CHEMICAL MODIFICATION OF CHITOSAN AND FORMULATION OF ITS NANOPARTICLES FOR PROTEIN DRUG DELIVERY SYSTEM

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ABSTRACT

The most common route of administration of proteins has been parenterals using invasive ways of administration but this route has many side effects like lack of patient compliance, cost, high drug levels etc. The development of an oral dosage form that improves the absorption of protein drugs is the most desirable formulation but one of the greatest challenges in the pharmaceutical field. The major barriers in developing oral formulations for peptides and proteins include poor intrinsic permeability, luminal and cellular enzymatic degradation and chemical and conformational stability. A number of innovative oral drug delivery approaches have been recently developed, including the drug entrapment within polymer nanoparticles. The various advantages of oral delivery of proteins are like ease of administration, can be used as cure in primary stages of disease eg. Thrombosis, no internal bleeding, patient compliance and economical. Our formulation development approach is to develop the formulations targeted to bypass the stomach with an aim to release the drug in the intestine; with extended residence time in the GIT and for this polymer like Chitosan is used. To improve the lipophilic property of chitosan, to modify biodegradation pattern of polymer and for oral delivery of proteins (Insulin and serratiopeptidase), modification is done using lactic-acid and polyethyleneglycol by copolymerization.

Keywords: Insulin, Serratiopeptidase, Lactic-Acid, Polyethyleneglycol and Chitosan

INTRODUCTION

Proteins also known as polypeptides are organic compounds made up of amino acids arranged in a linear chain and folded into a globular form. The amino acids in a polymer are joined together by the peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. Serving as the basic structural molecule of all the tissues in the body, protein makes up nearly 17 % of the total body weight. Insulin, Somatotropin Like other biological macromolecules proteins are essential parts of organisms and participate in virtually every process within cells. The oral route is considered to be the most convenient and desired route of drug delivery, especially when repeated or routine administration is necessary. Generally, peptides and proteins such as insulin cannot be administered via the oral route due to rapid enzymatic degradation in the stomach, inactivation and digestion by proteolytic enzymes in the intestinal lumen, and poor permeability across intestinal epithelium because of its high molecular weight and lack of lipophilicity. The oral bioavailability of most peptides and proteins therefore is less. Insulin is usually administered to diabetic patients through subcutaneous injection. However, the problems encountered with subcutaneous insulin injections are pain, allergic reactions, hyperinsulinemia, and insulin lipodystrophy around the injection site. Insulin if administered via the oral route will help eliminate the pain caused by injection, psychological barriers associated with multiple daily injections such as needle anxiety and possible infections. In light of the above distinct benefits, pharmaceutical technologists have been trying to design an oral delivery system for different proteins. Such is the interest in oral SRPT and Insulin delivery that some pharmaceutical companies are solely focused on it¹⁻⁶. A number of different methods have been explored to improve the oral bioavailability of proteins. A common method, has been, the use of penetration enhancers, like detergents, fatty acids or bile salts which are meant to permeabilize the mucus and epithelial layers and open the intercellular

tight junctions. Encapsulation of the protein within enteric capsules or nanospheres has also been used. In this present research work attempt has been made to develop oral dosage form for insulin using modified Chitosan polymer which is formed by copolymerization technique.

MATERIALS AND METHODS

Materials used

Insulin, Chitosan, Polyethylene glycols, Lactic acid, Sodium tripolyphosphate, Sodium Alginate and Anhydrous Calcium chloride

Selection of polymer for modification

Selection of polymer was done on the basis of solubility in water. Chitosan is a polysaccharide formed primarily of repeating units of b-(1-4)2-amino-2-deoxy-D-glucose (D-glucosamine). Generally, chitosan is soluble in aqueous medium in the presence of a small amount of acids such as AcOH, lactic acid, HCl and so on. Though the chitosan dissolves in aqueous acidic medium below pH 6.5, it precipitates above this pH by the addition of alkali solution like aq. NaOH. The application of chitosan was limited owing to the insolubility at neutral or high pH region. To improve the lipophilic property of chitosan, modification is required.

Preparation of Acetylated chitosan copolymer: (C1)

The reaction is involved between the NH₂ groups of chitosan and -OH group of acetic acid and removal of water takes place. First, chitosan was dissolved in aqueous acetic acid 1%. After that, the solution was stirred at 100°C to create a final solution of chitosan. The solution was placed in petri dishes overnight for film formation. The film obtained as final product, acetylated chitosan copolymer⁷.

Preparation of LA-g-chitosan copolymer: (C2)

The reaction is involved between the NH₂ groups of chitosan and -OH group of lactic acid and removal of water takes

place. First, chitosan was dissolved in a mixture of 1% acetic acid. Aqueous solution of 0.5% lactic acid was added into the chitosan solution. After that, the solution was stirred at 100°C to create a final solution of chitosan. The solution was placed in petri dishes overnight for film formation. The film obtained as final product, chitosan-g-LA copolymer⁸⁻¹⁰.

Preparation of Pegylated chitosan: (C3)

The reaction is involved between the NH₂ group of chitosan and -OH group of PEG and removal of water takes place. First, chitosan was dissolved in a mixture of 1% acetic acid. Aqueous solution of 1% PEG was added into the chitosan solution. After that, the solution was stirred at 100°C to create a final solution of chitosan. The solution was placed in petri dishes overnight for film formation. The film obtained as final product, chitosan-g-PEG copolymer¹¹⁻¹².

Preparation of Lactic acid and PEG-grafted-chitosan copolymer: (C4)

The reaction is involved between the NH₂ group of chitosan and -OH group of lactic acid and PEG and removal of water takes place. First, chitosan was dissolved in a mixture of 1% acetic acid. Aqueous solution of both 0.5% lactic acid and 1% PEG was added into the chitosan solution. After that, the solution was stirred at 100°C to create a final solution of chitosan. The solution was placed in petri dishes overnight for film formation. The film obtained as final product, chitosan-g-LA-PEG copolymer⁸⁻¹³.

Formulation of nanoparticles

Chitosan nanoparticles were prepared according to the ionic gelation of Chitosan with Pentasodium tripolyphosphate (TPP) anions. Plain chitosan and modified chitosan were used for formulating nanoparticles. Four types of samples were prepared:

I. Chitosan was dissolved in 1 % v/v acetic acid aqueous solution at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 % w/v). [N1]

II. Chitosan was dissolved in 1 % v/v acetic acid aqueous solution at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 % w/v) and 0.5 % v/v lactic acid aqueous solution at concentration of 0.5. [N2]

III. Chitosan was dissolved in 1 % v/v acetic acid aqueous solution at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 % w/v) and 1%v/v PEG. [N3]

IV. Chitosan was dissolved in 1 % v/v acetic acid aqueous solution at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 % w/v) and 0.5 % v/v lactic acid aqueous solution at concentration of 0.5 and 1% v/v PEG. [N4]

Aqueous solution of TPP 5ml with concentration (0.01 % w/v) was added through a syringe needle into 10ml chitosan solution under stirring at room temperature. The phenomena observed were in aqueous solution. Nanoparticles were formed spontaneously upon incorporation of 5ml TPP solution into 10ml chitosan solution. The above nano solutions N1, N2, N3, and N4 were adjusted to pH 6.5-6.8.

Percentage yield of chitosan nanoparticles

Percentage yield of nanoparticles was calculated using the following formula

$$\text{Percentage Yield} = \frac{\text{Amount of nanoparticles obtained (mg)}}{\text{Theoretical Amount (mg)}} \times 100$$

Loading of protein on nanoparticles

The lyophilized nanoparticles (100mg) were resuspended in distilled water under ultrasonication at room temperature. The known amount of protein solution was incubated overnight in the nanoparticles suspension. The encapsulation efficiency and loading capacity of nanoparticles were determined by the separation of nanoparticles from the aqueous medium containing non-associated protein by centrifugation at 1400 rpm for 10 min. The amount of free protein in the supernatant was measured by Assay method. The protein loading efficiency (LE) and the protein loading capacity (LC) of the nanoparticles were calculated as follows:

$$\text{Loading Efficiency (\%)} = \frac{\text{Total amount of protein} - \text{free protein}}{\text{Total amount of protein}} \times 100$$

$$\text{Loading Capacity (\%)} = \frac{\text{Total amount of protein} - \text{free protein}}{\text{Weight of nanoparticles}} \times 100$$

All measurements were performed in triplicate.

Formulation of BEADS¹⁴⁻¹⁸

Entrapment of chitosan nanoparticles loaded with protein in Alginate beads

Various concentrations of Sodium alginate solution were prepared in double distilled water and fixed amount (100mg) of chitosan nanoparticles loaded with protein were incorporated in the Sodium alginate solutions of various concentrations.

Evaluation of uncoated beads

Appearance

Surface morphology was observed by microscopy.

Beads size analysis

The dimensions of beads were measured with the help of Digital Vernier Calliper by taking 10 beads from each batch. Average size of bead was noted.

Percentage yield of beads

Percentage yield of beads was calculated using the following formula-

$$\text{Percentage Yield} = \frac{\text{Amount of beads obtained (mg)}}{\text{Theoretical Amount (mg)}} \times 100$$

In-vitro Drug release studies in pH 6.8 PBS

In-vitro Drug Release studies of protein loaded beads were carried out by using USP XXIII Dissolution Test Apparatus 1(Basket method) at 37± 0.5°C and 100 rpm speed. The weighed amount of protein Loaded Beads were placed in the baskets and then submerged into 900 ml dissolution flask containing 500ml of PBS pH 6.8 for 3 hrs. Aliquots of 2 ml were withdrawn and replaced with the same volume of fresh solution at each different time intervals. Aliquots withdrawn were filtered through Whatman filter paper number 42. The amount of drug released was analyzed by UV-visible spectrophotometric bio assay. The release studies were conducted in triplicates and mean values of cumulative % drug release were plotted versus time¹⁹⁻²⁰.

Content uniformity

Content Uniformity was determined by dissolving beads in pH 6.8 PBS for 5 hrs and UV absorbance was recorded to estimate the % drug content.

Table 1: Optimization of parameters for preparation of alginate beads. E, F, G and H for Insulin

Hardening Time (min)	Sodium alginate (% w/v)				CaCl ₂ (% w/v)	Drop Height(cm)	Drug loaded nanoparticles(mg)
	2	3	4	5			
5	E1	F1	G1	H1	2	5	100
10	E2	F2	G2	H2			
15	E3	F3	G3	H3			
20	E4	F4	G4	H4			

Table 2: Oral glucose tolerance test in non diabetic rats

Group (n = 6)	Treatment and Dose	Glucose Load	Evaluation Parameter
I Control	Distilled water	10% Glucose solution (2 g/kg, p.o) 150 min after vehicle/drug	Plasma glucose (mg/dl) at 0 th , 30 th , 90 th and 150 th mins. and for test, after a lag period of 150 mins.
II Standard	Insulin (0.525IU/kg, S.C.)		
III, IV, V and VI Test	Insulin Beads (1.0 IU/kg, p.o.)		

Table 3: Percent yield, loading efficiency and loading capacity of chitosan nanoparticles

Batch Code	Chitosan (1% w/v)	TPP (%w/v)	Chitosan: TPP	% Yield* (w/w)	LE* (%w/w)	LC* (%w/w)
NANO N1	C1	0.1	2.5:1	60±0.08	7.5±0.61	50±0.11
NANO N2	C2		5:1	73±0.38	20.5±0.09	56±0.19
NANO N3	C3		7.5:1	80±0.38	52±0.69	65±0.37
NANO N4	C4		10:1	87±0.04	49±0.45	79±0.56

* All values are expressed as mean, n = 3

Table 4: Percent yield, mean particle size and sphericity of beads of alginate

Batch Code	% Yield*(w/w)	Mean* Particle Size (mm)	Sphericity*
A1, A2, A3, A4	No Uniform Beads		
B1	Uneven beads		
B2	98±0.17	1.210±0.022	Spherical
B3	96±0.25	1.103±0.024	Spherical
B4	96±0.19	1.075±0.011	Spherical
C1	Uneven beads		
C2	97±0.19	1.214±0.019	Spherical
C3	96±0.57	1.213±0.045	Spherical
C4	98±0.73	1.204±0.069	Spherical
D1	Uneven beads		
D2	98±0.18	1.240±0.015	Spherical
D3	97±0.22	1.226±0.016	Spherical
D4	97±0.29	1.221±0.018	Spherical

* All values are expressed as mean ± SD, n = 3

Table 5: % Drug Release in pH 6.8 phosphate buffer from batches IB2-IB4

Time (mins)	*Batch Code. % Drug Release in pH 6.8 phosphate buffer		
	IB2	IB3	IB4
0	0	0	0
15	14.78±0.71	12.31±0.19	11.71±0.65
30	19.71±0.12	17.65±0.71	15.89±0.31
45	24.12±0.46	21.78±0.48	19.51±0.52
60	29.16±0.71	27.78±0.11	25.87±0.62
90	35.71±0.81	33.98±0.45	31.75±0.74
120	40.78±0.64	38.61±0.41	35.84±0.16
150	45.61±0.98	43.81±0.26	40.84±0.32
180	54.89±0.11	48.79±0.16	44.78±0.99

*All values are expressed as Mean ±SD, n = 3

Table 6: % Drug Release in pH 6.8 phosphate buffer from batches IC2-IC4

Time (mins)	Batch Code* % Drug Release in pH 6.8 phosphate buffer		
	IC2	IC3	IC4
0	0	0	0
15	17.89±0.63	15.81±0.19	14.05±0.76
30	26.71±0.81	24.78±0.61	23.91±0.34
45	34.87±0.16	31.71±0.19	29.36±0.17
60	43.91±0.12	40.98±0.79	38.14±0.54
90	52.13±0.16	49.81±0.23	46.84±0.14
120	61.89±0.34	58.12±0.74	54.12±0.79
150	72.12±0.15	70.97±0.64	66.98±0.12
180	82.16±0.98	80.91±0.58	78.01±0.74

*All values are expressed as Mean ±SD, n = 3

Table 7: % Drug Release in pH 6.8 phosphate buffer from batches ID2-ID4

Time (mins)	*Batch Code. % Drug Release in pH 6.8 phosphate buffer		
	ID2	ID3	ID4
0	0	0	0
15	23.43±0.12	17.56±0.39	15.43±0.75
30	38.55±0.29	33.77±0.12	26.22±0.69
45	52.23±0.40	48.23±0.85	43.65±0.33
60	62.65±0.81	59.67±0.48	53.78±0.15
90	71.77±0.28	67.98±0.07	59.54±0.44
120	78.45±0.14	71.21±0.67	66.78±0.11
150	81.77±0.38	78.12±0.38	70.90±0.18
180	85.98±0.29	80.11±0.12	75.19±0.54

*All values are expressed as Mean ±SD, n = 3

Table 8: % Drug Release of optimized batch ID2 in pH 6.8 PBS at 0 and 45 days

Time (min)	% Drug release in PBS pH 6.8. ID2	
	0 day*	45 days*
0	0	0
15	23.43±0.12	21.35±0.79
30	38.55±0.29	35.72±0.46
45	52.23±0.40	50.23±0.49
60	62.65±0.81	59.85±0.64
90	71.77±0.28	68.23±0.29
120	78.45±0.14	76.54±0.12
150	81.77±0.38	78.24±0.58
180	85.98±0.29	82.39±0.59

* All values are expressed as Mean ±SD, n = 3

Table 9: Oral glucose tolerance test in non diabetic rats

Treatment and dose (mg/dl)	Time (mins) (Monitoring after lag period of 150 mins of administration of beads)			
	0	30	90	150
Distilled water (10ml)	69.63±0.93	137.19±0.92	128.17±1.28	112.21±0.13*
Standard Insulin	70.10±1.81	130.01±0.89**	97.28±1.38**	89.28±0.71**
Test-1	65.06±1.67	132.28±3.12	114.32±1.28	104.48±1.06**
Test-2	65.86±1.91	130.9±0.68	115.04±1.38*	103.27±3.12*
Test-3	70.49±1.28	132.17±0.91	114.78±2.12*	97.17±4.28**
Test-4	67.98±1.28	130.74±0.76	106.18±4.12**	94.04±2.03**

All values are expressed as Mean ±SEM, n = 6, ** = Significance p<0.01 and * = Significance p<0.05

Figure 1: IR Spectra of acetylated chitosan (C1)

Figure 2: IR Spectra of LA-g- chitosan (C2)

Figure 3: IR Spectra of PEG-g- chitosan (C3)

Figure 4: IR Spectra of LA-PEG-g- chitosan (C4)

Figure 5: Percent yield, loading efficiency and loading capacity of chitosan nanoparticles

Figure 6: SEM images of chitosan nanoparticles

Figure 7: Percent yield, loading efficiency and loading capacity for Insulin

Figure 8: SEM images of surface section and T.S. of the core alginate bead

Figure 9: % Drug Release in pH 6.8 phosphate buffer from alginate uncoated beads (IB2-IB4)

Figure 10: % Drug Release in pH 6.8 phosphate buffer from alginate uncoated beads (IC2-IC4)%

Figure 11: % Drug Release in pH 6.8 phosphate buffer from alginate uncoated beads (ID2-ID4)

Figure 12: IR Spectra of Insulin formulation ID

Figure 13: % Drug Release of optimized batch ID2 in pH 6.8 PBS at 0 and 45 days

Figure 14: DSC thermogram of pure sodium alginate

Figure 15: DSC thermogram of pure calcium alginate

Figure 16: DSC thermogram of pure insulin

Figure 17: DSC thermogram of formulated alginate insulin loaded bead

DSC analysis

Thermograms were obtained using a Shimadzu DSC-60system (Shimadzu). Samples were, powder crimped in a standard aluminium pan and heated from 30 to 300 °C at a heating constant rate of 10 °C/min under constant purging of nitrogen at 10 ml/min.

Stability Studies

Stability studies of the optimized formulations were carried out to know whether the chemical change or degradation of the active ingredient has occurred. This may lower the therapeutic potency of active ingredient over storage period. Whether any toxic degradation product has formed which may be undesirable and gross changes in the physical form of the dosage form have occurred implying poor or substandard quality of ingredients. To assess the drug and formulation stability, stability studies were done according to ICH guidelines. Optimized formulation sealed in aluminum packaging coated inside with polyethylene, and various replicates were kept in the humidity chamber maintained at 40°C and 75% RH for 45 days. At the end of studies, samples were analyzed for the physical appearance, drug content and drug release studies.

In-vivo Animal activity study Oral Glucose Tolerance Test (OGTT)

Experimental animals- Albino rats of Wistar strain weighing 150-200 g were obtained. Animals of either sex were housed in groups of five under standard laboratory conditions with free access to standard pellet diet and water.

Procedure

The overnight fasted animals were divided into Three groups (n=6). The animals were given 10% Glucose Solution 0 minutes after drug treatment.

1. Group-I (Control): The animals of this group received Distilled water.
2. Group-II (Standard Insulin): The animals of this group received Standard Insulin 0.525 IU/kg S.C.
3. Group-III, IV, V and VI (Insulin Beads): The animals of this group received Insulin beads 1.0 IU/kg, p.o, 3 hrs before administration of standard insulin S.C. to Group-II. After the lag period of 150minutes of administration of beads the 0th, 30th, 90th and 150th minutes blood was collected by puncturing retro-orbital plexus under light ether anesthesia by using fine glass capillary. Plasma was separated by centrifugation. Plasma glucose level was determined by GOD/POD method²¹⁻²³.

Figure 18: Plasma glucose level in non diabetic rats at 0,30,90 and 150min and for beads after a lag period of 150mins of administration

RESULTS AND DISCUSSIONS

Characterization of modified polymers

IR spectra of acetylated chitosan copolymer

The IR Spectra of acetylated chitosan obtained on a FTIR with diffused reflectance assembly is shown in Figure 1. It is similar with that reported in literature.

IR spectra of LA-g-chitosan copolymer

The IR Spectra of LA-g- chitosan obtained on a FTIR with diffused reflectance assembly is shown in Figure 2. It is similar with that reported in literature.

IR spectra of PEG-g- chitosan copolymer

The IR Spectra of PEG-g- chitosan obtained on a FTIR with diffused reflectance assembly is shown in Figure 3. It is similar with that reported in literature.

IR spectra of LA-PEG-g- chitosan copolymer

The IR Spectra of LA-PEG-g- chitosan obtained on a FTIR with diffused reflectance assembly is shown in Figure 4. It is similar with that reported in literature.

Evaluation of chitosan nanoparticles

Percent yield, loading efficiency (LE) and loading capacity (LC)

As concluded in Table 3 and Figure 5. % yield, loading efficiency and loading capacity of the nanoparticles were affected by the variable chitosan concentration. This might be due to the concentration of TPP taken as constant to achieve the chitosan: TPP ratio between 6:1 to 10:1 for effective protein association and loading. Thus NANO (N4) batch having chitosan: TPP (10:1) ratio was selected for further studies as it showed highest % yield, loading efficiency and capacity Table 3 and Figure 5.

Surface morphology and particle size analysis by scanning electron microscopy (SEM)

The optimized batch of chitosan nanoparticles (NANO N4) was characterized by SEM. Scanning electron microphotograph of chitosan nanoparticles is given in fig.no.6. According to Figure 6, chitosan nanoparticles were irregular with size ranging from 100 to 520 nm. Size of nanoparticles: N1=309.9, N2=428.9, N3=392.2 and N4=519

Drug loading studies on optimized chitosan nanoparticles Loading Efficiency (LE) and Loading Capacity (LC) studies for Insulin

To optimize dose equivalent concentration of Insulin loaded on the nanoparticles various concentrations of Insulin solution from 10-40 IU/ml were incubated with the optimized batch of chitosan nanoparticles, the LE and LC was studied. The loading efficiency was greater than 70% for Insulin concentration 10 IU/ml. The loading efficiency significantly decreased below 65 % when Insulin concentration was increased above 20 IU/ml. This phenomenon might be attributed to the saturation of loading efficiency which was achieved at concentration of Insulin above 20 IU/ml; the more addition of insulin onto chitosan nanoparticles lead to the great decrease in loading efficiency (LE). Meanwhile, loading capacity increased from 36 % to 65 % as the Insulin concentration increased from 10 IU/ml to 20 IU/ml as given in Figure 7. Thus, Insulin concentration of 20 IU/ml was selected for further studies.

Evaluation of alginate beads

Morphology, percent yield and particle size analysis of beads

Morphology of uncoated beads was examined by microscopic study. SEM of surface section and T.S of the core sodium alginate beads is given in the Figure 8. Prepared beads were distinguished by its spherical nature. Particle size was determined by Digital Vernier Calliper. The Morphology, Percent yield and Particle size analysis of beads is given in Table 4.

Alginate uncoated beads batches A1-A4

In the batches A1-A4 no uniform beads were formed, this might be due to the less viscosity of the sodium alginate solution (2%w/v) as given in Table 4.

Alginate uncoated beads batches B1, C1, D1

In the batches B1, C1 and D1 uneven beads were formed, this might be due to the insufficient hardening time (5 minutes) in CaCl_2 solution as given in Table 4.

Insulin release from uncoated beads in pH 6.8 phosphate buffer

Percentage insulin release of alginate uncoated beads batches IB2-IB4

The release profiles in phosphate buffer solution pH 6.8 of Insulin loaded chitosan nanoparticles entrapped in IB2-IB4 batches of uncoated alginate beads are shown in Table 5 and Figure 9. As depicted in Figure 9, Insulin release of about 54.89%, 48.79% and 44.78% was observed in batches IB2, IB3 and IB4 respectively. The delayed release of insulin from the batches might be due to increase in the hardening time from 10 to 20 minutes respectively.

Percentage insulin release of alginate uncoated beads batches IC2-IC4

The release profiles in phosphate buffer solution pH 6.8 of Insulin loaded chitosan nanoparticles entrapped in IC2-IC4 batches of uncoated alginate beads are shown in Table 6 and Figure 10. As depicted in fig.no.10, Insulin release of about 82.16%, 80.91% and 78.01% was observed in batches IC2, IC3 and IC4 respectively. The release might be attributed to the water uptake/ swellable nature of alginate beads. The release profile of Insulin batch IC4 given in Table 6 and

Figure 10. The delayed release of insulin from the batch IC4 might be due to increase in the hardening time from 10 to 20 minutes.

Percentage insulin release of alginate uncoated beads batches ID2-ID4

The release profiles in phosphate buffer solution pH 6.8 of Insulin loaded chitosan nanoparticles entrapped in ID2-ID4 batches of uncoated alginate beads are shown in Table 7 and Figure 11. As depicted in Figure 11, Insulin release of about 85.98%, 80.11% and 75.19% was observed in batches ID2, ID3 and ID4 respectively. The release might be attributed to the water uptake/ swellable nature of alginate beads. The release profile of Insulin batches ID2 and ID3 above 80% and ID4 given in Table 7 and Figure 11 showed release of 75%. This might be due to increase in hardening time.

Selection of optimized batch of insulin loaded chitosan nanoparticles entrapped uncoated alginate beads

From the release profiles in phosphate buffer solution pH 6.8 of Insulin loaded chitosan nanoparticles entrapped in batches of uncoated alginate beads ID2, ID3, IC2 and IC3 were selected as the optimized batches. The mean content uniformity of optimized batches ID2, ID3, IC2 and IC3 of insulin was found to be 85.21%, 80.22%, 82.35% and 80.90% respectively.

IR study of optimized formulation of Insulin ID2

IR studies for formulations were done to confirm presence of Insulin in uncoated sodium alginate beads. IR spectrum was obtained on FTIR with diffused reflectance assembly shown in Figure 12. The encapsulation of insulin in ID2 formulation was confirmed from the presence of IR spectra.

Stability studies

Intermediate stability studies were carried out for 0 and 45 days on optimized formulations. The samples were stored at $40^\circ\text{C} \pm 2^\circ\text{C}$ and $75\% \pm 5\%$ RH to assess their stability. The protocol of stability studies were in compliance with ICH guidelines for stability testing intended for the global market. No sticking of beads was observed for both optimized formulations. The dissolution profiles for 0 and 45 days that were placed for Stability studies are studied.

Stability studies of Insulin

Content uniformity of insulin

The mean content uniformity of optimized insulin ID2 after 0 and 45 days was found to be 85.14% and 80.12% respectively.

In-vitro Drug release studies of optimized batch ID2 in pH 6.8 PBS at 0 and 45 days

The release profiles in PBS pH 6.8 of optimized Insulin formulation ID2 after 0 and 45 days are shown in Table 8 and Figure 13 As depicted in Figure 13, insulin release of about 85.98% and 82.39% was observed at the end of 180 minutes for both 0 and 45 days respectively. This indicates that the optimized Insulin formulation ID2 was stable till 45 days.

Differential Scanning Colorimetric Analysis

The differential scanning calorimetry was carried out for the pure sodium alginate, calcium alginate, pure insulin and selected formulation of alginate insulin loaded beads to study compatibility or any interaction of drug and polymer in matrix beads and is shown in fig.no.14, 15, 16 and 17

respectively. The thermogram of beads indicated endothermic peak at 83.2 °C for insulin corresponding to its melting point. There was no significant change in the position of peak and its intensity. Thus, DSC study showed no interaction between the drug and polymers.

In-vivo Animal activity study

Oral Glucose Tolerance Test (OGTT)

Insulin-loaded bead were administered orally to overnight fasted Non diabetic rats. The reduction of the initial blood glucose levels versus time after intragastric insulin beads and subcutaneous administration of insulin is depicted in Figure 18 and Table 9. After subcutaneous administration of insulin the blood glucose level significantly ($p < 0.01$) decreases to 130.01, 97.28, and 89.28 mg/dl as compared with control group 137.91, 128.17 and 112.21 mg/dl after 30, 90 and 150 min. respectively.

After the administration of intragastric insulin beads the blood glucose level, the decrease started after the lag period of 150 minutes and decreased up to 30th min as compared with control group while after that significant ($p < 0.05$) decrease was showed 114.32 and 104.48 mg/dl in T1, 115.04 and 130.27 mg/dl in T2, 114.78 and 97.17 mg/dl in T3 and 106.18 and 94.04 mg/dl in T4 as compared with control group 128.17 and 112.21 mg/dl after 90 and 150 min. respectively.

From above observation it has been concluded that the intragastric insulin beads showed onset of action after lag period of 150 minutes at 30th min and the better results were shown by T4.

The internal insulin decrease was found to be 0.3IU. 41µg/dl which was the decrease in glucose level after 150th min of administration of standard insulin (0.52 IU) and for 1 IU was equivalent to 78.8µg/dl. Thus the decrease in glucose level on administration of insulin beads T1, T2, T3 and T4 after the lag period of 150 minutes were calculated. Table.no.10 shows the % glucose drop after administration of standard insulin and test insulin beads T1, T2, T3 and T4.

REFERENCES

1. Ieva E, Trapani A, Cioffi N. Analytical characterization of chitosan nanoparticles for peptide drug delivery applications, *Anal Bioanal Chem* 2009; 393: 207–215. <http://dx.doi.org/10.1007/s00216-008-2463-4>
2. <http://en.wikipedia.org/wiki/Protein> (Access Date - 9 February 2013)
3. <http://en.wikipedia.org/wiki/Insulin> (Access Date - 13 February 2013)
4. Yan W. Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate, *International Journal of Pharmaceutics* 2005; 295: 235–245. <http://dx.doi.org/10.1016/j.ijpharm.2005.01.042>
5. Morishita M, Peppas NA. Is the oral route possible for peptide and protein drug delivery? *Drug discovery today* 2006; 11: 905-910. <http://dx.doi.org/10.1016/j.drudis.2006.08.005>

6. Arhewoh IM. Optimizing oral systems for the delivery of therapeutic proteins and peptides, *African Journal of Biotechnology* 2005; 4 (13): 1591-1597.
7. Sashiwa H, Shigemasa Y. Chemical modification of chitin and chitosan 2: preparation and water soluble property of N-acylated or N-alkylated partially deacetylated chitins, *Carbohydrate Polymers* 2009; 39: 127-138. [http://dx.doi.org/10.1016/S0144-8617\(98\)00167-2](http://dx.doi.org/10.1016/S0144-8617(98)00167-2)
8. Yang H. Synthesis and characterization of chitosan-g-poly-(D,L-lactic acid) copolymer, *Chines chemical letters* 2005; 16: 123-126.
9. Bhattaraj N. Chitosan and lactic acid-grafted chitosan nanoparticles as carriers for prolonged drug delivery, *International Journal of Nanomedicine* 2006; 181-187. <http://dx.doi.org/10.2147/nano.2006.1.2.181>
10. Natalija G, Makuska R. Synthesis and study of water-soluble chitosan-O-poly (ethylene glycol) graft copolymers, *European polymer journal* 2009; 40: 685-691.
11. Amiji MM. Synthesis of anionic poly(ethylene glycol) derivative for chitosan surface modification in blood-contacting applications, *Carbohydrate Polymers* 1997; 32: 329-332. [http://dx.doi.org/10.1016/S0144-8617\(97\)00006-4](http://dx.doi.org/10.1016/S0144-8617(97)00006-4)
12. Yao F. A study on cytocompatible poly(chitosan-g-l-lactic acid), *Polymer* 2003; 44: 6435-6441 [http://dx.doi.org/10.1016/S0032-3861\(03\)00676-1](http://dx.doi.org/10.1016/S0032-3861(03)00676-1)
13. Xiong FU, Yan. Water-soluble chitosan nanoparticles as a novel carrier system for protein delivery, *Chinese science bulletin* 2007; 52: 883-889. <http://dx.doi.org/10.1007/s11434-007-0127-y>
14. Cuisine Innovation – www.cuisineinnovation.fr (Access Date - 17 February 2013)
15. http://www.scitopics.com/The_oral_delivery_of_therapeutic_proteins.html. (Access Date - 19 February 2013)
16. Mandal S. Development and evaluation of calcium alginate beads prepared by sequential and simultaneous methods, *Brazilian journal of pharmaceutical sciences* 2010; 46: 785-793.
17. Gazori T, Mohammad RK, Ebrahim A, Parisa Y, Alireza N, Ismaeil H. Evaluation of Alginate/Chitosan nanoparticles as antisense delivery vector: Formulation, optimization and in vitro characterization 2009; 77: 599-606.
18. Takka S, Aybige G. Evaluation of chitosan/alginate beads using experimental design: formulation and *in vitro* characterization. *American association of pharmaceutical scientist* 2010; 11(1): 460-466.
19. Patel FM. Release of metformin hydrochloride from ispaghula - sodium alginate beads adhered cock intestinal mucosa. *International Journal of Current Pharmaceutical Research* 2011; 3(3).
20. Jain D, Panda AK, Majumdar DK. Eudragit S100 entrapped insulin microspheres for oral delivery. *American association of pharmaceutical scientist Pharm Sci Tech* 2005; 1-27.
21. S.R. Keller. Insulin Tolerance Test protocol. *Cell* 1997; 88: 561-572.
22. S.V. Tembhurne, D.M. Sakarkar. Hypoglycemic effects of fruit juice of *murraya koenigii* (l) in alloxan induced diabetic mice. *International journal of pharma tech research CODEN (USA) IJPRIF* 2009; 1: 1589-1593.
23. Panchal II. Design, synthesis and biological evaluation of some substituted sulphonyl urea/ guanidine derivatives as hypoglycemic agents. *J.chem. pharm* 2010; 609-617.

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