APPLICATION OF DESIGN THINKING APPROACH FOR THE DEVELOPMENT OF PEG-4000 COATED CHITOSAN NANOPARTICLES LOADEDWITH GALANTAMINE HYDROBROMIDE FOR IMPROVEDTREATMENT OF NEURODEGENERATIVE DISORDERS

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Abstract

Alzheimer's disease is a neurodegenerative disorder of old age, that severely affect the memory, and disrupts their social and behavioral skills and the drug treatment for this disease is very limited because of the restriction of blood brain barrier. This research work is aimed to develop apolymeric nanoparticle coated with PEG 4000 to enhance the permeability of Galantamine Hydrobromide across the blood brain barrier for improvement in the therapeutic efficacy. Galantamine Hydrobromide loaded Chitosan nanoparticles coated with PEG 4000 with varying concentration of drug polymer ratio were prepared by ionic gelation method using sodium tri poly phosphate. The prepared nanoparticles were subjected to evaluation of various physico- chemical properties like transmission electron microscopy, determination of surface charge and particle size using zetasizer, drug content, entrapment efficiency and *invitro* diffusion studies. The batch of the nanoformulation

with the ideal characters was subjected to *invivo* studies for determination of anti-amnesic effect in scopolamine induced amnesic mice model. The formulation, PEG-CNGH-1 indicated morphologically spherical nanoparticles with a particle size of 68.2±12.4 nm and a positive surface charge of +44.5±0.4 mV.The behavioural studiesand the acetylcholine esterase levels revealed that the PEG-CNGH-1 had improved the therapeutic effect of galatamine hydrobromide in polymeric nanoparticle formulation.

Keywords:

Nanoparticles, ionic gelation, Alzheimer's disease, Galantamine Hydrobromide, Acetylcholine esterase

Introduction

Empathizing the elderly patients with Alzheimer's disease, which is a neurodegenerative disorder that severely affect the memory, and disrupts their social and behavioral skills[1]. AD is characterized by the accumulation of the beta-amyloid peptide and the hyperphosphorylation of the tau protein in the brain. The most widely accepted hypothesis on the etiopathogenesis of this disease proposes that the aggregates of the beta-amyloid (βA) peptide are the main triggers of tau hyperphosphorylation and the subsequent degeneration of affected neurons. In support of this view, fibrillar aggregates of synthetic βA peptide induce tau hyperphosphorylation and cell death in cultured neurons[2].

Galantamine Hydrobromide (GH) is a competitive selective acetyl cholinesterase inhibitor[[3, 4] used to treat mild to moderate Alzheimer's disease. GH inhibits the action of acetyl cholinesterase (AChE), the enzyme that is responsible for the destruction of acetylcholine in vivo, one of the neurotransmitters in the brain. Reduced levels of acetylcholine in the brain were reported in AD and hence by blocking AChE activity, GH increases the concentration of acetylcholine in the brain. It is an approved drug for the treatment of AD in more than 70 countries. The extensive pharmacokinetic studies of this drug carried out in animals reveals, the very poor distribution in brain. To define the problem in brief the distribution of the GH in brain is lesser.

The idea of the present work is to develop PEG 4000 coated chitosan nanoparticles containing GH (PEG-CNGH) for enhancement of the distribution of the AD drug GH in brain.

A prototype of the PEG 4000 coated chitosan nanoparticles will be prepared and to test the invitro properties and to assess the improvement in the overall therapeutic activity through the study of behavioral parameters in scopolamine induced neurodegenerative animal model.

1. Materials and Methods

Chemicals

GH was received as a gift sample from Ranbaxy, Mumbai and Chitosan was purchased from Sigma Aldrich, USA. All other chemicals, reagents and solvents were of analytical grade.

Animals

Male Swiss albino mice, 8-10 week's old and weighing 22-25 g were in-breed in the animal house of School of Pharmaceutical Sciences, Vels University, Chennai. They were kept under standard

conditions of temperature and humidity in the Animal House Facility and provided withstandard pellet feed (Lipton, India) and water *ad libitum*. The protocols adopted for the animal

studies in this work were duly approved by Institutional Animal Ethics Committee (IAEC) of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Compatibility studies

The compatibility of the drug with the polymer and the surfactant were studied by recording the spectrum using Fourier Transform Infra Red Spectroscopy (FTIR).The sample of GH was triturated with dry, finely powdered potassium chloride (KCl) in a proportion of 1 to 200. A portion of the GH-KCl mixture in the hydraulic dies and subjected for compression under high pressure. FTIR spectra were recorded on Shimadzu Fourier Transform In FTIR Spectrophotometer by scanning from 400 to 4000 cm^{-1}. Identification was done by comparing the obtained spectrum to reference spectrum. Similarly, the FTIR of pure polymer and formulated nanoparticles were also recorded.

Preparation of nanoparticles containing GH:

The PEG-CNGH were prepared by ionotropic gelation [5, 6] of the positively charged quaternary amine groups in chitosan by the interaction of the negatively charged sodium tri poly phosphate (TPP), as indicated in the procedure explained by Wang et al and shah et al. In this procedure, chitosan solution (0.2% w/v) was prepared by dissolving 0.2 g of chitosan in 100ml of 1% v/v acetic acid. TPP solution of 0.05% w/v was prepared by dissolving 0.5g of TPP in 1litre of distilled deionized water. GH was added to the chitosan solution and then 200 mg of PEG 4000 was added. The solution was stirred at 1500rpm for 30min on magnetic stirrer. TPP solution was added drop by drop (2ml/hr) and stirring was continued for 30min. The NP suspension is then

centrifuged at 15,000 rpm for 10 min using high-speed centrifuge (Sigma) andthe supernatant nanosuspension was collected and stored at 2^{o}C. The detailed formula of the three batches is given in the table.1.

Table 1: Formulation of PEG 4000 coated chitosan nanoparticles containing GH

Formulation	GH (mg)	Chitosan (mg)	PEG-4000 (mg)	1% Acetic acid (ml)	0.05%TPP solution (ml)
PEG-CNGH-1	100	200	200	100	2
PEG-CNGH -2	100	400	200	100	2
PEG-CNGH -3	100	600	200	100	2

Physicochemical characterization:

The optimized nanoparticles containing GH were characterized by studying various physico-chemical properties[[7].

i) Transmission Electron Microscopy (TEM): TEM analysis of the prepared formulations was carried out to understand the morphology of nanoparticles. A drop of NPs suspension containing 0.01% of phosphotungstic acid was placed on a carbon film coated on a copper grid for TEM. TEM studies were performed at 80 kV. The copper grid was fixed into sample holder and placed in vacuum chamber of the transmission electron microscope and observed under low vacuum, and TEM images were recorded.

Particle size: The size of the prepared nanoparticle was determined by using Photon Correlation Spectroscopy (PCS). All samples were diluted with ultra purified water and the analysis was performed at a scattering angle of 90° and at a temperature of 25ºC. The mean diameter for each sample and mean hydrodynamic diameter was generated by cumulative analysis in triplicate.

ii) Zeta potential: Nanoparticles were characterized with Zeta potential using a Zeta Sizer. The zeta-potential measurement was carried out in an aqueous dip cell in an automatic mode by placing diluted samples in the capillary measurement cell and cell position is adjusted. The measurements were performed using laser doppler electrophoresis and the net velocity of the nanoparticles in the liquid that resulted on the application of electric field was measured.

iii) Surface morphology: The surface morphology of the particles was studied using Transmission Emission Spectroscopy (TEM) set at 200 kV by placing an air dried nanoparticle

suspension on copper electron microscopy grids.

iv) Percentage entrapment efficiency: The entrapment efficiency of PEG-CNGH was determined by centrifugation of the drug loaded nanoparticles at 25000 rpm for 40min. The supernatant were collected and assayed for GH by UV- Visible spectrophotometer at 289nm. Entrapment efficiency was calculated as follows:

$$EE\% = \frac{\text{Total amount of drug added} - \text{Non-bound drug}}{\text{Total amount of drug added}} \times 100$$

v) In vitro release studies: The in vitro release profile of the prepared PEG-CNGH was studied by diffusion across an artificial membrane. In the donar compartment nanosuspension containing

known concentration of drug was placed and in the receptor compartment buffer was placed and constantly agitated using a magnetic stirrer at 37^{o}C. Samples were withdrawn from the receptor compartment at predetermined time intervals and replaced with same volume of fresh buffer. The estimation of released GH in the samples was carried out at 289nm by using UV-Vis spectrophotometer. The experiment was carried out in triplicate and the values were reported as mean value ±standard deviation.

***Invivo* studies**:

The protocols adopted for the animal studies were submitted to IAEC and duly approved (X11/Vels/Col/30/SEA/IAEC/23.09.11).Experiments were performed with colony inbreed strains of Swiss albino mice (male) weighing 22-25g at the age of 5-6 weeks. The animals are kept under standard conditions maintained at 23-25^{o}C, 12h light /dark cycle and provided food and water *ad libitum*. The animals are acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into groups consisting each of six animals. Three groups of mice each group consisting of six animals was taken for the study. The group 1 animals treated with phosphate buffered saline will be administered perorally, whereas, the group2 and group 3 received i.p administration of plain GH solution (1.5mg/kg) and GH nanoparticles (dose equivalent to 1.5mg/kg of GH) for 10 days followed by administration of scopolamine (0.4mg/kg, i.p.) on the eleventh day.

***i) Step Down Inhibitory avoidance*:**

The apparatus is a 50 cm × 25 cm × 25 cm acrylic box, in which the floor is made up of parallel

1.0 mm diameter stainless steel bars spaced 1.0 cm apart. A 7.0 cm wide, 2.5 cm high, 25.0 cm long platform is present in the centre floor. In the training session, immediately after stepping down, placing their four paws on the grid the animals received a 0.4 mA, 2.0 s scrambled foot shock. In test sessions no foot shock was given and step-down latency is measured with a cut off time of 300s. One-trial step-down inhibitory avoidance in rats and mice involves the activationof two separate memory types respectively, short-term memory (STM) system, and a long-term memory (LTM) system. Therefore, retention tests were also carried out 90 min after training to evaluate STM [8].

ii) Morris water maze task:

The Morris water maze task was carried out as reported earlier[9]. The experimental apparatus consisted of a circular water tank of diameter of100 cm and with the height of 35 cm, containing water at 28 ^{o}C to a depth of 15 cm and made opaque by incorporation of powdered milk. A platform of diameter 4.5 cm and with a height of 14.5 cm, was submerged 0.5 cm below the water surface and placed at the midpoint of one quadrant. After several trials, the test was conducted on the 5th day after administration of scopalamine. In each training trial, the time required to escape on to the platform was recorded.

iii) Acetylcholinesterase enzyme:

The whole brain AChE activity was measured[10] in 20% of brain homogenate in phosphate buffer (0.1M; pH 8). The rate of reaction was determined by the formation of yellow color due tothe reaction of acetylthiocholine with [5,5'-dithiobis (2-nitro benzoic acid)]. The rate of formation of thiocholine from acetylcholine iodide in the presence of brain cholinesterase was measured using a spectrophotometer at 412 nm.

2. Statistical Studies

All values of the *invivo* studies are expressed as mean ± S.D. The data obtained were subjected to analysis of variance (ANOVA) followed by students t-test. P values >0.05 were considered significant.

3. Results

As per the protocol of the design thinking the prototype of galantamine loaded nanoparticles were tested

and the data were presented here:

4.1.Compatibility studies:

The FTIR spectrum of pure GH with chitosan and PEG 4000 were recorded and given in Figure 1. The peaks in the IR spectrum of GH were compared with that of the prepared nanoparticles.

The C-N, C-O and C-H stretchings of GH at1197, 1353 and 3418 remain unaltered in its combination with chitosan as well as PEG-CNGH. This indicates that chemical interaction had occurred between the GH and other ingredients including the chitosan and PEG 4000.

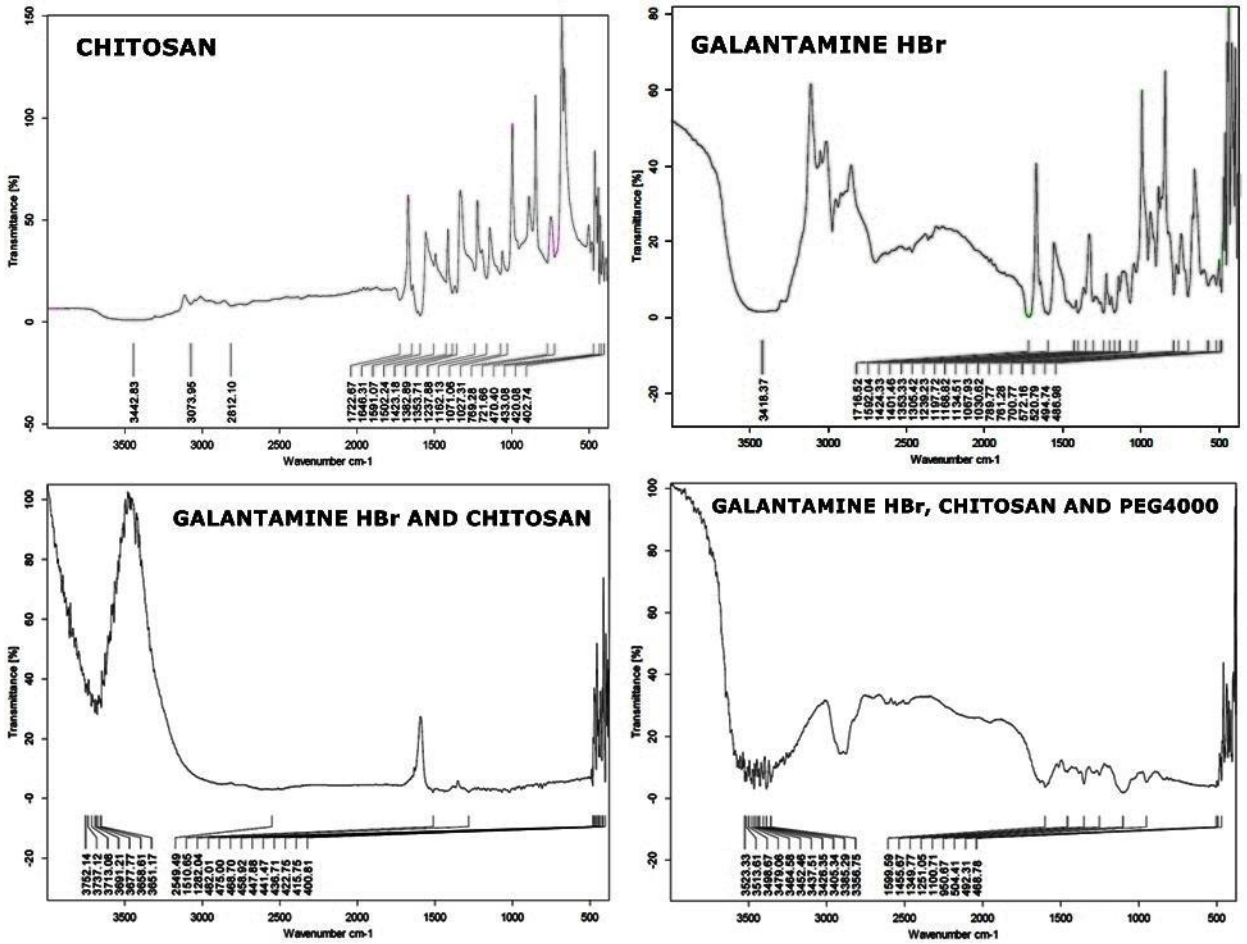

Figure 1. FTIR studies of Galantamine HBr with Chitosan and PEG 4000

Characterization of Nanoparticles:

TEM Analysis: The prepared PEG-CNGH exhibited spherical shape and the surfaces were smooth. The particles were discrete and no symptoms of agglomeration were identified. The TEM images of PEG-CNGH-1, 2 and 3 were given in the figures 2, 3 and 4, respectively.

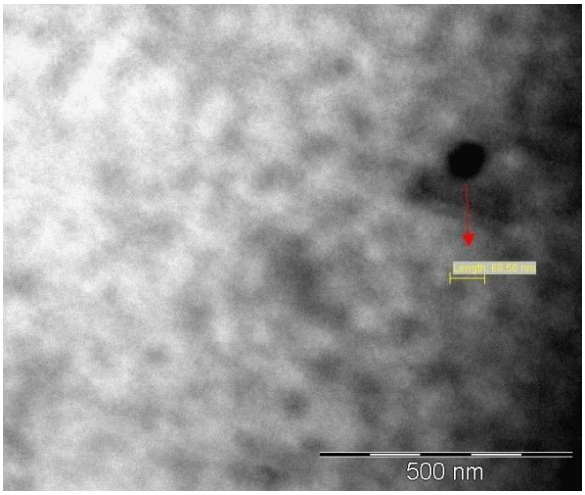

Figure 2. TEM image of PEG-CNGH-1

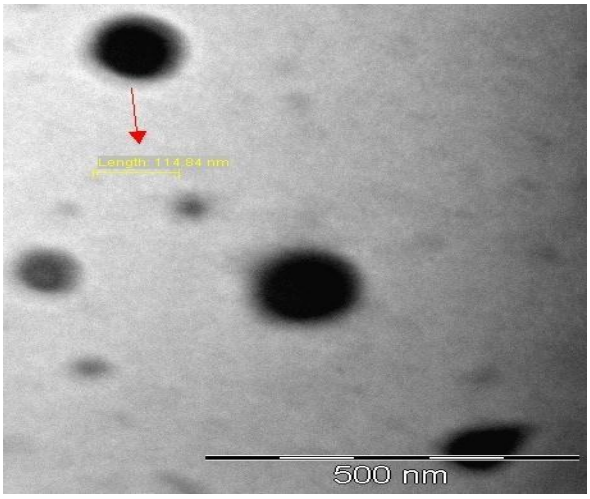

Figure 3. TEM image of PEG-CNGH-2.

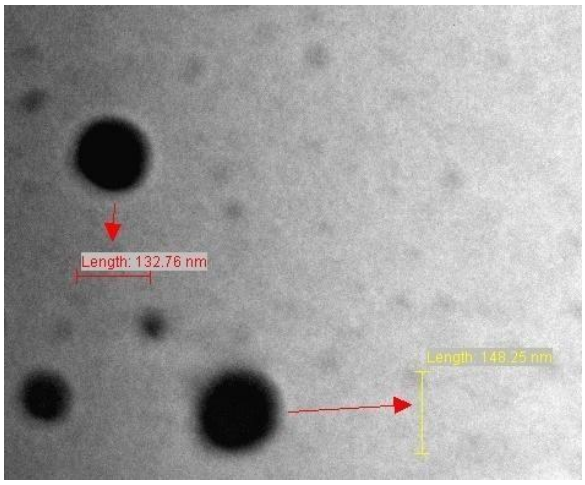

Figure 4. TEM image of PEG-CNGH-3.

Particle size

The prepared PEG-CNGH with varying concentration of chitosan indicates an increase in particle size with an increase in the polymer ratio. The formulation PEG-CNGH-1 with drug: polymer ratio of 1:2 indicated the least particle size among the three formulations. The formulation PEG-CNGH-1, 2 and 3 had exhibited particle size of 68±12nm, 112±20nm and150±26nm, respectively.

Zeta Potential

Minor increases in zeta potential of PEG-CNGH were recorded with increase in the concentration of chitosan. The details of the zeta potential of the PEG-CNGH were given in the table 2. The higher zeta potential indicates that the colloidal system is more stable.

Table 2: Physicochemical characterization of PEG-CNGH prepared by ionic gelation method.

Formulations	Average particle size (nm)	Zeta potential (mV)	Average drug content (mg/ml)	Average entrapment efficiency (%)
PEG-CNGH-1	68.2±12.4	+44.5±0.4	1.6±0.2	45.8±1.6
PEG-CNGH-2	112.3±20.2	+50.3±0.3	1.7±0.3	48.6±2.7
PEG-CNGH-3	150.7±26.4	+54.5±0.4	1.9±0.3	53.3±3.5

Drug content and entrapment efficiency:

A parallel increase in the drug content and the entrapment efficiency of the prepared nanoparticles were observed with increase in polymer concentration. The content and the entrapment efficiency of the prepared nanoparticles were given in the table-2

***In vitro* release of GH from PEG-CNGH:**

The in vitro release of PEG-CNGH was studied using diffusion cells with dialysis membrane having pore size 2.4 nm, molecular weight cut-off between 12,000–14,000. The invitro release ofPEG-CNGH was determined using phosphate buffer (pH 7.4) as the release media to simulatethe condition in the brain. The drug release from the nanoparticles was found to be 97.2%, 87.5%and 91.3% for PEG-CNGH-1, 2 and 3 formulations, respectively, after 24 h . Hence it issuggested that, formulation PEG-CNGH-1 posses the ideal characters required for the release of GH from the nanoparticles.

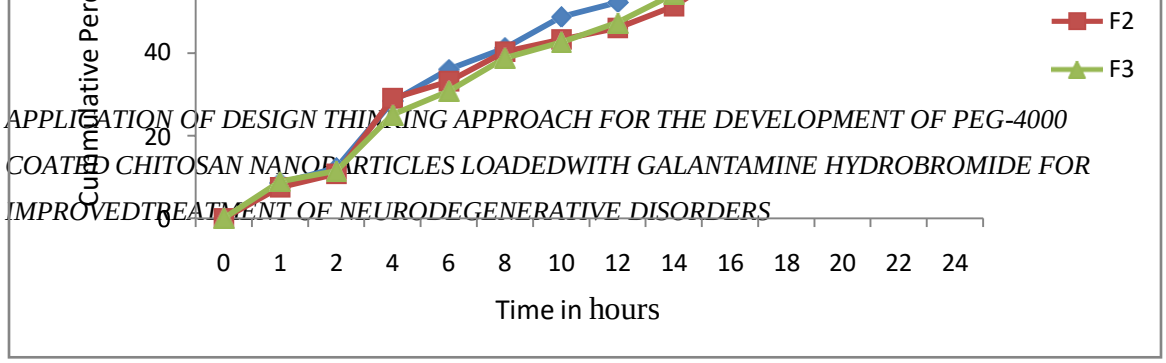

Figure 5. *In vitro* release profile of GH fromPEG-CNGH-1,2 and 3 in pH 7.4 phosphatebuffer.

***In vivo* studies:**

i) Effect of PEG-CNGH on water maze:

The change in escape latency after the administration of scopalamine is shown in Table 3. The amnesia induced animals received phosphate buffered saline showed the maximum escape latency time. A decreased time to escape on to the escape platform was recorded for amnesic animals treated with plain GH solution as well as PEG-CNGH-1. Among, the GH treated and PEG-CNGH-1 treated groups the later shown significant decrease in escape latency time.

ii) Effect of EAG on step-down inhibitory avoidance

The cognitive dysfunction and the types of memory in one-trial step-down inhibitory assessment are depicted in Table 3. There was a significant difference between the GH treated group and the PEG-CNGH-1 treated group in STM ($p<0.01$). The PEG-CNGH-1 treated group had exhibited significant

Table 3. Effect of PEG-CNGH-1 on short term memory by step down inhibitory avoidance,water maze and acetylcholinesterase enzyme levels.

Group	Treatment	Behavioral parameters		
		Step-down latency in sec	Water maze-Escape latency in sec	**AChE (µmol/min/mg)**
I	Scopalamine (1mg/kg.b.w.i.p) + 1 ml Phosphate buffered saline(p.o)	143.2 ± 6.10	75.00 ± 5.27	20.56± 0.74
II	Scopalamine (i.p) + 1ml of 2.5mg/kg/day.b.w of GH in PBS(i.p)	189.0 ± 10.24^{b}	49.50 ± 3.51^{a}	12.56± 0.55^{a}
III	Scopalamine (i.p) +1ml of PB PEG-CNGH-1 (i.p) equivalent to 2.5mg/kg/day.b.w of GH	231.0 ± 8.31^{a,e}	30.50 ± 1.89^{a,e}	9.55 ± 0.25^{a,e}

Values are expressed as mean ± SEM of 6 animals. Superscript letters represents the statisticalsignificance done by ANOVA, followed by Tukey's multiple comparison tests.

$^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$, indicates the significance on comparison of group II and IIIwith group I.

$^{d}P < 0.05$, $^{e}P < 0.01$, indicates the significance on comparison of group III with Group II.

5. Discussion

The pharmacokinetic studies of this drug carried out in animals reveals, the very poor distribution in brain. The activity of GH can be considerably improved if the amount of the GH reaching brain could be increased. Various methods had been tried by the researchers to improve the penetration of GH across BBB and increasing the concentration of GH in brain. The 'prodrug' approach for improving the permeability of GH across BBB was patented [11].

In the method adopted for the preparation of nanoparticles in the present study was reported by Sunil Shah et al [6], which utilizes hydrophilic polymers. Nanoparticles were prepared byionotropic gelation method, which is a spontaneous phase separation process arising from electrostatic interaction, when oppositely charged macromolecules are mixed together. The preparation of nanoparticles is dependent on the number of positively charged groups left on the dissolved chitosan molecules. Because of sufficient protonated amine groups remaining, the process of the ionic crosslinking occurs more easily for chitosan with high degree of deacetylation. A similar principle has been used by other research groups for the preparation of nanoparticles useful in the encapsulation and controlled release of peptides, proteins.

PEG is widely used as a coating material due to its proven safety potential attributed by its hydrophilicity, nontoxicity, absence of antigenecity, and immunogenicity. Nanoparticles with right coating can quickly slip through human mucus preventing the adherence of nanoparticles and viruses in the protein meshwork in the mucus, allowing them to become long circulating particles in physiological fluids.

The nature of interaction between the drug and CS as well as drug, CS and PEG-4000 was studied using FTIR spectrometry. The major peaks of GH remained unaltered when combined with CS and exhibited almost similar peaks when combined with CS and PEG-4000. From the above data it can be concluded that no incompatibility exists between GH and other ingredients used for the development of nanoparticles.

The physicochemical properties of nanoparticles are considered to be very important in determining the physiological functions and stability of drug-loaded nanoparticles. The particle size and surface charge are the most significant determinants. The increase in the concentration of the polymer ratio caused an increase in the particle size. It was observed that particle size increases on coating with PEG due to the intermolecular hydrogen bonding between the electropositive quaternary amine groups of CS with electronegative hydroxyl groups of PEG.

The surface charge is the critical parameter on the stability of the suspensions and adhesion of particle systems onto biological surfaces. The zeta potential was found to be higher whencompared with the zeta potential of the chitosan nanoparticles as reported by Rajendran et al [12]and Shahrooz Saremi et al [13]. The higher zeta potential of the prepared chitosan nanoparticlesindicates that the nanosuspension are electrically stable.

From the TEM images GH loaded chitosan nanoparticles coated with PEG 4000, it was observed that nanoparticles were morphologically spherical with the sizes ranging from 60nm to 150 nm.

The increased viscosity of the medium with increasing concentration of CS may be responsible for the enhancement in the encapsulation efficiency. However the increase in entrapment efficiency and drug content were indicated with proportional increase in particle size and hence, little interest was shown in further increment of the polymer ratio.

The release profile of GH nanoparticles revealed that the drug release is slow at pH7.4. Generally the drug release is due to the diffusion of drug molecules through the matrix or due to the degradation of polymer matrix. The slow release rate was also attributed to the PEG coating due to the presence of surface crosslinking of PEG.

The administration of scopolamine has been reported to impair memory retention when given to mice shortly before training in a dark avoidance task [14, 15]. The capability of different cholinergic agonist drugs was well demonstrated to reverse the amnesic action of scopolamine in animal models, specifically mice and rats. However, the neuropathology of dementia of the Alzheimer disorder is not confined only to the cholinergic system [16], still this model had been widely used by the researchers to assess the neuropharmacological action of drugs.

The increase in latency time of step down inhibitory avoidance and decrease in the escape latency time of Morris water maze test had clearly exhibited the improvement in the anti-amnesic activity of GH loaded in polymeric nanoparticles coated with PEG4000. This also suggests that the nanoformualtion(PEG-CNGH-1) might have increased the penetration of GH across the BBB and caused the improvement in the activity. The biochemical estimation of AChE, also supported the above claim, as it indicates a significant decrease in the AChE levels in brain when compared with administration of similar dose of plain GH solution in PBS.

6. Conclusion

The empathy obtained from the literature about the difficulty in treatment of the AD patients due to poor availability was well defined and it was ideated to develop PEG 4000 coated Galantamine Hydrobromide loaded chitosan nanoparticles. The prototype of the nanoparticles was successfully developed and tested both invitro and invivo. The study provides pharmacological evidence for

improvement in the activity of GH, when loaded polymeric nanoparticles. The developed nanoparticles can serve as tool for delivery of drugs with poor lipid solubility. However, further studies are required to evaluate the increase in the distribution of drug in brain and toxicology of polymeric nanoformulations in brain.

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