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Design and Characterization of Insulin Nanoparticles for Oral Delivery

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ABSTRACT

Insulin is a polypeptide hormone and usually administered for treatment of diabetic patients subcutaneously. The aim of this study was to investigate the efficiency of insulin loaded chitosan-xanthan gum nanoparticles for oral delivery. Various formulations of nanoparticles (F1-F8) were prepared by Ionotropic gelation method using chitosan and xanthan gum as polymers and sodium tripolyphosphate as cross-linking agent. The prepared formulations were characterized for percentage practical yield, entrapment efficiency, drug loading efficiency, swelling studies, *in vitro* release studies. Further studies like particle size analysis, zeta potential, polydispersity index, release kinetics, *in vivo* studies were performed for the optimized formulation F8 which was prepared using chitosan (0.1%w/v), xanthan gum (0.2%w/v) and sodium tripolyphosphate (0.2%w/v). The results showed that the entrapment efficiency was 65.6%, drug loading efficiency was 9.34% and swelling index at pH 1.2 was 42.97 and at pH 7.4 were 312.6. F8 Formulation showed drug release of 14.89% at second hour and 77% at eighth hour in acidic pH 1.2 & in phosphate buffer pH 7.4 respectively. The mean particle size was 81.2nm, Zeta potential was -22.04mV, polydispersity index was 0.15. Evaluation *in vitro* showed that insulin nanoparticles were accorded with zero order release kinetics and follows Highuchi model diffusion as drug release mechanism and from peppas plot the drug transport mechanism was found to be super case II transport as (n>0.89). The *in vivo* studies showed that maximum reduction of blood glucose levels was 52%. The results suggest that the Ionotropic gelation method using chitosan and xanthan gum polymers may provide a useful approach for entrapment of hydrophilic polypeptides. The synergistic hypoglycemic activity can be achieved from insulin loaded chitosan-xanthan gum nanoparticles since xanthan gum also exhibits the hypoglycemic activity.

Key words: Nanoparticles, Ionotropic gelation, Diabetes mellitus, Insulin, Chitosan, Xanthan gum.

INTRODUCTION

Diabetes is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin or because cells do not respond to the insulin that is produced. Parenteral route is the only route for administration of insulin (Avadi MR *et al.,* 2011*)*. More over with subcutaneous injection of insulin it causes local discomfort due to frequent injections, Hypoglycemia due to overdose of insulin (Mahkam M 2010).

From the beginning of pharmaceutical era, the oral route has always dominated over any other routes of drug delivery. This can be accredited to the numerous advantages of the oral route, such as ease of administration, patient compliance and economical production methods. Production of pharmaceutically active proteins, such as insulin, in large quantities has become feasible (Awadhesh kumararya *et al.,* 2008 *and* Liang HF *et al.,* 2004). The oral route is considered to be the most convenient and comfortable means for administration of insulin for less invasive and painless diabetes management, leading to a higher patient compliance (Krauland AH *et al*., 2004).

Nevertheless, the intestinal epithelium is a major barrier to the absorption of hydrophilic drugs, as they cannot diffuse across epithelial cells through lipid-bilayer cell membranes to the blood stream. Therefore, attention has been given to improving the Para cellular transport of hydrophilic drugs (Lamprecht A *et al*., 2006 and Kotze AF *et al*., 1998).

A variety of intestinal permeation enhancers including chitosan (CS) have been used for the assistance of the absorption of hydrophilic molecules (Ward PD *et al.,* 2000 and Agnihotri SA *et al.,* 1995). Therefore, a carrier system is needed to protect protein drugs from the harsh environment in the stomach and small intestine, if given orally. IN recent years, many strategies have been developed to enhance oral protein delivery. Among these

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approaches, nanoparticulate systems have attracted interest in drug delivery due to the fallowing reasons. Firstly nanoparticles are able to protect active agents from degradation (Florence AT *et al*., 1995).secondly they can improve the drug mucosal transport (Lowe PJ and Temple CS 1994) and transcytosis by M-cells, and thirdly particulate system provide controlled release properties for encapsulated drugs (Janes KA *et al*., 2001). In recent years, ion gelation or polyelectrolyte complex formation (PEC) has drawn increasing attention for producing nanoparticles containing peptides (Galindo-Rodríguez SA *et al.,* 2005). The nanoparticles prepared by this method have several characteristics favorable for cellular uptake and colloidal stability, including suitable diameter and surface charge, spherical morphology, and a low polydispersity index indicative of a relatively homogeneous size distribution (Mao HQ *et al.,* 2002). In addition, this method has the advantage of not necessitating aggressive conditions such as the presence of organic solvents and/or sonication during preparation; therefore, minimizing possible damage to proteins and peptides during ion gelation formation (Bayat A *et al*., 2008). Additionally, CS nanoparticles (NPs) enhanced the intestinal absorption of protein molecules to a greater extent than aqueous solutions of CS *in vivo* (Mao HQ *et al*., 2002). Xanthan gum was anionic polymer which has synergestic hypoglycemic activity along with insulin .The insulin loaded NPs coated with mucoadhesive CS and Xanthan gum may prolong their residence in the small intestine, infiltrate into the mucus layer and subsequently mediate transiently opening the tight junctions between epithelial cells while becoming unstable and broken apart due to their pH sensitivity and/or degradability. The insulin released from the broken-apart NPs could then permeate through the Para cellular pathway to the bloodstream, its ultimate destination.

MATERIALS

Insulin was Gift sample from Sigma Aldrich, Mumbai, Chitosan from Otto's laboratory, Chennai. Xanthan gum was gift sample from Vivi Med Labs, Hyderabad. Glacial acetic acid, Sodium tripolyphosphate and Streptozotocin from SRL Chemicals, Chennai.

METHODOLOGY

Accurately weighed quantity of insulin was taken in a test tube, subsequently insulin was dissolved by adding few drops of 0.1N Hcl to the test tube. To prepare an Organic Phase, weighed quantity of chitosan was dissolved in required quantity of glacial acetic acid (1%v/v) solution. The chitosan solution was stirred at 2200 rpm with a mechanical stirrer at room temperature 25^0C. To this add previously prepared drug solution and allowed for stirring. To prepare an aqueous phase, various ratios of xanthan gum were taken and dissolve in required quantity of distilled water. The organic phase of chitosan solution along with insulin was kept under mechanical stirring at 2200 rpm.

To the above solution add aqueous phase of xanthan gum solution drop wise using syringe and add different concentrations of sodium tripolyphosphate allow for continuous stirring until to evaporate the organic solvent result in formation of solution. It was centrifuged for 15 min at 17,000 rpm, 4^0C and removes the supernatant and kept the precipitate in lyophilizer to form powder form of nanoparticles for 24 hrs.

Composition of Different Formulations of Nanoparticle

Table.1 Formulations of Oral Insulin Nanoparticles

S.No	Ingredients	F1	F2	F3	F4	F5	F6	F7	F8
1	Insulin (mg)	5	5	5	5	5	5	5	5
2	Chitosan (%w/v)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
3	Xanthan gum (%w/v)	0.05	0.10	0.15	0.20	0.05	0.10	0.15	0.20
4	Sodium tripolyphosphate (%w/v) (mL)	(0.1)	(0.1)	(0.1)	(0.1)	(0.2)	(0.2)	(0.2)	(0.2)
5	Glacial acetic acid (1%v/v) (mL)	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
6	Distilled water (mL)	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

Evaluation of insulin loaded chitosan - xanthan gum nanoparticles

Percentage Practical Yield

Percentage practical yield of the nanoparticles was done by taking the total amount of ingredients before formulation and total amount of the nanoparticles formed. Each measurement was repeated for three times.

$$\text{Percentagepractical yield} = \frac{\text{Theoretical yield (mg)}}{\text{Practical yield(mg)}} \times 100$$

Determination of Entrapment Efficiency

For determination of insulin entrapment efficiency, 10 ml of insulin nanoparticle suspension was centrifuged at 17,000 rpm for 15 min. Insulin concentration in the supernatant was measured by UV spectrophotometry at 271 nm. Insulin entrapment efficiency was expressed as the ratio of the insulin amount measured in the supernatant to the total insulin amount added. Each measurement was repeated for three times.

$$\text{Entrapment efficiency (\%)} = \frac{Tp - Tf}{Tp} \times 100$$

Where,

Tp = Total drug used to prepare nanoparticles

Tf = Free drug in the supernatant

Determination of Drug Loading Efficiency

The Drug loading efficiency of nanoparticles was determined by first calculated the drug present in

nanoparticles and weight of the dried recovered nanoparticles. The loading efficiency was obtained by following formula. Each measurement was repeated for three times.

$$\text{Loading efficiency (\%)} = \frac{\text{weight of drug in nanoparticles}}{\text{Weight of nanoparticles recovered}} \times 100$$

Swelling Studies

To measure the swelling, preweighed the dry drug free nanoparticles were immersed in various buffer solutions (pH 1.2 and pH 7.4) at 37^0C. After excess water on the swollen samples was measured at various time intervals. The procedure was repeated until there was no further weight increase. The degree of selling was calculated according to the following formula. Each measurement was repeated for three times

$$SW(\%) = \frac{(Ws - Wd)}{Wd} \times 100$$

Where, Ws and Wd represent the weight of swollen and dry samples, respectively.

***In Vitro* Release Studies**

Weighed accurately 4mg of nanoparticles were dispersed in 20mL 0.1N Hcl taken in a sealed jacketed beaker at 37^0C. The suspension was stirred continuously at a constant rate using magnetic stirrer with bead. Aliquots of 1mL were taken at specific time intervals up to 2hr. The drug content in the dissolution medium was determined by centrifugation of nanoparticles at 17,000 rpm for 15 min and then the supernatant was collected and analyzed by using UV-Visible spectrophotometer at 271nm. Each measurement was repeated for three times.

After 2hrs the nanoparticles were transferred with 20mL of phosphate buffer at pH 7.4. The suspension was stirred continuously at a constant rate using magnetic stirrer, aliquots of 1mL were taken at specific time intervals up to 8hrs.

Drug content in the dissolution medium was determined using the sedimenting the nanoparticles by centrifugation at 17,000 rpm for 15min, then the supernatant was collected and analyzed by using UV-Visible spectrophotometer at 271nm. Each measurement was repeated for three times.

The % drug release from nanoparticles was calculated using the formula

$$\% \text{ Drug release of insulin} = \frac{\text{Cummulative drug dissolved}}{\text{Total amount of drug present in the nano particles}} \times 100$$

Particle Size

The nanoparticles after lyophilization are then collected and analyzed by Malvern Zeta Sizer for particle size analysis.

Zeta Potential

Zeta potential is a key indicator for evaluating the stability of dispersed colloid system. The higher of the absolute value of zeta potential, the bigger the electrostatic repulsion between each nanoparticle in solution, and can result in more stable nanoparticles in solution and maintains a homogeneous, transparent and stable state for a long time without deposition of nanoparticles.

Polydispersity Index

Polydispersity index was done for lyophilized nanoparticles. Polydispersity (non-uniform size distribution) was calculated by the following formula

Polydispersity Index= $(D_{0.9} - D_{0.1}) / D_{0.5}$

Where $D_{0.9}$, $D_{0.5}$ and $D_{0.1}$are the particle diameters determined at the 90th, 50th and 10th percentile of undersized particles respectively. High polydispersity index value indicates the high level of non-uniformity and is used to characterize the nanoparticles as monodisperse, homogeneous and heterogenous systems.

Surface Morphology

Scanning electron microscopy is an excellent tool for physical observation of morphological features of nanoparticles. It is helpful to examine the shapes and for qualitative assessment of morphology of nanoparticles.

Release Studies

Data obtained from *in vitro* release studies are fitted to various kinetic equations.

The kinetic models are,

- **Zero order equation : ($Q=k_0t$)**
- **First order equation : {$\ln (100 - Q) = \ln Q - k_1t$}**
- **Higuchi equation : ($Q= kt_{1/2}$)**
- **Peppasequation:** $\frac{Mt}{M\infty} = k.t_{nw}$

Further, to find out the mechanism of drug release, first 60% drug release is fitted in Korsmeyer and Peppas equation (Q = kpt n). Where, Q is the percent of the drug release at time 't' and k_0 and k_t are the coefficients of the equations and 'n' is the release exponent. The 'n' value is used to characterize different diffusion mechanisms.

***In Vivo* Studies**

Induction of Diabetes

Diabetes was induced in rats (group II, III, IV& V) by intraperitoneal injection of streptozotocin (40mg/kg body weight for three consecutive days) dissolved in citrate buffer pH 4.5.The rats were considered diabetic when the fastened glucose level exceed 160mg/dl at 2 weeks following the streptozotocin treatment. Prior to administration of oral insulin-loaded nanoparticles, the animals were fasted for 48hrs.

Injection of Nanoparticle Solution:

The rats were restrained in supine position. The insulin loaded nanoparticles with dose of 25 IU/Kg body weight of rats was administered through mouth using feeding needle. During experiment 0.5ml aliquot of blood was collected from the retro-orbital vein at 1, 2, 3, 4, 6, 8hrs following dosing. Blood serum was separated by centrifugation at 8000-10,000 rpm for 15 min. The serum glucose levels were determined by auto-analyzer using

glucose-B-test kit.

Pharmacological Studies

Healthy adult male wistar rats were randomly divided into 5 groups (n=4) as normal, control, standard, treatment with nanoparticles, treatment with plain insulin.

RESULTS AND DISCUSSION

Table.2 Determination of insulin loaded chistosan-xanthan gum nano particles oral activity

RESULTS	F1	F2	F3	F4	F5	F6	F7	F8
Percentage practical yield	85.0	86.0	86.6	88.5	87.5	88.0	88.5	89.0
% Entrapment Efficiency	46.2	50.1	55.3	60.1	49.3	56.2	61.3	65.2
Drug Loading Efficiency (%)	13.5	11.6	10.6	9.77	14.0	12.8	11.4	9.34
Swelling Studies	196.12	205	218	235	241	256	271	312

Figure.1 % Cumulative Amount of Drug Release from Formulations F1-F8

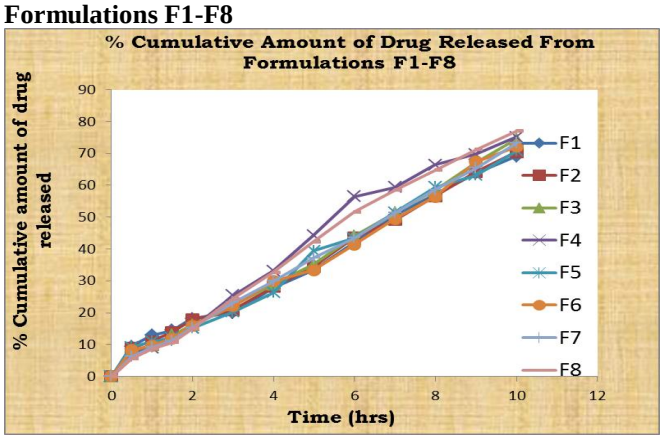


Figure.2 Percentage Reduction of Blood Glucose Levels

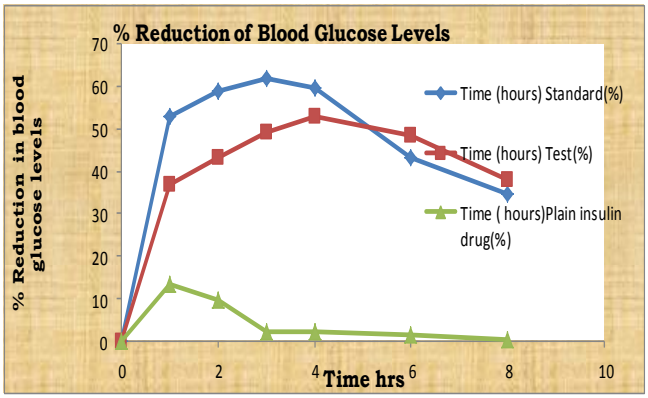


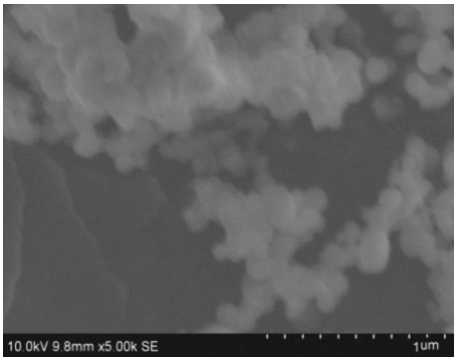
Table.3 Kinetic profile for F8 formulation

Formulation	Zero order plot	First order plot	Higuchi plot	Peppas plot	Peppas plot Slope n value
F8	0.995	0.931	0.936	0.796	1.163

Table.4 Particle size Analysis

Formulation	Particle size	Zeta potential	Poly-dispersity Index
F8	81.2nm	-22mV	0.15

Figure.3 Surface Morphology



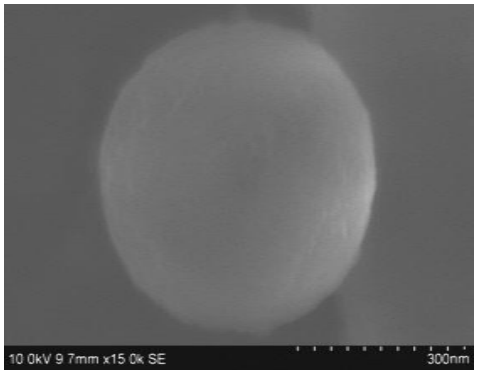


Table.5 Percentage Reduction of Blood Glucose Levels

Time (hours)	Standard (%)	Test (%)	Plain insulin Drug (%)
0	0	0	0
1	52.85±4.04	36.88±2.64	13.22±2.15
2	58.77±6.57	43.21±1.78	9.48±1.52
3	62.00±4.31	49.39±1.56	2.14±2.12
4	59.71±3.85	52.94±1.49	2.14±2.41
6	43.17±1.53	48.51±2.54	1.26±1.63
8	34.62±4.27	38.11±1.53	0.35±2.67

Percentage Practical Yield

The % practical yield of nanoparticles obtained from F1-F8 varied from 85-89%. The % practicalyield increases from F1-F8 it may be due to increase in concentration of Xanthan gum and NaTPP (0.2%W/V). The low %practical yield in formulations may also be attributed due to loss of polymer solution during various steps of processing such as sticking of polymer solution to glass containers etc.

Entrapment Efficiency

The electrostatic interactions between positively charged group of chitosan and negatively charged groups of Arabic gum play an important role in the entrapment efficiency of insulin in nanoparticles. These results have shown that increasing the concentrations of chitosan, Xanthan gum and sodium tripolyphosphate or amount of insulin used in nanoparticle preparation are important factors in Entrapment efficiency. The entrapment efficiency for the nanoparticles ranges from 46.2-65.2%.The highest entrapment efficiency for F8 formulation is due to the increasing concentration of NaTPP as crosslinking agent along with Xanthan gum concentration.

Drug Loading Efficiency

The drug loading efficiency for the formulations F1-F8 was found to be 9.34-14.05%.

Swelling Studies

The results of swelling behavior of the eight formulations were shown in table no 20,21. The results showed that there was slower rate of swelling in pH 1.2and greater rate of swelling in phosphate buffer pH7.4 is due to the presence of Xanthan gum with increasing ratios, since it has pH value of >6.5 and therefore the polymer complexes may swells in pH medium higher than 6.5.

***In Vitro* Release Studies**

In vitro release studies were performed for formulations F1-F8. The studies for Insulin nanoparticles were performed at pH conditions 1.2 and 7.4. In general a burst effect of Insulin release was expected in acidic medium pH (1.2) due to the solubility of chitosan and Insulin in acidic environment but, the results obtained showed that there was retarded release of Insulin in acidic medium this may be mainly due to the poly-electrolyte complexes formed between chitosan and Xanthan gum prevents its release and chitosan has solubility in acidic medium but in nanoparticles it was surrounded by negatively charged Xanthan gum and due to all these reasons insulin release and degradation from acidic and enzymatic environment is prevented with increasing ratio of Xanthan gum from formulations F1 to F8. The solubility of chitosan and Insulin in phosphate buffer was lower than that in acidic medium and so burst effect was not observed. Xanthan gum has pH value of 7-7.5 aqueous solution and therefore the polymer chain may swell in pH greater than 6.5 and obtain more porosity in the structure of nanoparticle resulting in more Insulin release. In overall the results showed that the maximum release of Insulin 77% was obtained from F8 for a period of 8hrs since the formulation has high swelling index. From the above *in vitro* release studies F8 was considered as optimized formulation since it shows maximum drug release was observed for a period of 8hours in pH 7.4 and minimum drug release in acidic pH 1.2.

For Optimized Formulation

R^2 Values for F8 Formulation

From graphical method it is revealed that Formulation F8 followed zero order release and Higuchi model diffusion and from Peppas plot the drug transport mechanism was found to be super case II transport as (n>0.89) Table.3). The surface morphology of the nanoparticles prepared by using chitosan with Xanthan gum (1:2) are centrifuged at 17,000 rpm and lyophilized. These nanoparticles are then analyzed for surface morphology using Scanning electron microscope and found that they are spherical shaped, with homogenous morphology (Figure.3). *In vivo* release studies was performed for the optimized formulation F8, by comparing standard, test, and plain insulin, the results shows that maximum reduction of blood glucose levels with standard drug was up to 62% at 3rd hour, for test formulation it was up to 52% at 4th hour, for plain insulin it was up to 13.22% at 1st hour (Figure.2).

CONCLUSION

A systematic study involving the enhancement of oral bioavailability of insulin was made by preparation of nanoparticles using Ionotropic gelation technique. Various formulations of nanoparticles (F1-F8) were prepared using chitosan and xanthan gum as polymers and sodium tripolyphosphate as cross linking agent. The optimized formulation F8 was prepared using chitosan (0.15%w/v), xanthan gum (0.4%w/v) and sodium tripolyphosphate (0.2%w/v). The results showed that the, entrapment efficiency was 65.6%, drug loading efficiency was 16.34%, swelling index at pH 1.2 was 42.97 & at pH 7.4 was 312.6. F8 Formulation showed drug release of 14.89% at second hour and 77% at eighth hour in acidic pH 1.2 & in phosphate buffer pH 7.4 respectively. For the optimized formulation F8 mean particle size was 81.2nm, Zeta potential was -22.04mV, polydispersity index was 0.15. Evaluation *in vitro* showed that insulin nanoparticles were accorded with zero order release kinetics and follows Highuchi model diffusion as drug release mechanism and from peppas plot the drug transport mechanism was found to be super case II transport as (n>o.89).The *invivo* studies showed that maximum reduction of blood glucose levels was 52%. Results showed that chitosan nanoparticles are able to enhance permeation of Insulin as hydrophilic models through intestinal epithelium. *In vivo* study with nanoparticles demonstrated a prominent hypoglycaemic effect, suggesting that the polymers could effectively protect insulin from enzymatic degradation, from variable pH of GIT tract. The synergistic hypoglycaemic activity can be achieved from insulin loaded chitosan-xanthan gum nanoparticles since xanthan gum also exhibits the hypoglycaemic activity.

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