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Chitosan-alginate capsules as oral delivery system for insulin: studies *in vitro* and *in vivo*

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ABSTRACT

The aim of this study was to assess chitosan:alginate capsules as gastric resistant systems for oral administration of insulin. Chitosan:alginate capsules of insulin were tested in simulated gastric and intestinal media and *in vivo*. The capsules released only about 20% of the insulin after 60 minutes of incubation in simulated gastric medium. On the other hand, almost all the encapsulated insulin was released after being incubated for 90 min in simulated intestinal medium. When capsules containing 20 IU and 40 IU insulin were given to rats by gavage, significantly reduced plasma glucose levels were observed (33.7 % and 51.7%, respectively) two hours after the treatment, which returned to normal after six hours. These results indicate that chitosan:alginate capsules are potential carriers for oral protein delivery.

Keywords: chitosan:alginate capsules; insulin; oral administration.

INTRODUCTION

Type 1 diabetic patients require insulin treatment for survival because of an insulin deficiency caused by autoimmune destruction of insulin-secreting β cells in the pancreas (Carino & Mathiowitz, 1999). Subcutaneous injection is currently the predominant route of delivery for insulin because of its poor bioavailability when administered by non-invasive means (Khafagy et al., 2007). However, alternative non-invasive routes (e.g. oral, colonic, rectal, nasal, ocular, buccal, pulmonary, and transdermal) have been investigated (Hoffman & Ziv, 1997; Trehan & Ali, 1998).

The oral delivery of proteins such as insulin has been a challenge for researchers. This is the most convenient and comfortable way of administering protein drugs and eliminates the pain of injection, the stress associated with multiple daily injections and possible infections (Lin et al., 2007). However, intestinal absorption of peptide and protein drugs taken by mouth is generally poor, owing to the extensive hydrolysis of these drugs by proteolytic enzymes in the gastrointestinal tract and/or their poor membrane permeability (Tozaki et al., 1997). Moreover, the liver is the primary site of insulin clearance. Approximately 50% of portal insulin is removed during first-pass transit, but this percentage varies widely under different conditions. Very high concentrations of insulin result in a decrease in its fractional uptake, and prolonged raised portal insulin levels also result in reduced clearance, due to receptor down-regulation. Removal of insulin from the circulation does not imply the immediate destruction of the hormone, as a significant amount of receptor-bound insulin is released from cells and reenters the circulation. (Duckworth et al., 1998; Mora et al., 2003).

Encapsulation of drugs in various non-toxic materials is a long-established way of producing sustained-release dosage forms. Among these non-toxic materials, chitosan (a polysaccharide obtained by chitin deacetylation) appears to be suitable for this purpose, due to its biocompatibility and biodegradability (this compound is also degraded by the microflora richly distributed throughout the colon) (Tozaki et al., 1997; Bugamelli et al., 1998). There have been several attempts at oral delivery of insulin using chitosan. Takeuchi et al. (1996) demonstrated the effectiveness of enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. Aiedeh et al. (1997) and Bugamelli et al. (1998) investigated systems chitosan microparticle systems, using ascorbyl palmitate as the surface crosslinker for controlled insulin release. In addition, Hari et al. (1996) developed calcium-alginate-chitosan beads containing insulin and tested them *in vitro*. These authors observed that during treatment with acid, no significant release of the contents occurred (Hari et al., 1996).

The present study was planned with the aim of analyzing chitosan:alginate capsules, intended for the oral delivery of insulin, by means of *in vitro* and *in vivo* tests.

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MATERIALS AND METHODS

Materials

The chitosan used in this study was obtained from Purifarma Ltda (São Paulo, Brazil). The degree of deacetylation was 74% and the nominal average molecular weight, 565 kDa. The viscosity-average molecular weight (M_n) was calculated from the intrinsic viscosity, (η), measured in 0.1 M CH_3COOH (pH 4.5) at 25 °C with a Brookfield Engineering Laboratories viscometer and Rheocal v1.0 software, by means of the Mark-Houwink relationship. The chitosan was found to have $M_n = 6.0 \times 10^5$. Sodium alginate was purchased from Vetec Química Final Ltda (Brazil) and insulin was donated by Biobrás S.A. (Brazil) All other reagents were of analytical reagent grade.

Preparation of Capsules

Chitosan was dispersed in 1% acetic acid solution (w/w) and the pH was adjusted to 5.9 with NaOH. 117 mM NaCl and 50 mM $CaCl_2$ were added to the dispersion. The alginate (1.2%) was dispersed in deionized water and crystalline porcine insulin (20 or 40 IU) was added to the solution (Hosny et al., 1998). The capsules were formed by dropwise addition of the alginate dispersion (1 mL) to the chitosan dispersion. The mixture was then slowly stirred for 5 min to reticulate the capsules. Finally, the capsules were collected, washed in deionized water and dried under vacuum at room temperature. The dried capsules were stored in a refrigerator until use.

In vitro release experiments

For the insulin release tests, capsules were suspended in two media: dilute HCl, pH 1.5 (simulated gastric medium - SGM) and PBS, pH 6.8 (simulated intestinal medium - SIM). Samples were maintained under constant stirring in a thermostatic bath at 37.5 ± 0.5 ºC and aliquots of the media were withdrawn at various times (0, 15, 30, 60 min for SGM and 0, 15, 30, 60, 75 and 90 min for SIM). The insulin content was determined at 285 nm with a Shimadzu UV-1601 spectrophotometer. The method was found to show good linearity for standard insulin solutions in the concentration range used (2.50 UI mL^{-1} – 25.0 UI mL^{-1}) (Bugamelli et al., 1998).

Morphological Analyses

The capsules were characterized by stereoscope (Leica®MZAPO) equipped with Leica Qwin Image Analysis Systems software. The capsules were put on a glass slide and size was measured using the diameter according to Feret at 0º.

In order to evaluate the swelling and degradation of capsules in the two media, samples of capsules were suspended in SGM for 60 min and photographed. The same capsules were then suspended in SIM (pH 6.8) for 90 min and photographed.

Insulin Loading

The total amount of insulin entrapped was assessed by dissolving the capsules in 0.2M phosphate buffer (pH 7.4) and measuring the absorbance at 285nm, as described above.

In vivo experiments

The experiments were conducted with male *Wistar* albino rats (180-220g), housed at 22 ± 2ºC in a 12:12h (?) light-dark cycle, with free access to food and water. The experimental protocol was approved by the Institutional Ethics Committee (Res. 341/06 CEP-UNIVALI) and carried out in accordance with current Guidelines for the Care of Laboratory Animals and the Ethical Guidelines for Investigations of Experimental Pain in Conscious animals (Zimmermann, 1983). Fasted animals were divided into four groups, each group consisting of seven (7) animals. The first group of animals (I) were treated with a dose of chitosan capsules containing 20 IU of insulin (suspended in PBS), while group II received capsules containing 40 IU of insulin, by gastric intubation (gavage). The control group (III) received saline and group IV were injected with monocomponent porcine insulin (0.5 IU/kg, i.p.) (Barichello et al., 1999). Blood samples were taken from the tail vein, 0, 1, 2, 4 and 6 h after the treatment. The serum glucose level was measured to assess the effectiveness of the insulin. After the experiment, the animals were killed by exposure to CO_2.

Blood glucose was assayed by the glucose-oxidase method (Diatewa et al., 2004).

Statistical Analysis

Each result was expressed as the mean ± standard deviation. For group comparisons, one-way layout analysis of variance was used. Significant differences between the means were evaluated by the Dunnett test. A *P* value of <0.05 was considered significant.

RESULTS

The chitosan:alginate capsules had a diameter of 2.5 ± 0.2 mm and a narrow size distribution. The method of preparation resulted in capsules of practically spherical shape, with a rough surface, as shown in Figure 1a. The empty capsules were transparent and smooth while the insulin-containing capsules were opaque, with a somewhat altered surface (Figure 1b).

The degradation of capsules was confirmed by optical photomicrographs. The initially smooth capsules containing insulin showed a changed surface morphology after 30 minutes of incubation in SGM. However, as shown in Figure 1c, even after incubation all the capsules retained their spherical shape.

After 90 minutes of incubation in SIM, a dramatic change was observed in the capsules, as shown in Figure 1d. Optical photomicrographs confirmed that the capsules no longer retained their spherical shape, swelling and dissolving very quickly.

***In vitro* release studies**

The percentage of insulin released into SGM is plotted against time in Figure 2. Approximately 20 % of the insulin load was released during incubation in SGM for 60 min. On the other hand, in SIM, the release of insulin was several times faster than in SGM and all the insulin encapsulated was released in about 90 min.

***In vivo* studies**

When capsules of insulin containing 20 IU were given by gavage, a significant reduction (33.7%) in the serum glucose level was detected, two hours after administration of the capsules, with a return to normal values after six hours (Figure 3). By increasing the dose of insulin to 40 IU, the blood glucose level showed a sharper decrease (51.7 % of the initial value) two hours after administration. The blood glucose again returned to the normal level after six hours (Figure 3). Intraperitoneal injection of insulin (0.5 IU/kg) produced a progressive fall in the serum glucose level (Figure 3), which decreased by 69.6% relative to the initial value, two hours after administration.

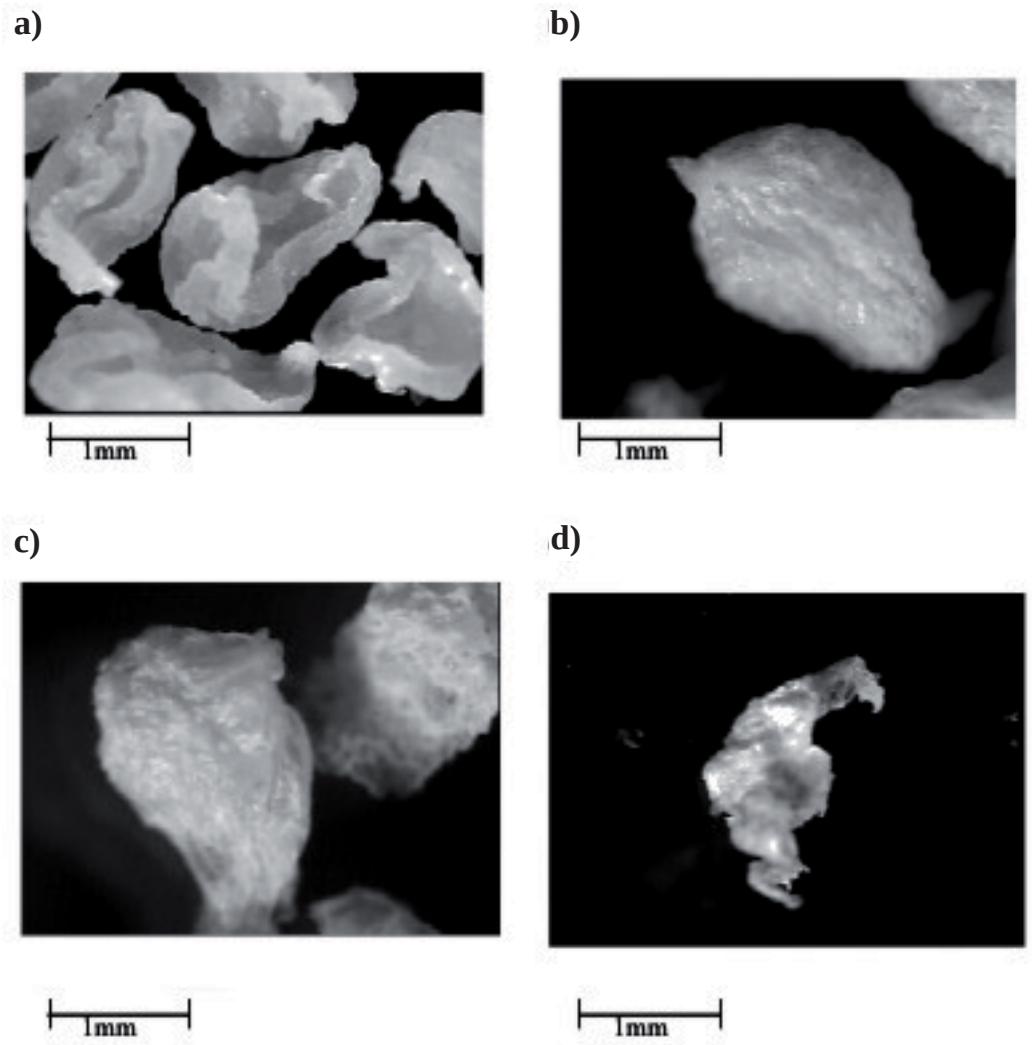

Figure 1. Photomicrographs of the chitosan:alginate capsules. **a)** Empty capsules ; **b)** capsules containing 20 IU crystalline insulin; **c)** capsules containing 20 IU crystalline insulin, after 30 min of incubation in SGM; **d)** capsules containing 20 IU crystalline insulin, after 60 min of incubation in SGM and 90 min of incubation in SIM.

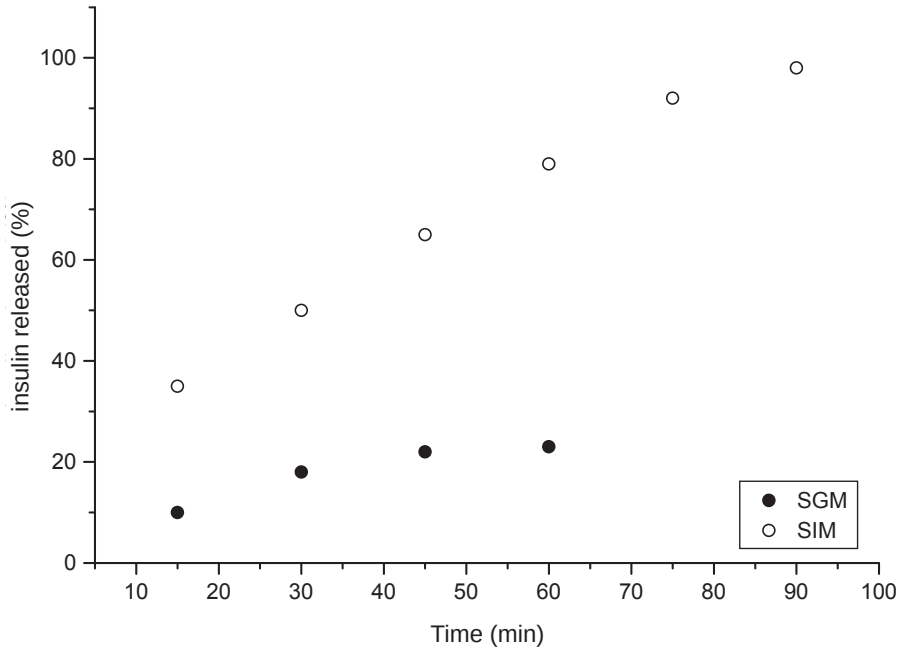

Figure 2. Insulin release profiles from chitosan: alginate capsules in SGM and SIM, at 37.5 ± 0.5 ºC.

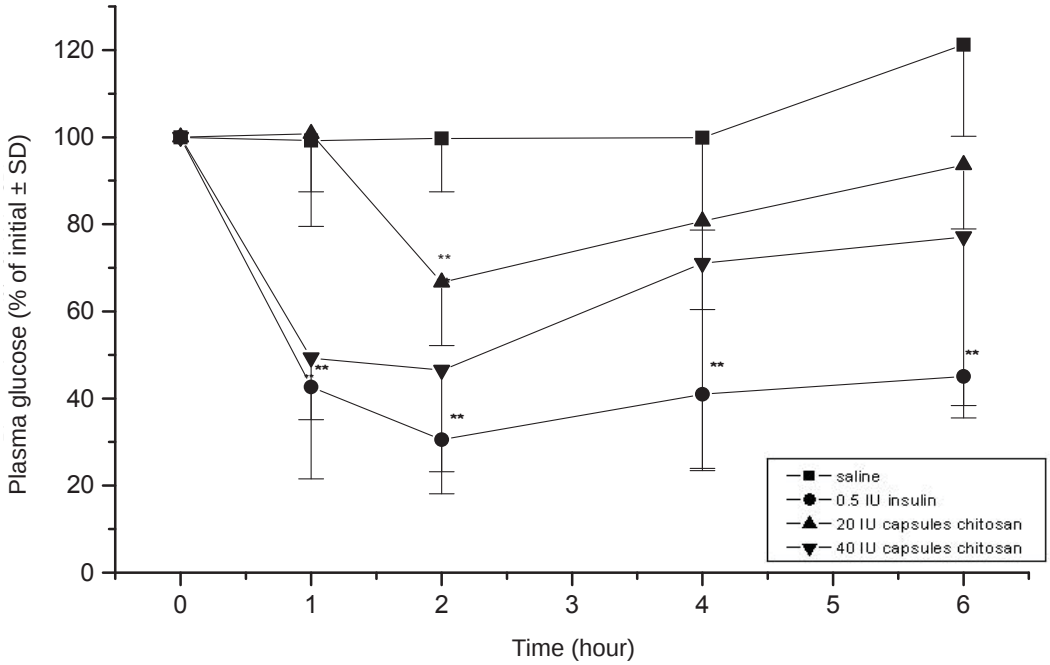

Figure 3. Effect of insulin given by i.p. injection (0.5 IU kg^{-1}) and orally in chitosan:alginate capsules form (total dose 20 and 40 IU) on the plasma glucose level (percent of initial ± SEM).

DISCUSSION

Oral administration of insulin requires protection of the protein against degradation within the gastrointestinal tract. The use of the crystalline form of insulin allowed a high efficiency of insulin encapsulation. Ninety-five % of the initial amount of insulin was found to be associated with the capsules. This behavior can be related to the poor solubility of the crystalline form of insulin; thus, it remains dispersed and not dissolved in the alginate dispersion. These results are in agreement with those obtained by Aiedeh et al. (1997) and Bugamelli et al. (1998), who used the method

of water/oil emulsion to prepare chitosan microparticles. On the other hand, these results are different from those reported by Hari et al. (1996) in experiments using insulin injection and chitosan: alginate capsules.

The release of insulin from the chitosan:alginate capsules depended on the penetration of the dissolution medium into the capsules, their swelling and consequent dissolution, insulin dissolution and diffusion through the swollen capsules. The swelling of the chitosan:alginate capsules depended in turn on the pH of the medium. The membranes are formed as a result of electrostatic interactions between the polycation (chitosan) and polyanion (alginate). These interactions are influenced by the degrees of protonation of the $-NH_2$ groups of chitosan and $-COO^-$ of alginate, which depend on the pH of the medium. This may explain the results obtained when insulin was released from the capsules placed in SGM and SIM media in the *in vitro* studies.

Lee et al. (1996) reported that chitosan-alginate capsules show a maximum swelling volume at pH 9.0. As the pH was reduced, the swelling volume fell until pH 3.0 and a slight increase in swelling volume at pH 1.0 may be ascribed to dissolution due to some hydrolysis of the alginate. At a lower pH, a decrease in the degree of alginate dissociation occurred and dissolution of chitosan could have taken place, increasing protein liberation (Lee et al., 1996).

We also observed that as the insulin dissolved, so did the capsules. These results agree with those of Martins et al. (2007), who observed that at higher pH, a higher diffusion rate of insulin from the microspheres occurred (chitosan-coated alginate microspheres released 80% of the insulin).

According to Daily & Knorr (1988), phosphate may disrupt the alginate-calcium matrix above pH 5.5 by chelating the calcium ions. At this pH, the affinity of phosphate for calcium is higher than that of alginate and the solubility of the calcium phosphate complex is higher.

The *in vivo* results indicate that encapsulation of insulin into chitosan:alginate capsules allows the preservation of its biological activity, because serum glucose decreased in proportion to the insulin content in the capsules.

Insulin uptake and degradation is a feature of all insulin-sensitive tissues. At physiological concentrations, uptake is mediated primarily by the insulin receptor with a smaller contribution from nonspecific processes. In diabetes, clearance rates are decreased (Duckworth et al., 1998). Insulin disappearance from the circulation is more rapid in diabetic rats than in non-diabetic rats (Philippe et al., 1981), and it has a short plasma half-life (4-6 min) (Duckworth et al., 1998). After administration of the capsules with 40 IU to diabetic rats, a hypoglycemic effect was observed in the treated animals, similar to that in animals treated with insulin (Figure 3). The plasma glucose was reduced by 51.7% of basal values. This system of encapsulation of insulin prolongs its biological effect to at least 2 h after oral administration. These results are in agreement with Ubaidulla et al. (2007), who showed that insulin-loaded chitosan succinate microspheres orally administered to diabetic rats decreased their plasma glucose levels (55%). This result may be attributed to the improved stability of insulin in the gastrointestinal tract when it is encapsulated in chitosan:alginate, which protects insulin from degradation by extreme pH and various enzymes.

The results of the *in vitro* experiments showed that the chitosan:alginate formulation produced gastric-resistant capsules. On the other hand, the capsules containing insulin were effective in decreasing serum glucose in rats. Further experiments are in progress with diabetic rats to improve the relative hypoglycemia of the oral insulin formulation.

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RESUMO

Cápsulas de quitosana-alginato como sistema para administração oral de insulina: estudos in vitro e in vivo

O objetivo deste estudo foi analisar as cápsulas de quitosana:alginate como um sistema gastro-resistente para a administração oral de insulina. Cápsulas de quitosana:alginato contendo insulina foram testadas em meio gástrico e intestinal simulado e *in vivo*. As cápsulas de quitosana:alginato liberaram somente 20% de insulina durante 60 minutos no meio gástrico simulado. Por outro lado, grande parte da insulina encapsulada foi liberada após a incubação no meio intestinal simulado dentro de 90 minutos. Quando as cápsulas contendo 20 UI e 40 UI de insulina foram administradas em animais, houve uma redução significante da glicose plasmática (33,7% e 51,7%, respectivamente) duas horas após a administração das cápsulas, retornando ao normal após seis horas. Estes resultados indicam que as cápsulas de quitosana:alginato podem ser potenciais carreadores para a administração oral de proteínas.

Palavras-chave: administração oral; cápsulas de quitosana:alginato; insulina.

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