JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 1345–1351

Received March 27, 2007
Accepted June 11, 2007
DOI 10.1211/jpp.59.10.0003
ISSN 0022-3573

Development and in-vivo evaluation of insulin-loaded chitosan phthalate microspheres for oral delivery

Udhumansha Ubaidulla, Roop Kishan Khar, Farhan Jalees Ahmed and Amulya Kumar Panda

Abstract

Novel chitosan phthalate microspheres containing insulin were prepared by emulsion cross-linking technique. The feasibility of these microspheres as oral insulin delivery carriers was evaluated. The pH-responsive release behaviour of insulin from microspheres was analysed. The ability of chitosan phthalate-insulin microspheres to enhance intestinal absorption and improve the relative pharmacological availability of insulin was investigated by monitoring the plasma glucose and insulin level of streptozotocin-induced diabetic rats after oral administration of microspheres at insulin dose of 20 IU kg^{-1}. In simulated gastric fluid (pH 2.0), insulin release from the microspheres was very slow. However, as the pH of the medium was changed to simulated intestinal fluid (pH 7.4), a rapid release of insulin occurred. The relative pharmacological efficacy for chitosan phthalate microspheres (18.66 ± 3.84%) was almost four-fold higher than the efficacy of the chitosan phthalate-insulin solution administration (4.08 ± 1.52%). Chitosan phthalate microspheres sustained the plasma glucose at pre-diabetic level for at least 16 h. These findings suggest that the microsphere is a promising carrier as oral insulin delivery system.

Introduction

Diabetes mellitus is a disorder caused by decreased production of insulin or by decreased ability to use insulin, leading to increased glucose levels in the blood. More than 30 million people around the world suffer from insulin-dependent diabetes mellitus (Chun-Lei & Ying-Jie 2004). The most common therapy is insulin injected subcutaneously two to four times a day, which is painful and inconvenient, resulting in poor patient compliance (Pillai & Panchagnula 2001). Therefore, other routes of administration have been developed—nasal, buccal, rectal, vaginal, pulmonary and transdermal (Trehan & Ali 1998). Oral administration is the most convenient route and has physiological advantages (i.e. insulin undergoes a first hepatic bypass, thus ensuring a primary effect by inhibiting hepatic glucose output) (Krauland et al 2004). However, oral delivery of insulin is limited due to acid and enzymatic degradation in the gastrointestinal tract, low epithelial permeability and instability under formulation conditions (Lee et al 2000). Research literature is available that addresses various approaches, such as alternative routes, absorption enhancers, protease inhibitors, chemical modification and dosage forms, which have been examined to overcome the delivery problem of insulin via the gastrointestinal tract (Lee & Yamamoto 1990; Mackay et al 1997). There has recently been increasing interest in developing a new carrier able to protect insulin from acid and enzymatic degradation in the gastrointestinal tract and to facilitate its transport from the gut lumen to the blood compartment.

Chitosan [poly (1,4-β-D-glucopyranosamine)] is a polysaccharide derived from naturally occurring chitin in crab and shrimp shells by alkaline deacetylation. This polymer has been investigated extensively for several potential applications in the pharmaceutical field, such as the development of various delayed drug delivery systems. This is due to its appealing intrinsic characteristics, which include biodegradability, biocompatibility, bioadhesiveness and ability to open epithelial tight junctions to allow an increase in the paracellular transport of macromolecular drugs (Thanou et al 2001a; Hejazi & Amiji 2003). Notable studies have investigated the oral delivery of insulin by using chitosan microspheres, microparticles and nanoparticles as a carrier (Pan et al 2002 a, b). However, chitosan suffers from high solubility

Department of Pharmaceutics, Faculty of Pharmacy, Hamdard University, New Delhi, India

Udhumansha Ubaidulla, Roop Kishan Khar, Farhan Jalees Ahmed

National Institute of Immunology, New Delhi, India

Amulya Kumar Panda

Correspondence: U. Ubaidulla, Department of Pharmaceutics, Faculty of Pharmacy, Hamdard University, New Delhi, India.
E-mail: ubaidnkl@gmail.com

in gastric pH 2.0 and low solubility in physiological pH 7.4, which limits its use as a carrier in oral delivery of insulin. Recently, a chitosan derivative, chitosan phthalate, has attracted more attention in oral drug delivery systems because of its low solubility in acidic pH and high solubility in basic pH. Aiedeh & Taha (1999) reported that chitosan phthalate polymer protects the drug molecule from acidic pH 2.0 and most of the drug molecule releases in alkaline pH 7.4. In our earlier studies, we found that chitosan phthalate microspheres protect insulin from acid and enzymatic degradation in the gastrointestinal tract (Ubaidulla et al 2007).

In this study, the feasibility of chitosan phthalate microspheres as oral delivery carriers of insulin was evaluated by investigating the pH-responsive release behaviour of insulin in the physiological pH range and in-vivo pharmacological activity of insulin loaded chitosan phthalate microspheres in diabetic rats.

Materials and Methods

Materials

Chitosan phthalate polymer was synthesized in house using a method reported earlier (Aiedeh & Taha 1999). The degree of phthalate substitution was 16%. Porcine insulin (specific activity of 23 IU mg^{-1}) was obtained from Abbott Laboratories (Abbott Park, IL). Pepsin and trypsin were purchased from Sigma (St Louis, MO). Light liquid paraffin, glutaraldehyde (25% v/v), Span 80, sodium sulfate (Na_2SO_4), sodium dihydrogen phosphate (NaH_2PO_4) and phosphoric acid (H_3PO_4) were procured from Merck (Bombay, India). Micro BCA kit was purchased from Pierce (Rockford, IL). All other reagents were of analytical grade.

Preparation of microspheres

Chitosan phthalate microspheres were prepared by an emulsion cross-linking method (Ubaidulla et al 2007). Chitosan phthalate was dissolved in 15 mL of distilled water. The aqueous phase was extruded through a syringe (No. 20) in 100 mL of the external oily phase of liquid paraffin containing 0.2% Span 80 and stirring was carried out using a propeller stirrer (Remi, India) at 1500 rev min^{-1}. After 15 min, 1 mL of glutaraldehyde (25% v/v aqueous solution) was added drop-by-drop to the emulsion and stirring was continued. The emulsion was left stirring for 1 h and then was centrifuged (Remi, India) at 2000 rev min^{-1} for 15 min. The microspheres were separated by filter paper and washed with petroleum ether. The microspheres were then dried under vacuum and stored at 4–8°C before use in further studies.

Insulin loading of microspheres

The insulin loading of microspheres was performed using a passive absorption technique (Jameela et al 1994) by incubating 1% (w/v) chitosan phthalate microspheres and 0.5–2.0% (w/v) insulin in PBS pH 7.4 under shaking at 25°C. After incubation for 12 h, the suspension was centrifuged (12 000 rev min^{-1} for 15 min) to remove the unloaded insulin. The degree of loading was determined by quantifying non-bound insulin in supernatant with Micro BCA technique. The loading efficiency (LE) and loading capacity (LC) of the microspheres was determined by using Equations 1 and 2:

$$LE = (\text{Total amount of insulin} - \text{insulin in supernatant})/\text{total amount of insulin} \quad (1)$$

$$LC = (\text{Total amount of insulin} - \text{insulin in supernatant})/\text{weight of microspheres} \quad (2)$$

Scanning electron microscopy (SEM)

The shape and surface characteristics of the microspheres were studied by scanning electron microscopy (Leo 435VP; UK). The samples for SEM analysis were mounted on metal grids using double-sided adhesive tape and coated with gold under vacuum before observation.

Particle size analysis

The size of the microspheres was measured by using a laser light scattering technique (Mastersizer 2000; Malvern instruments, UK). The microsphere suspensions (dispersed in paraffin oil) were placed in a stirred tank and were circulated through the cell, which was placed in the path of the laser beam. The scattered light was received on a detector consisting of photosensitive rings that detected microsphere diameters.

Zeta potential

The microspheres were dispersed in de-ionised water at pH 6.0 and surface charge (zeta potential) was measured by using a Malvern 3000 HS zetasizer (Malvern Instruments, UK).

In-vitro release study

The in-vitro release of insulin from microspheres was carried out in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) media (without enzymes) using a shaker (Lab-Therm, Kuhner, Switzerland) under physiologic conditions at 37°C with a shaking frequency of 50 rev min^{-1}. A sample of chitosan phthalate microspheres was suspended in 100 mL dissolution medium. At scheduled time intervals, the release medium was withdrawn and replaced with the same volume of fresh medium. Samples were centrifuged at 12 000 rev min^{-1} for 10 min and supernatant insulin concentration was analysed by Micro BCA technique.

Circular dichroism (CD)

The samples were prepared using a method reported earlier (Hinds et al 2005). CD spectra were recorded on a J-710 spectropolarimeter (Jasco, Japan) equipped with a J-710 for

windows software (Jasco). A 0.1-cm path cell and 200–250 nm for far-UV CD and a 1.0-cm path cell and 250–300 nm for near-UV CD were used. The instrument was calibrated using an aqueous solution of D-10-camphorsulfonic acid (0.6 mg mL^{-1}). The data were expressed as mean residue ellipticity [θm] according to the expression:

$$[\theta m] = \theta / ncl \times 100 \quad (3)$$

Where θ is the observed ellipticity (m deg), n is the total number of residues of the protein, l is the optical path length (cm) and c is the protein concentration (g mL^{-1}).

In-vivo studies

The animals used for in-vivo experiments were adult Wistar male albino rats, 230–250 g, provided by the Central Animal House of our school. The study protocol was approved by the institutional Animal Ethics Committee, Hamdard University, New Delhi, India.

Diabetes was induced by an intravenous injection of 45 mg kg^{-1} of streptozotocin (Sigma, MO) dissolved in 0.1 M citrate buffer solution (pH 4.0). The glucose level was measured before the experiment and rats that had a glucose level of only 280–380 mg dL^{-1} were used. The diabetic rats were fasted for 12 h before experiments. Rats were randomly divided into four groups, and each group (n = 6) was housed in cages. The following formulations were administered: 1, subcutaneous insulin solution (insulin dose 2 IU kg^{-1}); 2, chitosan phthalate-insulin solution (insulin dose 20 IU kg^{-1}); and 3, chitosan phthalate microspheres (insulin dose 20 IU kg^{-1}). The dosed rats were fasted for 12 h and kept in restraining cages with free access to water. Twelve hours after application, the rats were fed. Blood samples were collected from the rats at different time intervals (0, 1, 2, 4, 6, 8, 12, 16, 20 and 24 h) after dosing and glucose level was measured by the glucose-oxidase method (Life scan strip; Johnson & Johnson Company, USA). The initial blood glucose levels were considered as 100%. Using this value, the percentage of glucose reduction at each time after dosing was calculated and plotted against time. Insulin levels in rat serum were determined using a commercially available kit (Active Insulin ELISA; Diagnostic System Laboratories, USA).

Statistical analysis

Relative pharmacological availability (PA %) was calculated by using Equation 4. The same equation was used in calculation of the relative bioavailability (F %); however, the AUC (area under curve) values were substituted instead of the AAC values (Nakamura et al 2004).

$$PA\ \% = (AAC_{oral}/AAC_{sc}) \times (Dose_{sc}/Dose_{oral}) \times 100 \quad (4)$$

where, PA is the pharmacological availability of the insulin and AAC_{oral} and AAC_{sc} are area above the curve of reduction in blood glucose levels for oral and subcutaneous administration, respectively. The area above the percentage glucose change versus time curve (AAC) was calculated by the trapezoid method.

Data were analysed by a one-way analysis of variance followed by Tukey's test at a 95% confidence interval to test for significant differences between formulations at each time point with regards to glucose and insulin levels.

Results and Discussion

Insulin loading of chitosan phthalate microspheres

Since peptide and protein drugs, such as insulin, should not be exposed to the harsh process involved in microsphere preparation, insulin loading was performed by a passive absorption technique with different percentage of insulin loading (0.5, 1, 1.5 and 2% w/v). The results showed that there was no effect on LC and LE of the microspheres with different percentage loading. When percentage insulin loading increased, a greater concentration of the insulin remained unbound. Therefore, 0.5% w/v insulin loading was selected as the optimal concentration because a small amount of insulin was unbound during the loading process compared with other percentage insulin loadings. The LC and LE of insulin were about 62% and 88%, respectively.

Morphology, particle size analysis and zeta potential

Scanning electron microscopy of chitosan phthalate microspheres is shown in Figure 1. The microspheres were spherical in shape with smooth surfaces. It can be observed that polymer cross-linking with glutaraldehyde was successful, which led to uniform formation of spheres. The free amino group of chitosan phthalate is cross-linked with the aldehyde group of glutaraldehyde to form Schiff base (imine group). The mean particle size of the microspheres was found to be $13.14 \pm 1.2\ \mu$m. Zeta potential measurement revealed a net positive charge on the chitosan phthalate microspheres and the average zeta potential was found to be $+10 \pm 1$ mV. The unreacted amino groups present in chitosan phthalate microspheres may be responsible for this positive zeta potential.

Release of insulin from microspheres

Taking into account an average gastric transit time, drug release was investigated in SGF between 0 and 2 h and in SIF between 2 and 12 h. As shown in Figure 2, a small fraction of insulin (less than 20%) was released in SGF, verifying the protective nature of the chitosan phthalate microspheres. In contrast, the release of insulin was markedly increased in SIF. The results suggest that the present microsphere formulation is able to control the insulin release in SGF and almost completely releases drug in SIF. The microspheres in SGF did not swell under the release conditions, surface bound drug was mostly released. The result can be attributed to the fact that under acidic conditions (SGF), the carboxylic groups present in the system exist in non-ionized form and are poorly hydrophilic. In the case of SIF, the carboxylic groups exist in ionized form and hence are considerably hydrophilic. It may

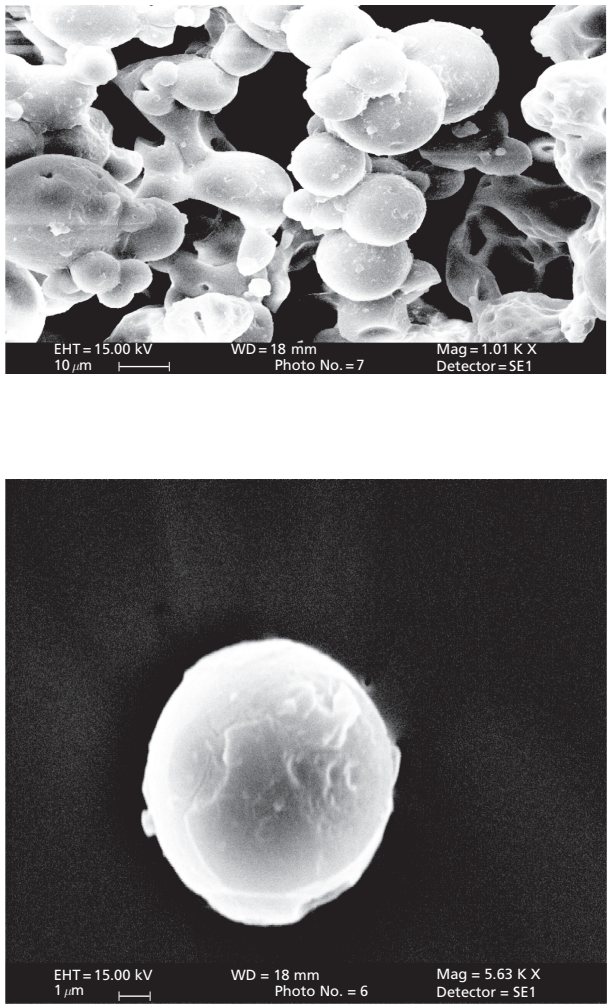

Figure 1 Scanning electron microscopy of chitosan phthalate microspheres.

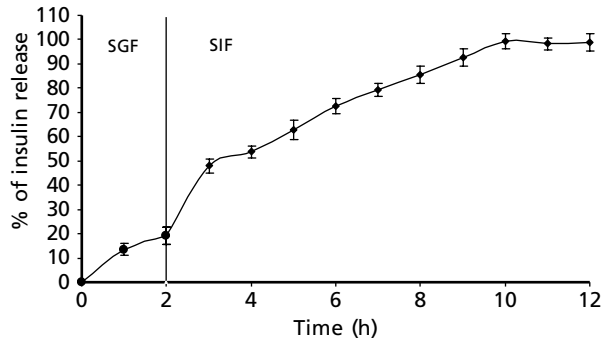

Figure 2 In-vitro release of insulin from microspheres in the presence of SGF (pH 2.0, circles) and SIF (pH 7.4, diamonds). SGF, simulated gastric fluid; SIF, simulated intestinal fluid. Data are means ± s.d., n = 3.

be noted that similar findings were observed in swelling studies. The fast release of insulin in SIF was attributed to the higher permeability of buffers into the microspheres, which resulted in more swelling of the polymers.

Further, the in-vitro release data in SIF were analysed by Ritger & Peppas (1987) model. The initial 60% cumulative release data were used to estimate the diffusional exponent, n, by using Equation 5:

$$M_t/M_\infty = kt^n \quad (5)$$

where, M_t is the amount of drug released at time t, M_∞ is the nominal total amount of drug released, k is the kinetic constant and n is the diffusional exponent that is used to characterize the release mechanism. By applying the least-squares method to the release data at 95% confidence level, we estimated the values of n and correlation coefficient r. The n value was found to be 0.51 ± 0.04 with good correlation coefficient r (0.99). The n value indicates that the release of insulin from microspheres follows Fickian diffusion behaviour.

Circular dichroism (CD)

Figure 3A shows the far-UV CD spectra of the 12th-hour in-vitro released sample and native insulin. The in-vitro release sample showed two minima at 208 and 223 nm; no significant difference was observed when compared with CD spectra of native insulin (PBS, pH 7.4, at a concentration of 0.1 mg mL^{-1}). The far-UV CD spectrum gives information about the secondary structure of insulin, since the polypeptide backbone absorbs in this region according to the type of secondary structure formed—either α-helix or β-sheet. Figure 3B, the near-UV spectra (250–300 nm), shows the tertiary structure conformation of insulin. It gives a single minimum at 274 nm, owing to the presence of chromophores which absorb in this region—aromatic amino acid, tyrosine (Quaglia et al 2003). The result indicated that the structure of the encapsulated insulin is not altered.

In-vivo evaluation of insulin loaded chitosan phthalate microspheres

The plasma glucose concentration–time profile after insulin administration to diabetic rats is shown in Figure 4. The percentage reduction of blood glucose level (pharmacological response) after subcutaneous injection at 1 h was found to be $43.82 \pm 4.82\%$ and the effect was maintained only for 6 h. When insulin-loaded chitosan phthalate microspheres were orally administered to the diabetic rats, a decrease in plasma glucose levels was observed. The results were significantly different when compared with the chitosan phthalate-insulin control solution ($P < 0.001$). Pharmacokinetic parameters of glucose levels after dosing are shown in Table 1. The chitosan phthalate microspheres produced a minimum glucose level of $51.54 \pm 3.42\%$ at 6 h and the reduction in glucose level was maintained over a prolonged period of time. This result may be attributed to the improved stability of insulin in the gastrointestinal tract using chitosan phthalate

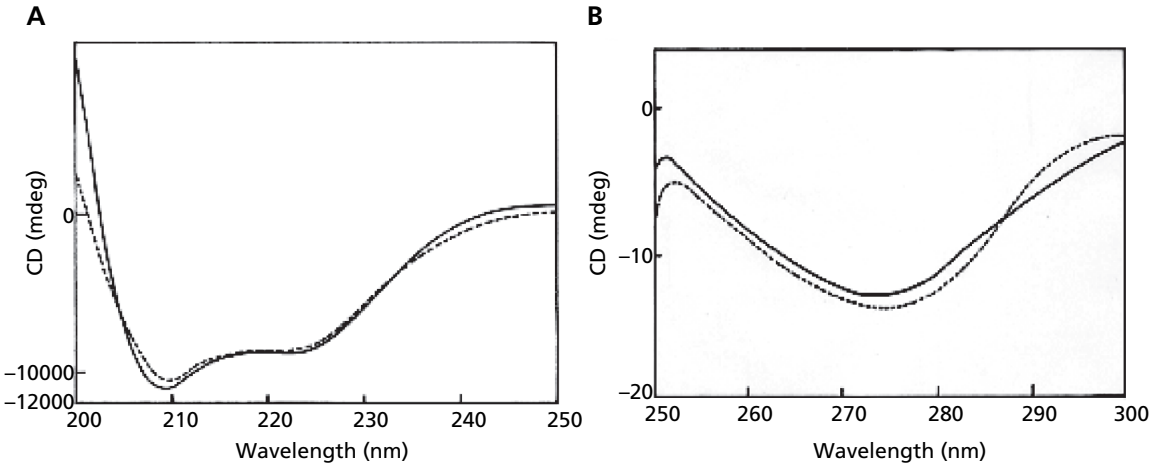

Figure 3 Far-UV CD spectra (A) and near-UV CD spectra (B) of insulin (—) and released sample (---).

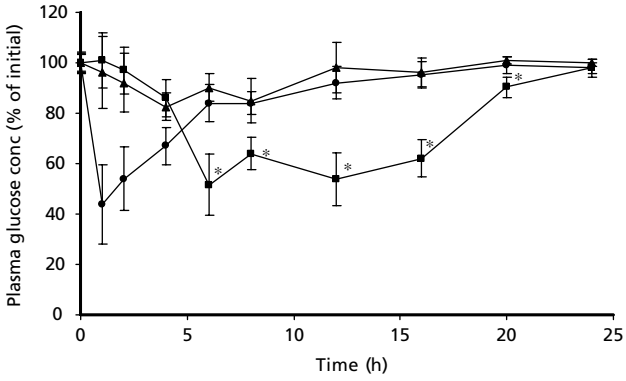

Figure 4 Effect on plasma glucose concentrations after the administration of different dosage forms to diabetic rats: subcutaneous insulin (circles); chitosan phthalate-insulin solution (triangles); insulin-loaded chitosan phthalate microspheres (squares). Data represent the mean ± s.d., n = 6 per group. *$P < 0.01$, chitosan phthalate microspheres versus oral solution and subcutaneous administration.

microspheres, which protect insulin from degradation by gastric pH and various enzymes. Orally administered chitosan phthalate-insulin solution at the same dose showed slight reduction in blood glucose level due to degradation of insulin by proteolytic enzymes in the gastrointestinal tract. The relative pharmacological efficacy for chitosan phthalate microspheres (18.66 ± 3.84%) was almost three-fold higher than the efficacy of the oral insulin administration (4.08 ± 1.52%).

Figure 5 compares the plasma insulin levels after administration of chitosan phthalate microspheres, chitosan phthalate-insulin solution and subcutaneous injection. The plasma insulin concentration increased from 6 h after administration; a maximum plasma insulin concentration of 412.24 ± 68.98 μIU mL^{-1} was observed with chitosan phthalate microspheres, and the bioavailability of insulin was 14.82 ± 2.56% (Table 2). A reduction in blood glucose levels accompanied the absorption of insulin. The hypoglycaemic effects were observed between 6 and 24 h after the oral administration of chitosan phthalate microspheres containing insulin. These results clearly showed the ability of chitosan phthalate microspheres to enhance the absorption of insulin from the gastrointestinal tract. The enhanced gastrointestinal absorption could be attributed to the positive charge of the microspheres being the factor responsible for interaction with the negative site of the tight junction and the subsequent widening of the paracellular transport route (Madara 1987). The poor solubility of chitosan at pH values > 6.5 hampers its usefulness as an absorption enhancer of protein and peptide drugs at neutral pH (Thanou et al 2001b), whereas in this study we demonstrate modification of chitosan (i.e. chitosan phthalate) with superior solubility at neutral pH. According to this strategy, the oral absorption of insulin can be dramatically increased by using chitosan phthalate microspheres.

Conclusions

Chitosan phthalate microspheres were prepared by emulsion cross-linking. Insulin was successfully entrapped into the microspheres. Microspheres were spherical with a narrow size distribution. Microspheres showed the greatest change in insulin release rate as the pH changed from low to high values and created the greatest protective effect for insulin in the simulated gastric condition. Furthermore, microspheres could release insulin in its active form in-vivo and they were able to improve the intestinal absorption of insulin to a greater extent than insulin-chitosan phthalate solution. The microspheres offer the potential for improved oral bioavailability and sustained delivery of insulin. Further, ongoing research indicates that this microsphere is useful for oral controlled delivery of other proteins for several therapeutic applications.

Table 1 Main pharmacokinetic parameters for plasma glucose levels after oral administration of chitosan phthalate microspheres and chitosan phthalate-insulin solution, as well as after subcutaneous injection of insulin to rats

Parameter	Subcutaneous injection	Chitosan phthalate-insulin solution	Chitosan phthalate microspheres
Insulin dose (IU kg^{-1})	2	20	20
Minimum glucose level (% of the initial level)	43.82 ± 4.82	82.44 ± 5.62	51.54 ± 3.42
Time point of minimum glucose level (h)	1.00 ± 0.18	4.00 ± 0.45	6.00 ± 0.27
$AAC_{0\rightarrow24}$	329.79 ± 12.22	134.66 ± 8.41	615.46 ± 11.67*
Relative pharmacological efficacy (PA%)	—	4.08 ± 1.52	18.66 ± 3.84*

Results are expressed as mean ± s.d., n = 6. *$P < 0.001$, chitosan phthalate microspheres versus oral solution and subcutaneous injection.

Table 2 Pharmacokinetic parameters for plasma insulin levels after oral administration of chitosan phthalate microspheres and chitosan phthalate-insulin solution, as well as after subcutaneous injection of insulin to rats

Pharmacokinetic parameter	Subcutaneous injection	Chitosan phthalate-insulin solution	Chitosan phthalate microspheres
Insulin dose (IU kg^{-1})	2	20	20
C_{max} (μIU mL^{-1})	469.63 ± 52.44	139.65 ± 24.87	412.24 ± 68.98
t_{max} (h)	1.00 ± 0.06	2.00 ± 0.39	6.00 ± 0.14
AUC (μIU h mL^{-1})	3162.86 ± 92.36	1819.85 ± 89.74	4687.36 ± 116.78*
Relative bioavailability (F %)	—	5.75 ± 0.92	14.82 ± 2.56*

Results are expressed as mean ± s.d., n = 6. *$P < 0.001$, chitosan phthalate microspheres versus oral solution and subcutaneous injection.

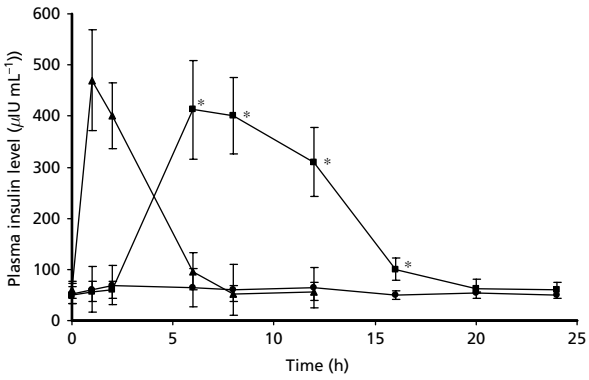

Figure 5 Plasma insulin concentrations after the administration of different dosage forms to diabetic rats: subcutaneous insulin (triangles); chitosan phthalate-insulin solution (circles); insulin-loaded chitosan phthalate microspheres (squares). Data represent the mean ± s.d., n = 6 per group. *$P < 0.01$, chitosan phthalate microspheres versus oral solution and subcutaneous administration.

References

Aiedeh, K., Taha, M. O. (1999) Synthesis of chitosan succinate and chitosan phthalate and their evaluation as suggested matrices in orally administered, colon specific drug delivery systems. *Arch. Pharm. Pharm. Med. Chem*. **332**: 103–107

Chun-Lei, L., Ying-Jie, D. (2004) Oil-based formulations for oral delivery of insulin. *J. Pharm. Pharmacol*. **56**: 1101–1107

Hejazi, R., Amiji, M. (2003) Chitosan-based gastrointestinal delivery system. *J. Control. Release* **89**: 151–165

Hinds, K. D., Campell, K. M., Holland, K. M., Lewis, D. H., Piche, C. A., Schmidt, P. G. (2005) PEGylated insulin in PLGA microparticles. In vivo and in vitro analysis. *J. Control. Release* **104**: 447–460

Jameela, S. R., Misra, A., Jayakrishnan, A. (1994) Cross-linked chitosan microspheres as carriers for prolonged delivery of macromolecular drugs. *J. Biomater. Sci. Polym. Ed*. **6**: 621–632

Krauland, A. H., Guggi, D., Bernkop-Schnurch, A. (2004) Oral insulin delivery: the potential of thiolated chitosan-insulin tablets on non-diabetic rats. *J. Control. Release* **95**: 547–555

Lee, V. H. L., Yamamoto, A. (1990) Penetration and enzymatic barriers to peptide and protein drug delivery. *Adv. Drug. Del. Rev*. **4**: 171–207

Lee, V. H. L., Kashi, S. D., Grass, G. M., Rubas, W. (2000) Oral route of protein and peptide drug delivery. In: Lee, V. H. L. (ed.) *Peptide and protein drug delivery*. Marcel Dekker, New York, pp 691–740

Mackay, M., Phillips, J., Hastewell, J. (1997) Peptide drug delivery: colonic and rectal absorption. *Adv. Drug. Del. Rev*. **28**: 253–273

Madara, J. L. (1987) Intestinal absorptive cell tight junctions are linked to cytoskeleton. *Am. J. Physiol*. **253**: C171–C175

Nakamura, K., Murray, R. J., Joseph, J. I., Peppas, N. A., Morishita, M., Lowman, A. M. (2004) Oral insulin delivery using P(MAA-g-EG) hydrogels: effects of network morphology on insulin delivery characteristics. *J Control. Release* **95**: 589–599

Pan, Y., Li, Y. J., Zhao, H. Y., Zheng, J. M., Xu, H., Wei, G., Hao, J. S., Cui, F. D. (2002a) Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin *in vivo*. *Int. J. Pharm*. **249**: 139–147

Pan, Y., Zheng, J. M., Zhao, H. Y., Li, Y. J., Xu, H., Wei, G. (2002b) Relationship between drug effects and particle size of insulin loaded bioadhesive microspheres. *Acta Pharmacol. Sin*. **23**: 1051–1056

Pillai, O., Panchagnula, R. (2001) Insulin therapies: past, present and future. *Drug Discov. Today*. **6**: 1056–1061

Quaglia, F., Rosa, G. D., Granata, E., Francesca, U., Fattal, E., Rotonda, M. I. L. (2003) Feeding liquid, non-ionic surfactant and cyclodextrin affect the properties of insulin loaded poly (lactide-co-glycolide) microspheres prepared by spray drying. *J Control. Release* **86**: 267–278

Ritger, P. L., Peppas, N. A. (1987) A simple equation for description of solute release II. Fickian and anomalous release from swellable devices. *J Control. Release* **5**: 37–42

Thanou, M., Verhoef, J. C., Junginger, H. E. (2001a) Chitosan and its derivatives as intestinal absorption enhancers. *Adv. Drug. Del. Rev*. **50**: S91–S101

Thanou, M., Nihot, M. T., Jansen, M., Verhoef, J. C., Junginger, H. E. (2001b) Mono-N-carboxymethyl chitosan (MCC), a polyampholytic chitosan derivative, enhances the intestinal absorption of low molecular weight heparin across intestinal epithelia *in vitro* and *in vivo. J. Pharm. Sci*. **90**: 38–46

Trehan, A., Ali, A. (1998) Recent approaches in insulin drug delivery. *Drug Dev. Ind. Pharm*. **90**: 589–597

Ubaidulla, U., Panda, A. K., Sultana, Y., Ahmad, F. J., Khar. R. K. (2007) Chitosan phthalate microspheres for oral delivery of insulin: preparation, characterization and *in vitro* evaluation. *Drug Deliv*. **14**: 19–23