Enhancement of Nasal Absorption of Insulin Using Chitosan Nanoparticles

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Purpose. To investigate the potential of chitosan nanoparticles as a system for improving the systemic absorption of insulin following nasal instillation.

Methods. Insulin-loaded chitosan nanoparticles were prepared by ionotropic gelation of chitosan with tripolyphosphate anions. They were characterized for their size and zeta potential by photon correlation spectroscopy and laser Doppler anemometry, respectively. Insulin loading and release was determined by the microBCA protein assay. The ability of chitosan nanoparticles to enhance the nasal absorption of insulin was investigated in a conscious rabbit model by monitoring the plasma glucose levels.

Results. Chitosan nanoparticles had a size in the range of 300–400 nm, a positive surface charge and their insulin loading can be modulated reaching values up to 55% [insulin/nanoparticles (w/w): 55/100]. Insulin association was found to be highly mediated by an ionic interaction mechanism and its release *in vitro* occurred rapidly in sink conditions. Chitosan nanoparticles enhanced the nasal absorption of insulin to a greater extent than an aqueous solution of chitosan. The amount and molecular weight of chitosan did not have a significant effect on insulin response.

Conclusions. Chitosan nanoparticles are efficient vehicles for the transport of insulin through the nasal mucosa.

KEY WORDS: absorption enhancement; chitosan; insulin; nanoparticles; nasal delivery.

INTRODUCTION

Nowadays, several mucosal surfaces such as the nasal, pulmonary, and peroral mucosae are being extensively explored as alternative routes for the systemic administration of macromolecular drugs. Among them, the nasal mucosa is receiving a great deal of attention due to its particularly high permeability and the easy access of the drug to the absorption site (1). The efficacy of the nasal route for the absorption of large peptides, such as insulin is low (2,3). Several absorption enhancers, including surfactants, protease inhibitors, solutions of bioadhesive polymers or bioadhesive microspheres have been proposed to overcome this limitation (4–9). In general, drug absorption enhancement is accompanied by mucosal damage (10–12). An exceptional behavior has, however, been observed for the polysaccharide chitosan (13). The mechanism of action of chitosan was suggested to be a combination of bioadhesion and a transient widening of the tight junctions between epithelial cells (14).

We have recently developed a new type of chitosan nanoparticles obtained by a very mild ionotropic gelation procedure, and have reported their excellent capacity for the association of proteins, such as BSA, tetanus toxoid and diphteria toxoid (15–16) or oligonucleotides (17). Based on the acceptability of chitosan for nasal administration, it was our idea to explore the potential of chitosan nanoparticles as a delivery vehicle for nasal administration of proteins and peptides. The hypothesis behind this idea was that chitosan nanoparticles would intensify the contact between the protein and the nasal absorptive mucosa, thus leading to an increased protein concentration at the absorption site.

Therefore, our first aim was to associate efficiently the model peptide insulin to chitosan nanoparticles. We chose the model peptide insulin because of the simplicity of measuring its therapeutic response, i.e., blood glucose concentration. Insulin-containing nanoparticles were prepared using different formulation conditions and characterized for their physicochemical properties and *in vitro* release behavior. Secondly, their ability to enhance the nasal absorption of insulin was studied by determining the decrease in the plasma glucose levels following nasal instillation.

MATERIALS AND METHODS

Materials

Two types of chitosan, in the form of hydrochloride salt (Seacure® 210 Cl and Protasan® 110 Cl) were purchased from Pronova Biopolymer, A.S. (Norway). Their main physicochemical characteristics are summarized in Table I. Pentasodium tripolyphosphate (TPP) and bovine insulin were supplied by Sigma Chemical Co. (USA). Ultrapure water (MilliQ Plus, Millipore Iberica, Spain) was used throughout. All other chemicals were reagent grade.

Methods

Preparation of Chitosan Nanoparticles

Chitosan nanoparticles were prepared according to the procedure previously developed by our group, based on the ionotropic gelation of chitosan with TPP anions (15). Ionotropic gelation happens when the positively charged amino groups in chitosan interact with the negatively charged TPP. Chitosan 210 Cl and 110 Cl were dissolved in purified water at 0.10%, 0.15%, 0.20%, and 0.25% (w/v). TPP was also dissolved in purified water at various concentrations in order to obtain a final ratio chitosan/TPP of 8/1, 7/1, 6/1, 5/1 and 4/1 (w/w). The nanoparticles were formed spontaneously upon the incorporation of a variable volume of the TPP solution into 3 ml of the chitosan solution, under magnetic stirring at room temperature.

For the association of insulin to chitosan nanoparticles, insulin was dissolved in 0.01N NaOH (5 mg/ml) and then incorporated in the TPP solution. The concentration of insulin

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Table I. Physicochemical Characteristics of Chitosan (Hydrochloride Salts)

	Chitosan 110 Cl (Protasan® 110 Cl)	Chitosan 210 Cl (Seacure® 210 Cl)
Molecular weight (× 1000)	< 50	130
Deacetylation degree (%)	87	>70
Intrinsic viscosity (mpa, 25°C)	10	20–200
pH of chitosan solution	4.81	4.63

in the TPP solution was calculated in order to obtain nanoparticles with a 20%, 30%, 40%, and 50% (w/w) of insulin based on chitosan.

Nanoparticles were concentrated by centrifugation at 10,000 × g on a 10 μl glycerol bed, for 40 min. Supernatants were discarded and nanoparticles were resuspended in purified water for further *in vitro* characterization or in acetate buffer (pH 4.3) or phosphate buffer (pH 6.4) for the *in vivo* studies.

Characterization of Nanoparticles

The morphological examination of the nanoparticles was performed by transmission electron microscopy (TEM) (CM12 Philips, Eindhoven, Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar® films for viewing by TEM.

The size and zeta potential of the nanoparticles were analyzed by Photon Correlation Spectroscopy and Laser Doppler Anemometry, respectively, using a Zetasizer® III (Malvern Instruments, Malvern, UK). For the determination of the electrophoretic mobility, samples were diluted with KCl 0.1 mM and placed in the electrophoretic cell where a potential of ±150 mV was established. Each batch was analyzed in triplicate.

Insulin Loading Capacity of the Nanoparticles

The association efficiency of the process was determined upon separation of nanoparticles from the aqueous medium containing the nonassociated insulin by ultracentrifugation at 16,000 × g at room temperature, for 30 min. The amount of free insulin was measured in the supernatant by the microBCA protein assay (Pierce, Rockford, USA). A calibration curve was made using the supernatant of blank nanoparticles. Each sample was assayed in triplicate. The insulin loading capacity of the nanoparticles and the insulin association efficiency were calculated as indicated below.

$$\text{Loading capacity} = \frac{\text{Total amount of insulin} - \text{Free insulin}}{\text{Nanoparticles weight}} \times 100$$

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

Some control experiments were performed in order to investigate the association mechanism between insulin and chitosan. With this purpose in mind, 4 mg of insulin, previously dissolved in NaOH, were incorporated into a suspension of 15 mg of blank chitosan nanoparticles in ultrapure water (final pH of the incubating medium, 7.2) or acetate buffer (final pH of the incubating medium, 4.4). After 15 min of incubation, nanoparticles were filtered (low-protein-binding filters, Sterile Millex®-HV, Millipore, Bedford, USA) and non-adsorbed insulin was measured by the microBCA protein assay.

In Vitro Release Studies

Insulin release was determined by incubating the nanoparticles in 1.5 ml of pH 7.4 and pH 6.4 phosphate buffer or pH 4.0 acetate buffer (53 μg/ml, 8 μg/ml and 26 μg/ml, respectively), at 37°C. The concentration of the nanoparticles in the release media was adjusted in order to assess sink conditions for insulin. An exception was made with the medium pH 6.4 phosphate buffer since the low solubility of insulin did not allowed us to work in sink conditions but only below the solubility limit for insulin (9.4 μg/ml). At appropriate time intervals, individual samples were filtered and the amount of insulin released from the nanoparticles was evaluated by the MicroBCA protein assay (Pierce, Rockford, USA). A calibration curve was made at each time interval using non-loaded nanoparticles. The experiments were performed in triplicate.

Animals and In Vivo Studies

Male New Zealand albino rabbits (2–3 kg) were provided with laboratory diet *ad libitum* and fasted 18 h before experiments. In order to avoid any influence of the anaesthesia in the insulin absorption (18,19) animals were kept conscious during the experiments.

The following formulations were instilled intranasally to the rabbits using a polyethylene tubing inserted about 3 cm into the nostril: (1) Control insulin solution, pH 4.3. (2) Insulin dissolved in a 0.72% chitosan 210 Cl solution, pH 4.3 (chitosan dose 0.43 mg/kg) (3) Insulin-loaded chitosan 210 Cl nanoparticles (chitosan doses: 0.16 and 0.35 mg/kg; insulin loading: 46% and 29%, respectively), pH 4.3. (4) Insulin-loaded chitosan 110 Cl nanoparticles (insulin loading: 47%), pH 4.3. (5) Insulin solution, pH 6.4. (6) Insulin-loaded chitosan 210 Cl nanoparticles (46% insulin loading), pH 6.4.

The required dose of insulin (5 IU/Kg) was administered in a volume range of 130–170 μl to the rabbits, depending on the insulin loading of the nanoparticles and animal weight. The amount of nanoparticles instilled varied between 0.40–0.64 mg/kg

Blood samples were collected from the ear vein 30 min prior the nasal administration (to establish baseline glucose levels) and at different times after dosing. Glycemia was determined in plasma samples by the glucose-oxidase method (Glucosa GOD-PAD kit, Spinreact S. A., Girona, Spain). Results are shown as the mean values of plasma glucose levels (± standard deviation) of 6 animals.

Statistical Analysis of In Vivo Data

The mean blood glucose levels determined in samples collected before insulin administration were taken as the baseline levels. Using these values, the percentage of glucose reduction at each time after dosing was calculated and plotted against

time. Data from different experimental groups were compared with the corresponding control groups (insulin solution pH 4.3 or pH 6.4) by the U-test of Mann and Whitney with significance level of $p < 0.05$.

RESULTS AND DISCUSSION

The main goal of the present work was to investigate the potential of chitosan nanoparticles for nasal delivery of peptides and proteins. Therefore, using insulin as a model peptide we expected to have information not only about the potential of chitosan nanoparticles for increasing the insulin nasal absorption, but also about the suitability of the nanoparticles formation process for preserving the insulin activity.

Insulin Association to Chitosan Nanoparticles

Preliminary studies were performed to identify the experimental conditions for the formation of chitosan nanoparticles. Results showed that for the types of chitosan selected in this study (chitosan 210 Cl and chitosan 110 Cl), nanoparticles can be obtained using 0.1 and 0.2% chitosan solutions and chitosan/TPP ratios of 6/1, 5/1 and 4/1 (20). The concentration of chitosan selected for the formation of insulin-loaded nanoparticles was 0.1% for chitosan 210 Cl and 0.2% for chitosan 110 Cl. For both types of chitosan the TPP concentration was adjusted in order to obtain a chitosan/TPP ratio of 6/1. TEM analysis revealed that, irrespective of their insulin loading, chitosan nanoparticles have a solid structure and a round shape.

As shown in Table II, chitosan nanoparticles displayed a particle size in the range of 300 nm to 400 nm and a positive zeta potential (from +54 mV to +25 mV) and a very high insulin association efficiency (>87%) leading to insulin loading values as high as 55%. Results indicated that size, zeta potential and loading capacity of the nanoparticles were affected by the insulin concentration in the TPP solution. More specifically, the incorporation of increasing amounts of insulin led to a slight enlargement of the nanoparticles, a decrease on their surface charge and an enhancement of their loading capacity.

These results agree well with the previously postulated mechanism of association of proteins to chitosan nanoparticles mediated by an ionic interaction between both macromolecules (16). Insulin dissolved in NaOH solution is negatively charged (pI 5.3) and, consequently, its electrostatic interaction with the positively charged amino groups of chitosan is favored. This mechanism of association is also supported by the observed partial neutralization of nanoparticles surface charges due to the association of increasing amounts of insulin. In addition, this ionic interaction mechanism may give an explanation to the influence of the chitosan type on the insulin loading capacity of the nanoparticles. Indeed, chitosan 110 Cl nanoparticles displayed higher zeta potential values and greater insulin loading efficiencies than chitosan 210 Cl nanoparticles.

In order to determine the extent in which insulin association with chitosan was mediated by an electrostatic interaction we incubated, at different pH values, blank chitosan nanoparticles with insulin. Results showed that 77.7 ± 0.9% of the insulin added as a NaOH solution was adsorbed onto chitosan nanoparticles suspended in ultrapure water, where insulin displays negative charge (final pH = 7.2), while only a 30.4 ± 5.9 % of insulin was adsorbed onto the nanoparticles suspended in acetate buffer, pH 4.0, where insulin displays a positive charge (final pH = 4.4). Therefore, it could be concluded that the association of insulin to chitosan nanoparticles is highly mediated by an electrostatic interaction although other mechanisms such as hydrogen binding or hydrophobic interactions could also be involved in the association process.

In Vitro Release of Insulin From Chitosan Nanoparticles

Figures 1A and 1B display the release profiles of insulin from nanoparticles made of chitosan 210 Cl and chitosan 110 Cl respectively, at different pH values. Results showed that insulin release from the nanoparticles occurred very rapidly at pH 7.4 and 4.0 and was not affected by the chitosan molecular weight (chitosan 210 Cl and chitosan 110 Cl). Release was slightly slowed down at pH 6.4, a result that could be attributed to the lower solubility of insulin at this pH (9.4 μg/ml) as compared to that at pH 7.4 (834 μg/ml) and at pH 4.0 (>1 mg/ml). In fact, as stated in the methods section, at this pH the low solubility of insulin did not allow us to work under sink

Table II. Physicochemical Properties of Chitosan 210 Cl and 110 Cl Nanoparticles (Chitosan/TPP Ratio 6/1) Prepared with Different Amounts of Insulin

Insulin concentration (mg/ml)*	Insulin/chitosan ratio (w/w)	Mean diameter (nm)	Zeta potential (mV)	Association efficiency (%)	Loading capacity (%)
210 Cl **					
0.00	0.0	370 ± 3	+40.0 ± 1.2	—	—
0.50	0.2	399 ± 5	+36.6 ± 0.4	87.4 ± 2.1	19.0 ± 2.5
0.75	0.3	390 ± 11	+35.6 ± 0.4	91.0 ± 2.0	29.0 ± 4.5
1.00	0.4	377 ± 7	+33.0 ± 0.3	92.0 ± 2.0	38.0 ± 4.0
1.25	0.5	407 ± 19	+25.3 ± 0.3	94.5 ± 1.5	46.0 ± 4.3
110 Cl **					
0.00	0.0	311 ± 6	+53.5 ± 1.0	—	—
1.50	0.3	317 ± 11	+40.3 ± 0.6	96.7 ± 1.4	39.0 ± 3.4
2.00	0.4	337 ± 14	+36.9 ± 0.3	94.7 ± 2.1	47.2 ± 4.2
2.50	0.5	352 ± 11	+32.9 ± 0.2	92.5 ± 4.5	55.0 ± 4.6

* Insulin concentration in the TPP solution.
** Initial chitosan concentration: 1 mg/ml for chitosan 210 Cl and 2 mg/ml for chitosan 110 Cl.

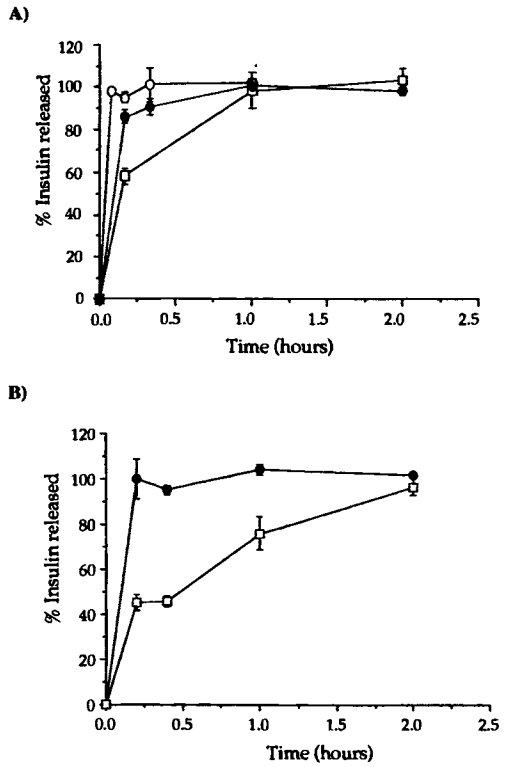

Fig. 1. Insulin release profiles from chitosan nanoparticles. A) chitosan 210 Cl nanoparticles containing 38% insulin, at pH 7.4 (○), 6.4 (□) and 4.0 (●). B) chitosan 110 Cl nanoparticles containing 47% insulin, at pH 6.4 (□) and 4.0 (●) (Mean ± SD, n = 3).

conditions. Also, the release rate of insulin from the nanoparticles was not dependent on the insulin loading (results not shown). This *in vitro* release behavior suggests that the interaction forces between insulin and chitosan are very weak and that insulin releases from the nanoparticles by a simple dissociation mechanism. Furthermore, the formation of irreversible complexes between insulin and chitosan can be discarded.

It was also interesting to observe that the release of insulin from chitosan nanoparticles in water was much slower (only a 30% of insulin was released in 2 hours) than in the above mentioned media. Consequently, it seems reasonably to accept that the fast release of insulin in buffered media is mainly governed by the dissociation of the ionic complex insulin-chitosan.

In Vivo Studies

Figures 2 to 4 exhibit the behavior of different formulations of insulin loaded-chitosan nanoparticles as well as insulin controls instilled intranasally to conscious rabbits. The efficacy of the formulations was assessed by measuring the plasma glucose concentration.

As shown in Fig. 2, the administration of acetate buffer pH 4.3 to rabbits did not cause any modification in blood glucose levels during the experimental period, indicating that the animals were not stressed by the administration procedure and blood sampling. The same figure shows that the administration of a 5 IU/kg insulin control solution (pH 4.3) resulted in a decrease of blood glucose levels of less than 15% at 30

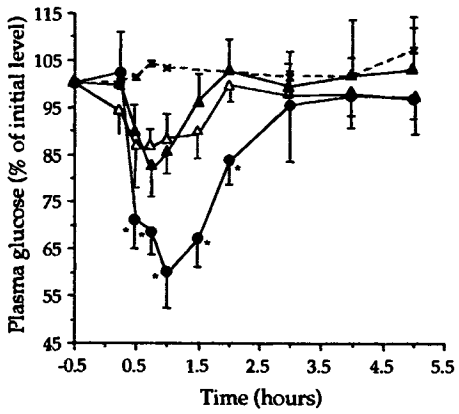

Fig. 2. Plasma glucose levels achieved in rabbits following nasal administration (at pH 4.3) of: acetate buffer (x); insulin acetate buffer solution (Δ); insulin-chitosan 210 Cl acetate buffer solution (▲); insulin-loaded chitosan 210 Cl nanoparticles suspended in acetate buffer (●). (Mean ± SD, n = 6). *Statistically significant differences from control insulin solution ($p < 0.05$)

min post-administration. However, the association of insulin to chitosan 210 Cl nanoparticles led to an increased systemic absorption of insulin. Indeed, the blood glucose level fell to 60% of basal levels at 1 hour post-nasal instillation of insulin-loaded chitosan 210 Cl nanoparticles (maximum glucose reduction). This decrease in plasma glucose levels was significantly different ($p < 0.05$) from that induced by the insulin control solution, for at least 2 hours. These results clearly evidence the ability of chitosan nanoparticles to enhance the nasal absorption of insulin. Furthermore, they indicate that insulin released from the nanoparticles in its active form, thus revealing that the nanoparticles preparation method was not harmful for this peptide. An explanation to this positive behavior of chitosan nanoparticles could be found on the demonstrated ability of chitosan to adhere to the mucosae and to open transiently the tight junctions (14).

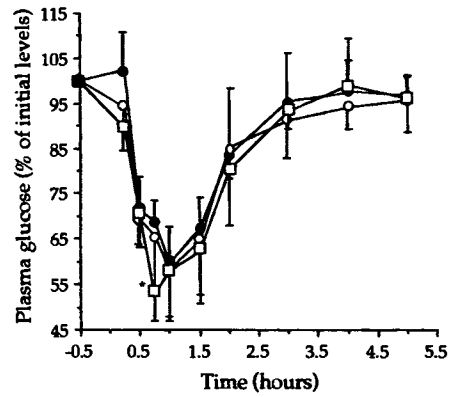

Fig. 3. Plasma glucose levels achieved in rabbits following nasal administration (at pH 4.3) of: insulin-loaded chitosan 210 Cl nanoparticles suspended in acetate buffer, 0.16 mg/kg chitosan (●) and 0.35 mg/kg chitosan (□), and insulin-loaded chitosan 110 Cl nanoparticles suspended in acetate buffer (○). (n = 6). (Mean ± SD, n = 6). *Statistically significant differences from insulin-loaded chitosan 210 Cl nanoparticles suspended in acetate buffer, 0.16 mg/kg chitosan ($p < 0.05$).

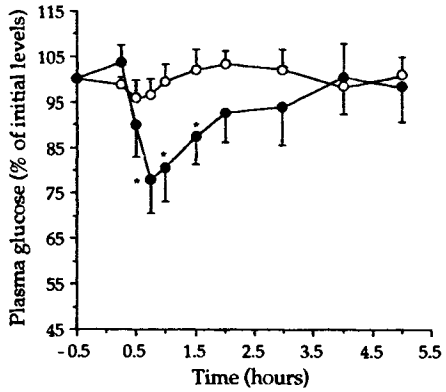

Fig. 4. Plasma glucose levels achieved in rabbits following nasal administration (at pH 6.4) of: insulin phosphate buffer solution (○); insulin-loaded chitosan 210 Cl nanoparticles suspended in phosphate buffer (●). (Mean ± SD, n = 6). *Statistically significant differences from control insulin solution ($p < 0.05$).

In order to investigate whether or not the nanoparticulate form of chitosan has a role in improving the nasal absorption of insulin, the effect of chitosan nanoparticles was compared to that of chitosan solutions. Rabbits were treated with a solution of chitosan 210 Cl (0.72%, w/v) containing the same dose of insulin as chitosan nanoparticles. The dose of chitosan administered in solution was 0.43 mg/kg, which is higher than that administered in the form of nanoparticles (0.16 mg/kg). Results depicted in Fig. 2 clearly show that the decrease in plasma glucose levels induced by the administration of insulin-loaded chitosan nanoparticles was significantly higher ($p < 0.05$) than that obtained following the instillation on the insulin-chitosan solution. It was also interesting to note that despite the previously reported capacity of chitosan glutamate solutions to increase insulin nasal absorption (13), results of the present study showed that insulin-chitosan Cl solutions induced a minor decrease in plasma glucose levels. These differences could be explained by the different experimental approach in both studies. In fact, in the previous work the animal model was different (anesthetized rats and sheeps *vs.* concious rabbits) as it was the chitosan type (chitosan glutamate *vs.* chitosan Cl) and the insulin type (semisynthetic sodium insulin vs. bovine insulin).

Once the penetration enhancement capacity of chitosan nanoparticles was demonstrated, it was important to determine if this capacity would be related to the amount of chitosan instilled. With this idea in mind, we administered the same dose of insulin (5 IU/kg) incorporated into different amounts of chitosan nanoparticles (containing 0.16 mg/kg and 0.35 mg/kg chitosan, loadings of 46% and 29% respectively). Results presented in Fig. 3 indicate that the intensity of the hypoglycemic effect was not influenced by the amount of chitosan used to deliver insulin. Consequently, these results led us to the assumption that the lowest dose of chitosan investigated in this study (0.16 mg/kg) was high enough to allow the transport of insulin through the epithelium.

Despite the effect of the chitosan molecular weight on the size, zeta potential and insulin loading of the nanoparticles, no differences were found in the *in vivo* efficacy of chitosan 210 Cl and chitosan 110 Cl nanoparticles (Fig. 3). It is possible that the differences between these two types of chitosan nanoparticles are not significant enough to have a consequence on the *in vivo* effects. The lack of significance of the chitosan molecular weight on its ability to enhance the nasal absorption of insulin has been previously shown for chitosan solutions (21).

Studies performed *in vitro* using Caco-2 cells have shown that the pH of chitosan solutions plays an important role in their ability to act as absorption enhancers (14,22). In order to investigate if this effect could have a consequence *in vivo* we compared the effect of insulin-loaded chitosan nanoparticles and insulin solutions following their intranasal instillation at pH 6.4 and pH 4.3. Results presented in Fig. 4 indicate that following instillation of insulin solution at pH 6.4, the hypoglycemic effect was negligible. This poor insulin absorption at pH 6.4 could be related to its low solubility and to its tendency to form high molecular weight aggregates, i.e., hexamers and octamers, at pH values close to its isoelectric point (4). Nevertheless, despite these unfavorable conditions, the nasal administration of insulin-loaded chitosan nanoparticles at pH 6.4 led to an important reduction of blood glucose levels as compared to the insulin solution. Statistical analysis of the data confirmed that the differences were significant at 45 min, 1 h and 1.5 h ($p < 0.05$). Even though the hypoglycemic effect of the insulin-loaded nanoparticles was lower at pH 6.4 than at pH 4.3, the increment in the response to insulin-loaded nanoparticles, as compared to the insulin control solutions, was similar at both pH values (18.6% at pH 6.4 and 17.4% at pH 4.3). The penetration enhancing effect of chitosan nanoparticles was then hardly affected by the pH.

In summary, results of this study show that chitosan nanoparticles are more efficient than chitosan solutions in improving the nasal absorption of insulin. The *in vivo* efficacy of chitosan nanoparticles was not affected by the molecular weight of the polymer or the amount of polymer instilled. In addition, and in contrast with chitosan solutions, chitosan nanoparticles were also efficient at a pH value close to the pKa of chitosan. The mechanism by which chitosan nanoparticles enhance the absorption of insulin remains unclear. However, we propose that chitosan nanoparticles could cause the tight junctions between epithelial cells to separate leading to an improvement of the insulin absorption, as it has been reported for chitosan solutions (14). In addition, based on the positive data obtained for chitosan nanoparticles as compared to chitosan solution, we speculate that nanoparticles could intensify the contact of insulin with the absorptive epithelium as compared to chitosan solutions. Finally, there is a possibility that chitosan nanoparticles cross the nasal epithelium, thus working as peptide carriers to the systemic circulation. Current experiments are being aimed at elucidating the mechanisms involved in the process.

CONCLUSIONS

Chitosan nanoparticles described in this paper have shown an excellent capacity for the association of insulin. Insulin-loaded chitosan nanoparticles displayed a high positive charge and a rapid insulin release kinetics, properties which render them very interesting systems for nasal drug delivery. Furthermore, chitosan nanoparticles were able to improve the nasal absorption of insulin to a greater extent than chitosan solutions.

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