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ScienceDirect
International Journal of Pharmaceutics 356 (2008) 259–266
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Nanoparticles of quaternized chitosan derivatives as a carrier for colon delivery of insulin: Ex vivo and in vivo studies

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Received 18 September 2007; received in revised form 14 November 2007; accepted 18 December 2007
Available online 5 January 2008

Abstract

The aim of the present study was to develop insulin nanoparticulate systems by using chitosan (CS), triethylchitosan (TEC) and dimethylethylchitosan (DMEC, a new quaternized derivative of chitosan) for colon delivery.

The nanoparticles were prepared by the polyelectrolyte complexation (PEC) method. Particle size distribution, zeta potential and polydispersity index of the nanoparticles were determined using dynamic light scattering technique. Transmission electron microscopy (TEM) was also used to observe the morphology of the nanoparticles. It was found that the nanoparticles carried positive charges and showed a size distribution in the range of 170–270 nm with spherical morphology and smooth surface structure. The amount of insulin loaded into the nanoparticles was determined by measuring the association efficiency and also the content of insulin in the nanoparticles. Insulin loading was found to be more than 80% for all of the nanoparticles. In vitro release studies showed a small burst effect at the beginning and then a sustained release characteristic for 5 h. Ex vivo investigations revealed better insulin transport across the colon membrane of rats for nanoparticles made with quaternized derivatives than those made of chitosan. In vivo studies in rats have showed enhanced colon absorption of insulin by using these nanoparticles compared to free insulin in diabetic rats. The insulin absorption from the rat's colon was evaluated by its hypoglycemic effect.

Keywords: Chitosan; Triethyl chitosan; Dimethylethyl chitosan; Insulin; Nanoparticles; Ex vivo and in vivo studies

1. Introduction

Significant advances in biotechnology and biochemistry have led to the discovery of a large number of bioactive molecules and vaccines based on peptides and proteins. However, these compounds still have to be administered by injection which is still a costly way and have a very low patient acceptability. It is well known that oral administration is the most convenient route for drug delivery but peptides and proteins have low bioavailability due to their instability in the GI tract and enzymatic degradation when administered orally (Owens et al., 2003). Moreover, oral administration of insulin would be the most physiological route due to the fact that insulin would be directly channeled from the intestine or colon to the liver to avoid peripheral hyperinsulinemic effects (Saffran et al., 1997).

Colon targeted drug delivery is useful in improving the absorption of peptide drugs via the GI tract (Saffran et al., 1986). Colon has been determined as an ideal site for peptide absorption. Site specific drug delivery to the colon is of special interest for drugs which are instable in the upper part of the gastrointestinal tracts. For example, the oral administration of protein and peptide drugs, such as insulin, is precluded because of small intestinal peptidase activity. The colon is thought to have lower enzymatic activity than other regions of the GI, hence if such

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doi:10.1016/j.ijpharm.2007.12.037

drugs can be delivered to this site, a greater efficiency of absorption would be expected (Ali Asghar and Chandran, 2006). Due to negligible activity of brush-border membrane, peptidase activity, and much less activity of pancreatic enzymes, the colon has been considered to be more suitable for delivery of peptides and proteins in comparison to the small intestine (Ikesue et al., 1991).

To date, various strategies have been developed to design a successful oral insulin delivery system, including co-administration with absorption enhancers (Radwant and Aboul-Enein, 2002; Mesiha et al., 1994), or enzyme inhibitors (Morishita et al., 1992; Bernkop-Schnurch, 1998), chemical modification (Asada et al., 1995), and lipid base carriers as liposomes (Takeuchi et al., 1996). Nevertheless, these approaches show low bioavailability and also some of them exhibit several negative effects such as irritation of the intestinal mucosal membrane and impairment of the membrane barrier. In recent years polymeric nanoparticles have been used as potential drug delivery devices because of their ability to protect proteins and peptides from degradation in the gastrointestinal tract by proteolytic enzymes (Krauland and Alonso, 2007; Vila et al., 2002; Takeuchi et al., 2001). This system can increase the stability of these compounds and possess useful controlled release properties. Damge et al. (1988) have shown that nanoparticles composed of polyalkylcyanoacrylate derivatives enhance the absorption of orally administered insulin.

Although a wide variety of techniques is currently used for producing polymeric nanoparticles, but most of these methods involve the use of organic solvents, heat or vigorous agitation and also formation of potentially toxic by-products from the use of these solvents which may be very harmful to biopharmaceuticals like proteins and peptides.

In recent years, polyelectrolyte complex formation (PEC) has drawn increasing attention for producing nanoparticles containing peptides (Mao et al., 2001). 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. In addition, this method has the advantage of not necessitating aggressive conditions like organic solvents and sonication during preparation, therefore minimizing possible damage to proteins and peptides during PEC formation (Mao et al., 2006). Recent studies have shown that polyelectrolyte complexation is a potentially useful technique for fabricating insulin delivery systems for peroral administration (Jintapattanakit et al., 2007).

Due to the hydrophilic properties of proteins and peptides, many different hydrophilic nanoparticles have been developed as drug carriers. Chitosan, a hydrophilic polysaccharide with excellent biocompatible and biodegradable properties has been used extensively in drug delivery (Agnihotri et al., 2004). However, its limited solubility, which is restricted to dilute acidic aqueous media (pH below 6.0), allows its application only in acidic condition but not in the intestinal tract where the pH values are higher than 6.5. Quaternized derivatives of chitosan have been evaluated to overcome this drawback of chitosan at neutral pH or weakly alkaline values (Thanou et al., 2001). Chitosan is positively charged at pH < 6.5 due to protonation of the amino groups and quaternized derivatives of chitosan are permanently positively charged (Curti et al., 2003). Insulin is negatively charged at a pH above its isoelectric point (apparent pI 6.4) (Mao et al., 2006). Hence insulin nanoparticles can easily be created by mixing oppositely charged molecules via electrostatic interaction between both entities.

The aim of this study is to introduce a nanoparticulate system using two new quaternized derivatives of chitosan, TEC and DMEC by PEC method for colon delivery of insulin. The nanoparticles have been characterized in terms of size, zeta potential, polydispersity index and association efficiency. Also in vitro release, ex vivo and in vivo studies were investigated to determine the efficacy of this system and the effective factors on absorption via this system.

2. Materials and methods

2.1. Materials

Chitosan (98.1% deacetylated, Mw 126 kDa, viscosity of 1% w/v solution, 22 mPas) was obtained from Primex, Iceland. Crystalline recombinant human insulin (28.3 IU/mg) was purchased from Eli Lilly, France; streptozotocin (SZ) from Pharmacia & Upjohn, USA; ketamine hydrochloride injection from Rotexmedica, Germany and chlorpromazine injection from Darou pakhsh, Iran. DMEC and TEC were synthesized in our laboratory as described by Bayat et al. (2006) and Avadi et al. (2003), respectively. All other materials were of pharmaceutical and analytical grades and were used as received.

2.2. Synthesis of chitosan derivatives

DMEC and TEC were synthesized by partial quaternization of chitosan as described by Bayat et al. and Avadi et al., respectively. We used low molecular weight chitosan (98.1% deacetylated) and synthesized DMEC by a two-step method (Bayat et al., 2006) and TEC in one step synthesis (Avadi et al., 2003). Under optimized conditions, degree of quaternization of about 52 and 51% were obtained for DMEC and TEC, respectively, which has been shown to be optimum for the permeation enhancing effect (Hamman et al., 2002).

2.3. Preparation of insulin nanoparticles

Chitosan (CS) was dissolved in 0.25% acetic acid aqueous solution at 1 mg/mL concentration under stirring and adjusting the pH to 5.5 with 1N NaOH. At this pH more than 90% of the amine groups of CS are protonated (Mao et al., 2006). As TEC and DMEC are water soluble, these polymers were dissolved in purified water at the same pH and concentration as the CS solution. Insulin (INS) solution (1 mg/mL) was prepared by dissolving the insulin powder in 0.01N HCl, and the pH was adjusted to 8.0 with 1N NaOH. Polyelectrolyte nanoparticles were prepared by adding 1 mL polymer solution to an equal volume of insulin solution in a beaker under gentle magnetic stirring at room temperature. Nanoparticles were separated from

aqueous phase by ultracentrifugation (Beckman L5-65) with 15,000 rpm at 4 °C for 30 min. The supernatant was collected and used for free insulin measurement (association efficiency) by HPLC, and the sediment was frozen at −70 °C for 1 h. Freeze drying was performed using the freeze dryer (LTE scientific LTD). Particles were dried for 48 h at a working pressure of 0.8 mbar corresponding to a temperature of −43 °C.

2.4. Characterization of the nanoparticles

Particle size of the nanoparticles was determined by photon correlation spectroscopy using a Zetasizer 3000HS (Malvern Instruments, Malvern, UK). Samples were diluted with purified water and the measurements were performed at a scattering angle of 90° and at a temperature of 25 °C. The diameter is calculated from the autocorrelation function of the intensity of light scattered from particles, assuming a spherical form for the particles.

The polydispersity index (PdI) is a measure of dispersion homogeneity and ranges from 0 to 1. Values close to 0 indicate a homogeneous dispersion while those greater than 0.3 indicate high heterogeneity.

The particle charge was quantified measuring the zeta potential by Laser Doppler Anemometry using a Zetasizer 3000HS (Malvern Instruments, Malvern, UK). Samples were diluted with purified water. For the measurements samples were placed in the electrophoretic cell where a potential of 150 mV was established. The results are presented as the mean of 3 determinations ± S.D.

The morphology of the nanoparticles was observed by transmission electron microscopy (TEM) using TEM-100CXII. Samples were placed onto a copper grid covered with nitrocellulose. They were dried at room temperature, and then were examined without negative staining.

2.5. Insulin loading of nanoparticles

2.5.1. Association efficiency

The association efficiency of the process was determined upon separation of the nanoparticles by ultracentrifugation at 15,000 rpm, 4 °C for 30 min from the aqueous medium containing non-associated insulin. The amount of free insulin in the supernatant was measured using HPLC. Twenty microliters were injected into a chromatograph (Waters, 2695) equipped with a UV detector, and C8 column (Chrompack, 5μ, 4.6 × 250 mm). The mobile phase was a mixture of 27% acetonitrile and 73% buffer containing 0.1 M KH_2PO_4 and 1% triethylamine adjusted to pH 3.0 with phosphoric acid (Dorkoosh et al., 2002). The flow rate was 1.5 mL/min, the wavelength was set at 214 nm. Insulin association efficiency of the nanoparticles was calculated according to the equation established by Fernandez-Urrusuno et al. (1999).

$$\text{Association efficiency} = \frac{\text{Total amount of insulin} - \text{Free insulin}}{\text{Total amount of insulin}} \times 100$$

All samples were measured in triplicate.

2.5.2. Insulin content in nanoparticles

The nanoparticles were immersed into 1N HCl aqueous solution to be completely degraded. The amount of entrapped insulin in the nanoparticles was determined by measuring insulin in the HCl phase after filtering through a 0.1 μm syringe filter by HPLC method.

2.6. In vitro release study

Insulin release from the nanoparticles was determined by incubating the nanoparticles in 20 mL of pH 6.8 phosphate buffer solution (PBS) at 37 °C. Since the pH of colon usually is in the range of 6.6–7.0 (Evans et al., 1988), pH 6.8 phosphate buffer solution (PBS) has been taken as release medium. The amount of nanoparticles in the release media was adjusted in order to assess sink conditions for insulin. Samples were withdrawn at predetermined time intervals and filtered through a 0.1 μm filter. The filtrates were analyzed for the amount of insulin using HPLC.

2.7. Ex vivo study

Transport of insulin across the colon membrane was determined according to the Barr and Riegelman method (Barr and Riegelman, 1970). First a section of about 5 cm of the ascending colon was removed from a male rat under ketamine (50 mg/kg) and chlorpromazine (10 mg/kg) anesthesia and washed with Krebs–Ringer bicarbonate solution with pH = 6.8. Then this section was gently inverted with a glass rod and a tube was inserted in one side of the section and tied securely with a tape. The other side of the lumen was tied and 2 mL of Krebs–Ringer bicarbonate solution were poured by means of a hypodermic needle into the tube. The gut sac was placed in a medium saturated with 95% O_2, 5% CO_2 and containing test sample in Krebs-Ringer bicarbonate solution at 37 °C. The test samples used include: (1) insulin (10 mg) + CS, TEC or DMEC (2%), (2) INS-CS, INS-TEC or INS-DMEC nanoparticles (equivalent to 10 mg of insulin) + CS, TEC or DMEC (2%), (3) INS-CS, INS-TEC or INS-DMEC nanoparticles (equivalent to 10 mg of insulin). In the absorption studies, an O_2 and CO_2 mixture was bubbled through the intestinal mucosal outer phase to obtain peristaltic movement. In certain time periods, 1 mL samples were drawn from inside of colon and the amount of transported insulin was measured by the aforementioned HPLC method.

2.8. In vivo study

Male Wistar rats (200–250 g) were rendered diabetic prior to the study by intraperitoneal injections of 60 mg/kg streptozotocine (SZ) in isotonic saline solution. After 72 h, rats with fasted blood glucose levels above 250 mg/dL were used for the experiments. The diabetic rats were fasted for 24 h before experiments, but had free access to water. The rats were anesthetized by an intraperitoneal injection of 50 mg/kg ketamine and 10 mg/kg chlorpromazine for surgical procedure with small incision in abdomen cavity. After drawing blood sample at time 0 min, the test samples were injected into the ascending colon portion. At

certain periods (30 and 60 min) blood samples were drawn from the portal vein and blood glucose concentrations were immediately measured using a blood glucometer. The test samples used were: (1) insulin (25 unit/kg) + CS, TEC or DMEC (2%), (2) INS-CS, INS-TEC or INS-DMEC nanoparticles (equivalent to 25 unit/kg of insulin) + CS, TEC or DMEC (2%), (3) INS-CS, INS-TEC or INS-DMEC nanoparticles (equivalent to 25 unit/kg of insulin).

3. Results

3.1. Preparation of insulin nanoparticles

Recent studies have shown the effects of pH on nanoparticle formation using the PEC method (Park et al., 1992). Since complex formation between proteins and polyelectrolyte polymers is primarily driven by coulombic interactions, the pH of the insulin solution will influence the properties of the resulting PEC nanoparticles. As reported previously, at the physiological pH of 7.4, human insulin carries two negative charges per molecule (Brange, 1987). It has been shown that desirable insulin nanoparticles could be formed when the pH of insulin solutions was between 8.0 and 8.5 (Mao et al., 2006). The pH of polymer solutions is also very important. The pH of chitosan solution was adjusted to 5.5 because at this pH more than 90% of the amine groups are protonated (Mao et al., 2006). Quaternized derivatives of chitosan have permanent and more positive charges but the pH of their solutions were adjusted to 5.5 like CS solution.

Therefore, for producing nanoparticles by PEC method, polymer solutions (1 mg/mL) with a pH of 5.5 were added to equal volume of insulin solution under gentle magnetic stirring. Insulin nanoparticles were formed spontaneously when the final system pH was about 5.5, 6.6 and 6.5 for INS-CS, INS-TEC and INS-DMEC nanoparticles, respectively.

3.2. Characterization of the nanoparticles

The freeze dried nanoparticles prepared by the PEC method were of a white powdered shape and insoluble in water, dilute acidic and alkali solutions. The particle size of nanoparticles is shown in Table 1. As in this table, the mean diameter of particles is 267.5, 175.2 and 171.5 nm for INS-CS, INS-TEC and INS-DMEC nanoparticles, respectively. Chitosan nanoparticles have larger size than quaternized derivatives nanoparticles because of the higher molecular weight of chitosan. During synthesis of the quaternized chitosan derivatives due to the harsh synthesis conditions the molecular weight of the derivatives is reduced which also results in smaller particles sizes.

The zeta potential of a nanoparticle is commonly used to characterize the surface charge of nanoparticles (Couvreur et al., 2002). It reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. The zeta potentials of all of the nanoparticles are listed in Table 1. As seen in this table the zeta potential of INS-CS, INS-TEC and INS-DMEC nanoparticles are 17.6, 25.1 and 26.2 mV, respectively. All of the nanoparticles are positively charged, but the charge value of DMEC and TEC nanoparticles are higher than that of the chitosan nanoparticles because of the stronger positive charges of the quaternary ammonium group. Commonly, the polydispersity index is an index of stability and dispersion homogeneity. As given in Table 1, all of the nanoparticles have a desired polydispersity index less than 0.3 indicating a homogenous size distribution.

3.3. Insulin loading of the nanoparticles

The association efficiency (AE) of the nanoparticles is shown in Table 1. Ideally, a successful nanoparticulate system should have a high drug loading capacity. As several studies have shown, the electrostatic interactions between the acidic groups of insulin and amino groups of chitosan and its quaternized derivatives play a dominant role in the association of the insulin to the nanoparticles (Calvo et al., 1997). As seen in Table 1, the AEs of INS-CS, INS-TEC and INS-DMEC nanoparticles are 84.31, 90.37 and 89.49%, respectively. All of the nanoparticles show a high AE, but TEC and DMEC nanoprticles have a slightly higher AE than CS nanoparticles most probably because of stronger positive charges.

The insulin contents in the nanoparticles show similar results as the AEs. As seen in Table 1, the insulin contents in INS-CS, INS-TEC and INS-DMEC nanoparticles are 81.95, 87.94 and 86.86%, respectively.

3.4. Transmission electron microscopy

Fig. 1 shows the TEM photograph of the nanoparticles. All of the nanoparticles exhibit spherical and sub-spherical shapes with smooth surface structure devoid of cracks. Besides, the majority of particles are separated from each other, suggesting that these nanoparticles are possibly stabilized against agglomeration by their strong surface charges.

3.5. Release of insulin from nanoparticles

The insulin release was studied as a function of time. INS-CS, INS-TEC and INS-DMEC nanoparticles containing above given insulin loads were used. It has been shown previously

Table 1
Properties and characteristics of the nanoparticles

Nanoparticles	Mean diameter (nm)	Zeta potential (mV)	Polydispersity	Association efficiency (%)	Insulin content (%)
INS-CS	267.5 ± 15.1	17.6 ± 3.9	0.253	84.31	81.95
INS-TEC	175.2 ± 10.3	25.1 ± 3.7	0.178	90.37	87.94
INS-DMEC	171.5 ± 9.6	26.2 ± 4.1	0.164	89.49	86.86

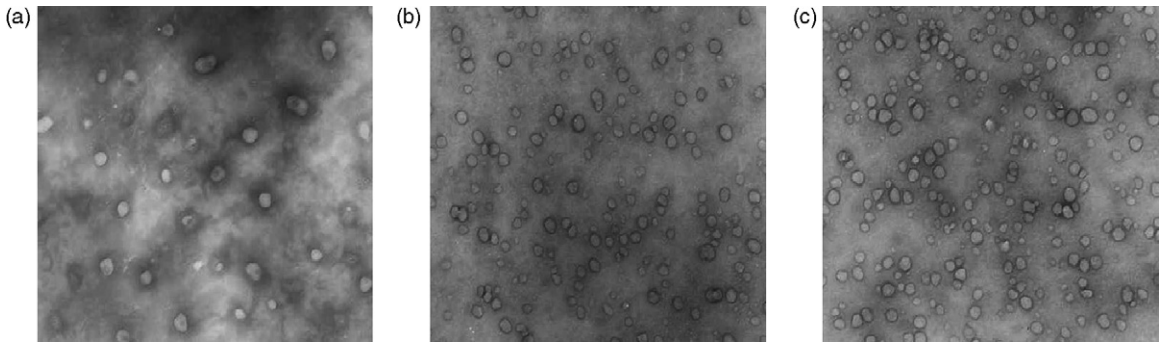

Fig. 1. Transmission electron microscopy of (a) INS-CS, (b) INS-TEC and (c) INS-DMEC nanoparticles.

that if the drug is loaded by the PEC method, the system has a relatively small burst effect and better sustained release characteristics (Fresta et al., 1995). The in vitro release profiles of insulin from chitosan and its derivatives nanoparticles at pH 6.8 is illustrated in Fig. 2. Insulin released from INS-CS, INS-TEC and INS-DMEC nanoparticles after 5 h in pH 6.8 phosphate buffer solution were 33.4, 44.3 and 47.3%, respectively. These results show that these systems have a small burst effect and then sustained release characteristics for 5 h, indicating a good insulin incorporation complexation. Insulin release from INS-TEC and INS-DMEC nanoparticles is higher than INS-CS nanoparticles because of more solubility of TEC and DMEC polymers in neutral and alkaline media.

3.6. Ex vivo study

In this study we measured the amount of insulin transported across the colonic barrier using the inverted gut-sac method. The amount of insulin transported from nanoparticles at pH 6.8 has been shown in Fig. 3. As seen in this figure after 120 min at 37 °C, the amount of insulin transported across the intestinal barriers with INS-TEC and INS-DMEC nanoparticles was significantly higher than free insulin, and presence of free polymers enhance the transport. Consequently, on the basis of these results, it was hypothesized that insulin is not able to transport across membrane and nanoparticulate systems facilitate the transport of insulin through the intestinal barriers and presence of quaternized derivatives of chitosan enhance the transport.

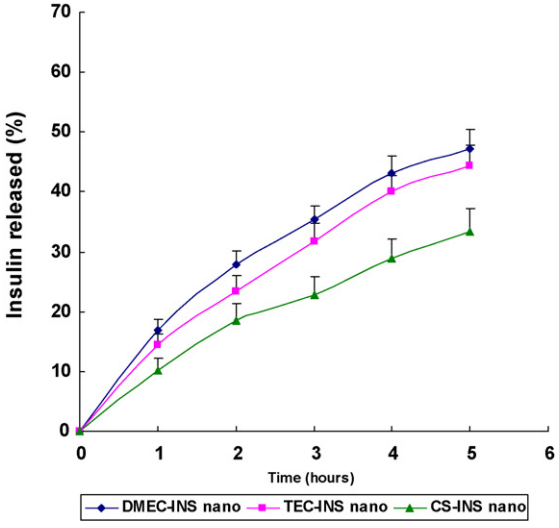

Fig. 2. In vitro release profiles of insulin from nanoparticles in PBS pH = 6.8.

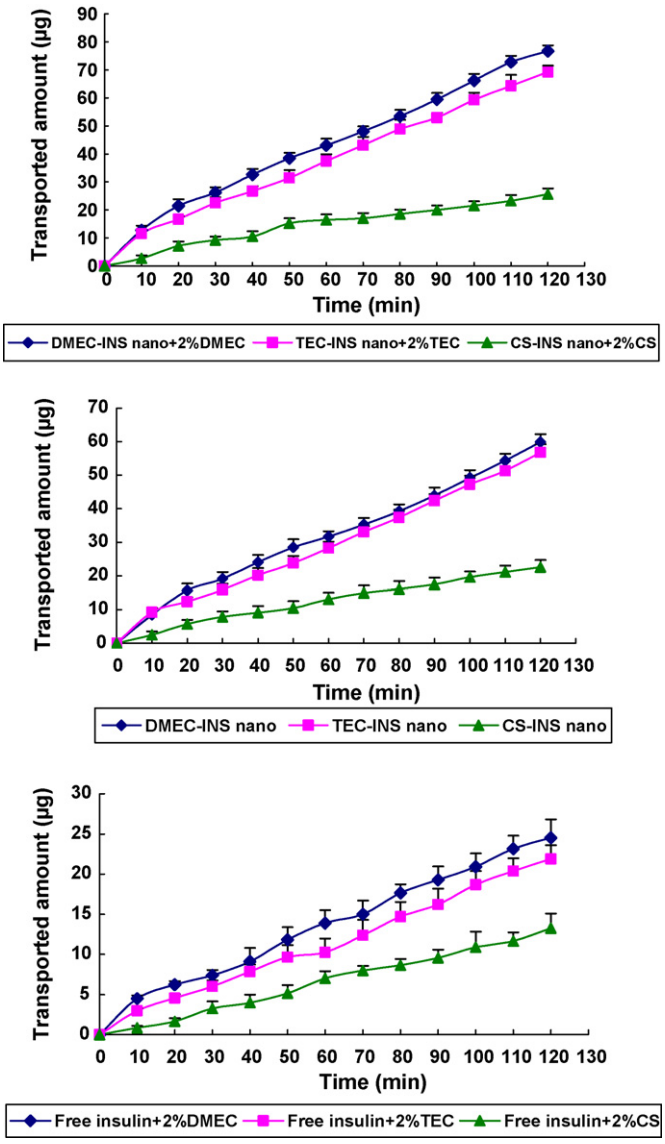

Fig. 3. Profile of the amount of insulin transported in medium pH = 6.8.

Table 2
Blood glucose concentration (mg/dL, $n = 4$)

Test sample	0 min	After 30 min	After 60 min
Insulin	395.7 ± 9.87	388.3 ± 25.3	399.2 ± 12.5
Inslin + DMEC 2%	405.8 ± 31.2	379.8 ± 25.8	365.5 ± 18.8
Insulin + TEC 2%	412.1 ± 26.5	396.5 ± 30.1	381.6 ± 35.2
Insulin + CS 2%	409.5 ± 27.1	401.2 ± 21.1	395.6 ± 18.4
DMEC-INS nano	401.2 ± 26.5	310.7 ± 35.5	264.5 ± 17.6
TEC-INS nano	405.6 ± 32.2	321.2 ± 28.1	285.4 ± 22.3
CS-INS nano	403.3 ± 29.1	365.6 ± 44.1	326.3 ± 29.4
DMEC-INS nano + DMEC 2%	389.3 ± 19.3	299.1 ± 22.6	232.3 ± 18.9
TEC-INS nano + TEC 2%	401.7 ± 31.1	310.2 ± 29.8	249.6 ± 33.5
CS-INS nano + CS 2%	414.5 ± 27.9	379.2 ± 31.2	311.6 ± 21.3

3.7. In vivo study

The mean blood glucose concentration after ascending colon administration of the nanoparticles and free insulin is shown in Table 2. Normal blood glucose concentration in the diabetic rats ranged from 395.7 ± 9.8 mg/dL at time 0 min to 399.2 ± 12.5 mg/dL after 60 min. As it has been shown in Table 2, there was no decrease in blood glucose level after colon injection of free insulin or free insulin at the presence of polymers. This demonstrates that insulin was not able to permeate from cell membrane on colon. In contrast, there was a significant change in blood glucose, when INS-TEC and INS-DMEC nanoparticles were injected into ascending colon. The presence of free polymer with these nanoparticles enhances the glucose lowering effect of the nanoparticles. As it is seen in the Table 2, the hypoglycemic effects of INS-CS nanoparticles are lower than INS-TEC and INS-DMEC nanoparticles due to larger particle size, less positive charge and also the less solubility of chitosan in neutral and alkaline media.

4. Discussion

The development of a delivery system for peptides and proteins that improves the oral absorption of these compounds whose bioavailability is very low because of instability in the GI tract and low permeability through the intestinal membrane is one of the greatest challenges in the pharmaceutical field. Despite numerous studies, oral bioavailability of insulin is quite low and normally insufficient for producing an effective systemic effect (Carino and Mathiowits, 1999; Owens et al., 2003). Therefore, developing an effective oral delivery system for these compounds has drawn increasing attention. A successful system should not only protect the drugs from enzymatic degradation but also increase the drug permeability within in the gastrointestinal barriers. To date, various drug delivery systems have been used to develop oral delivery of proteins and peptides, however, the systems have not shown sufficient bioavailability when administrated orally.

Colon targeted drug delivery is useful in improving the absorption of peptide drugs via the GI tract. Colon has been determined as an ideal site for peptide absorption. Site specific drug delivery to the colon is of special interest for drugs which are instable in the upper part of the gastrointestinal tracts. The colon is thought to have lower enzymatic activity than other regions of the GI, hence if protein and peptides like insulin can be delivered to this site, a greater efficiency of absorption would be expected. Most of the absorption in the colon occurs in the proximal one half of the colon, giving this portion the name, absorbing colon (Wilson and Basit, 2005).

In order to develop therapeutically effective oral insulin systems via colon delivery, one delivery strategy could be based on the encapsulation of insulin in nanoparticulate carriers. This would protect insulin against chemical and enzymatic degradation and potentially also enhance the selective uptake of these particles by various cells (Clark et al., 2001). Preparation of nanoparticles by the PEC 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 homogeneous size distribution. A major advantage of this method is that drug loading can be achieved without the aid of organic solvents or other harmful treatment. By this technique more than 80% encapsulation efficiency was achieved with chitosan and its quaternized derivatives.

In general, the in vitro and in vivo behavior of nanoparticles tends to be greatly dominated by their physicochemical properties such as particle size, surface charge and shape of particles (Sakuma et al., 1998; Sakuma et al., 1990). As it is found in this study, all of the nanoparticles show positive charges. This implies that insulin was encapsulated in the polymers projecting their positively charged chains towards the external aqueous medium, which will subsequently increase the interaction with the negatively charged cell surface and facilitate insulin uptake. It has already been demonstrated that positively charged particles are more absorbed than the neutral or negatively ones (Janes et al., 2001). Here we showed that particle size and presence of quaternized derivatives of chitosan are two main factors in transport and absorption of nanoparticles.

The profile of drug release from nanoparticles can be affected by method of preparation and also by ionic interaction between the drug and addition of auxillary ingredients. If the drug is loaded by incorporation method, the system has a relatively small burst effect and then sustained release characteristics. When the drug is involved in interaction with auxillary ingredients to form a less water soluble complex, then the drug release can be very slow with almost no burst effect. The release results show good incorporation of insulin in polymers with ionic interaction between insulin and polymers.

As it is found in this study, the release of insulin from nanoparticles in ex vivo is higher than in vitro which seems to be due to difference of the mechanism of release. In vitro release is less since drug is released mainly from surface of the nanoparticles and nanoparticles will not degrade completely in the in vitro media and remain relatively intact. However, ex vivo release is more since in this media, in addition to effect of medium, various tissue enzymes and mucin from mucus membranes will cause degradation of nanoparticles and release of drug is not only

from surface of nanoparticles but also from degraded nanoparticles.

It is clearly demonstrated that the low bioavailability of insulin is improved significantly by the nanoparticulate system (Krauland and Alonso, 2007; Vila et al., 2002; Takeuchi et al., 2001). In general, the gastrointestinal absorption of particulate materials involves either paracellular route or endocytotic pathway (Mohanraj and Chen, 2006). The paracellular route of absorption of nanoparticles utilizes less than 1% of mucosal surface area. It has been reported that hydrophilic polymers like chitosan and its quaternized derivatives by involving actin filaments of intestinal or colonic epithelium, are able to increase permeability of particles through the opening of tight junctions between epithelial cells and this property is closely related to the enhancement effect of peptide absorption (Borchard et al., 1996). Endocytotic pathway for absorption of nanoparticles is either by receptor-mediated endocytosis, that is, active targeting, or adsorptive endocytosis which does not need any ligands. This process is initiated by an unspecific physical adsorption of material to the cell surface by electrostatic forces such as hydrogen bonding or hydrophobic interactions (Florence and Hussain, 2001). Our results can propose that both paracellular and endocytosis are the main routes of absorption for nanoparticles and depends primarily on the size and surface charge of particles. It is clearly shown that insulin nanoparticles exhibit higher insulin transport across the barriers and also better absorption in colon, than free insulin. It seems that the mechanism of the absorption of the nanoparticles is via endocytotic pathway in colon, and presence of free polymers enhances the absorption by paracellular route. The highest transport and absorption of insulin and the most effectiveness in lowering the blood glucose level was achieved by INS-TEC and INS-DMEC nanoparticles in presence of free TEC and DMEC, respectively which can be translated to better up-take and interaction of nanoparticles with cell membranes and therefore higher transport of insulin nanoparticles. INS-CS nanoparticles exhibit low insulin transport and absorption because of large particle size, less positive charge and poor solubility of chitosan in neutral or alkaline pH. As particle size of INS-CS nanoparticles is more than 250 nm, it seems that particle size less than 200 nm is more suitable for endocytosis absorption of nanoparticles.

However, since the particle size of INS-TEC and INS-DMEC nanoparticles are less than 200 nm (175.2 and 171.5 nm, respectively) the endocytosis phenomenon is governing the absorption of these particles through colonic barriers.

5. Conclusions

Insulin nanoparticles using CS, TEC and DMEC were prepared by the polyelectrolyte complexation method. The remarkable advantage of this system is, preparing nanoparticles under mild conditions without any organic solvents and surfactants. All of the nanoparticles showed a positive charge that can facilitate insulin uptake, and have low burst effect and steady release behavior of insulin in vitro. DMEC and TEC nanoparticles have smaller particle size and higher insulin loading than CS nanoparticles. More insulin is incorporated in DMEC and TEC nanoparticles than in CS nanoparticles and also the transport and absorption of insulin in GI tract is higher. The blood glucose lowering effect of these nanoparticles after injection into ascending colon is much better than free insulin or CS nanoparticles. The results of this study indicate that nanoparticles prepared by quaternized derivatives of chitosan seem to be a promising vehicle for oral administration of proteins and peptides via colon absorption. However, an appropriate in vivo study is necessary to fully evaluate the potential of this system after oral administration of the drug delivery and colon absorption.

Acknowledgments

This research was partially supported by a grant from the research council of Tehran University of Medical Sciences. We are grateful to Mr. M. Banki, chairman of COSAR pharmaceutical company for his support and encouragement of this study.

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