug along with aluminum chloride showed slight improvement in their behavior. In contrast, the rats treat with formulations with Aluminum chloride, showed significantly decrease in time taken to reach platform as compared

with aluminum chloride treated rats. Groups arranged according to significance in time taken to reach platform: Group 1< Group 4< Group 3< Group 2

Activity of AchE

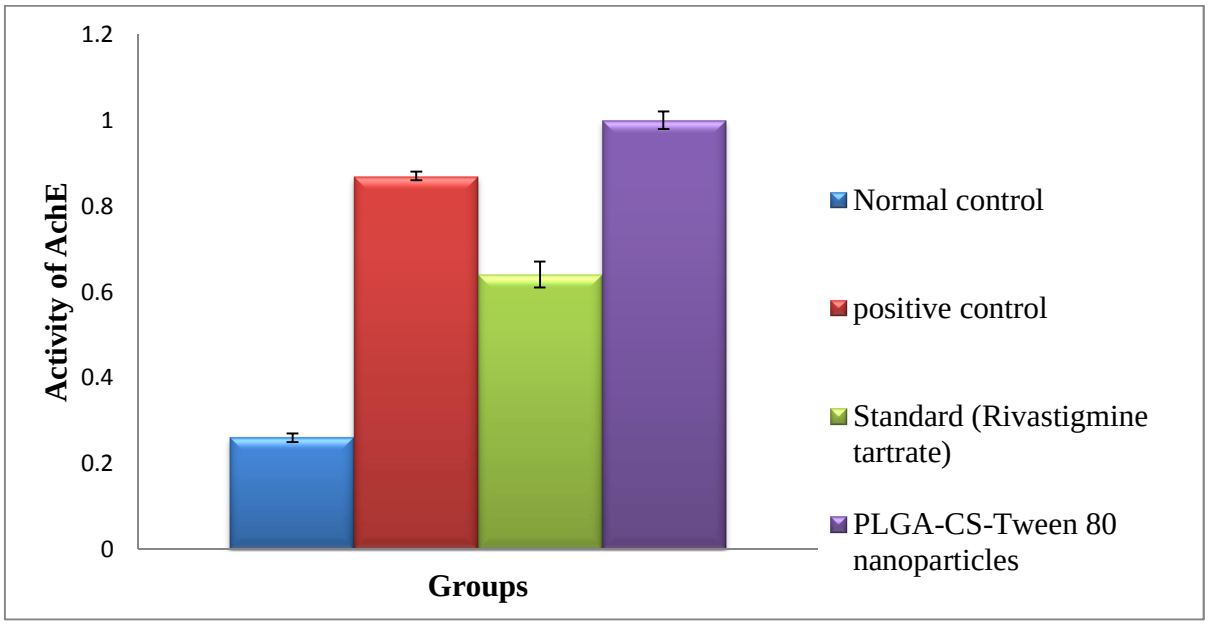

Fig. 9: Activity of AchE in brain region. Data represent means ± SEM (n = 6 animals each group) expressed as OD values/mg protein for activity of AchE

Currently, the cholinergic deficiency is considered to be one of the main reasons of dementia and cognitive deficits in AD. Based on this hypothesis, many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity through the cholinomimetic use of AchE inhibitors, Ach precursors and cholinergic antagonists. In the present work, after Morris water maze test, animals sacrificed and their brains removed, and evaluated for AchE activity was expressed as OD value/mg protein. After comparison with normal, standard and formulations treated groups, AchE activity in the positive control group was more, which indicates that an animal model was built successfully. When compared with standard group PLGA-CS-Tween 80 treated group demonstrated less AchE activity. It has been reported that the concentration of Ach rose with reduction of AchE activity under normal conditions, but both Ach and AchE concentration reduced under AD condition. The results showed that, compare to free RT, formulation treated group, inhibits AchE effectively, and the reduction of AchE concentration results in slower degradation of Ach. Therefore, the concentration of Ach rose in rat's brain, and cholinergic system could reach a new equilibrium between Ach and AchE, which improved memory and cognitive deficits of rats under AD.

CONCLUSION

RT loaded PLGA-CS-Tween 80 NPs were prepared using emulsification - solvent evaporation method, with narrow size distribution (<200 nm), higher entrapment efficiency and percentage drug release. The FTIR and DSC study demonstrated there was no interaction between drug and polymers and are compatible with each other. This study using CCD design, showed response of independent factors on dependent factors, with the help of response surface plots and polynomial equations. Using CCD design we could achieve higher entrapment efficiency and drug release with smaller particle size and polydispersity, with less number of experiments and could predict the values for particle size, polydispersity, entrapment efficiency and *in vitro* drug release. *In vitro* dug release found to follow biphasic drug release with Higuchi model. The SEM study showed that particles were spherical in shape with rough surface. The stability study for six months demonstrated that the formulations were stable at refrigerator (3-5°C) condition is the most suitable for storage of optimized PLGA-CS-Tweeen 80 nanoparticles. Administration of PLGA-CS-Tween 80 optimized formulation in Aluminium chloride treated animals results in noticeable improvement in learning and memory capacity and it antagonized the toxic effect of Aluminium chloride by

reduction in escape latency, compared to Standard drug solution treated animals. These results indicated that PLGA-CS-Tween 80 nanoparticles resulted in improvement of memory and learning efficiency into Aluminium chloride treated model of Alzheimer's disease. The results of AchE activity study showed that, compare to free RT, formulation treated group, inhibits AchE effectively, and the reduction of AchE concentration results in slower degradation of Ach. Therefore, the concentration of Ach rose in rat's brain, and cholinergic system could reach a new equilibrium between Ach and AchE, which improved memory and cognitive deficits of rats under AD. They could be effective in brain targeting and sustained release of RT for prolong period and could be a significant improvement for treating Alzheimer's disease.

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