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SCIENTIFIC PAPER

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INSULIN RELEASE FROM ALGINATE MICROSPHERES REINFORCED WITH DEXTRAN SULFATE

In a previous study, insulin was efficiently encapsulated in alginate microspheres using an emulsification/internal gelation process. However, these microspheres showed a high insulin release at gastric pH, exposing the protein to the harsh conditions of the stomach. In this study, our attempt was to improve insulin release profile by reinforcing the alginate matrix with dextran sulfate (DS). The size distribution was not altered by the presence of DS and the encapsulation efficiency increased to 100%. DS was also able to prevent insulin release at pH 1.2, protecting the insulin from an acidic environment. This effect was explained by an interaction between the permanent negatively charged groups of DS and insulin at low pH. When reinforced alginate microspheres were transferred to neutral pH, dissolution occurred within a few minutes. Increase of the adjuvant concentration did not improve the insulin release profile.

Key words: Alginate, Dextran sulfate, Gastric passage, Insulin, Internal gelation, Microspheres.

Continued progress in many fields of biotechnology has produced numerous kinds of engineered peptides and proteins as novel therapeutic drugs. The development of delivery formulations for these bioactive compounds has been recognized as a key technology to achieve successful therapeutic effects [1]. Oral delivery is the preferred route of administration because it offers several advantages over other routes. However, oral delivery is generally not an effective method due to the poor permeability across intestinal epithelia, acid–induced hydrolysis in the stomach, destruction by proteolytic enzymes in the gastrointestinal tract (GIT) and the chemical and physical instability of peptide and protein drugs [2]. This problem leads to unacceptably low oral bioavailability.

Among therapeutically active peptide molecules, insulin is of great interest for its wide use in the treatment of diabetes mellitus. This protein usually requires daily subcutaneous injections. The oral administration of insulin could be a more acceptable and convenient route of administration for chronic therapy and it could prevent the occasional hyperinsulinaemia observed by subcutaneous administration, since the principal organ in glucose homeostasis is the liver and this should logically be the prime target for intervention [3].

Several approaches to enhance the oral bioavailability of peptides have been or are currently being attempted, among them microencapsulation represents a promising concept [4]. Incorporating or encapsulating peptides in polymeric or non–polymeric microparticles should have the effect of protecting the drug against degradation by the proteolytic enzymes present in the gastrointestinal tract in the physical environment of the formulation itself and microparticles may release the peptide at or near the cellular membrane to optimize the driving force for passive permeation.

There are a wide variety of techniques available for producing microspheres, but many of these approaches, involve the use of organic solvents, heat or high shearing, procedures which are potentially harmful to the structure and consequently biological activity of proteins [5].

Alginate is a naturally occurring copolymer composed of α–L– guluronic and β–D–mannuronic acids that gels in mild conditions in the presence of divalent ions. It is biodegradable, biocompatible, non–toxic and has been used for the encapsulation of a wide variety of biologically active agents including proteins [6], enzymes [7], antibodies [8], cells [9] and DNA [10].

In a previous study insulin was encapsulated in alginate microspheres prepared by the emulsification/internal gelation technique [11]. A high encapsulation efficiency was obtained and insulin proved to be bioactive. However, a high insulin release, near 80%, was obtained at pH 1.2, meaning that the alginate microspheres by themselves could not retain insulin at this pH value.

The calcium alginate matrix is usually very permeable showing a low retention capacity of encapsulated molecules due to its pH dependent solubility [12]. Alginate microspheres are stable in acidic media, but easily swell in alkaline media followed by disintegration and erosion [13]. Numerous efforts have been made to control the erosion of alginate microspheres and extend the drug release. Coating microspheres with polycationic polymers such as chitosan [14,15] or poly–L–lysine [16,17] has been commonly investigated for controlled drug delivery. It has also been reported that multiple coatings, which are generally alternating polyanions and polycations

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Paper received: October 13, 2005
Paper accepted: December 30, 2005

(so–called "onion" capsules), may control the release profile of loaded drugs [18,19]. Finally, another strategy consists in blending different types of co–polymers with sodium alginate, such as, for example, cellulosic derivatives [20], acrylic polymers [21], pectin [22], chitosan [23], polyvinylpyrrolidone [24], chondroitin sulfate [25] or gelatine [26].

The purpose of this study was to reinforce alginate microspheres prepared by the emulsification/internal gelation method [27] and to evaluate the influence on the insulin release profile under simulated gastrointestinal conditions. A polyanionic adjuvant was chosen for reinforcement of the alginate matrix because it is compatible with alginate. Preservation of the optimal characteristics of size distribution and encapsulation efficiency was a concern, simultaneously with the retention of insulin release at gastric pH.

EXPERIMENTAL

Materials

Sodium alginate (Algogel® 3541) was obtained from Degussa Texturant Systems (Boulogne–Billancourt, France) and dextran sulfate from Sigma (Steinheim, Germany). Span® 80 was purchased from Fluka, Chemie GmbH (Buchs, Switzerland). Calcium carbonate (Setacarb 06) was supplied by Omya (Orgon, France) and paraffin oil was supplied by Vaz Pereira (Lisbon, Portugal). Insulin was obtained as a regular human insulin of recombinant DNA origin (Actrapid® 100 IU/ml) from Novo Nordisk A/S (Bagsvaerd, Denmark). All the other chemicals used were of analytical reagent grade.

Preparation of microspheres

The microspheres were prepared by emulsification/internal gelation as described in a previous study [11]. Briefly, a 2% (w/v) sodium alginate solution was prepared by dissolution of the polymer in insulin solution at 0.1% (w/v). Insulin solution was obtained by dilution of the Actrapid® formulation in zinc acetate aqueous solution at 0.4 mM. In some formulations, dextran sulfate (DS), at 0.5 or 1% (w/v), was dissolved in the alginate solution. Viscosity measurements of the alginate solution blended with the polyanionic adjuvant were performed using a rotational viscometer (Visco Star plus, Fungilab, S.A., Barcelona, Spain), at 21°C.

A suspension of 5% w/v ultrafine $CaCO_3$ was added to the polymer solution to obtain a calcium/alginate mass ratio (w/w) of 7.3%. After homogenization the mixture was dispersed into paraffin oil (30% internal phase ratio, v/v) containing 1% Span® 80 by stirring at 400 rpm using an Ika–Eurostar® mixer (Ika, Staufen, Germany) equipped with a marine impeller. The emulsion was formed in a round–bottomed cylindrical glass reaction vessel. After 15 min of emulsification, 20 ml of paraffin oil containing glacial acetic acid were added to the w/o emulsion to obtain an acetic acid/calcium molar ratio of 3.5 and stirring continued to permit calcium carbonate solubilization. The microspheres were recovered from the oily phase by using an acetate buffer at pH 4.5 (United States Pharmacopeia ŠUSPĆ XXVIII) and successively washed with this buffer until no more oil was detected by optical microscope observation.

The microspheres were frozen in an ethanol bath (Benchtop shell freezer, Freezone® model 79490, Labconco, Kansas City, MS, USA) at –50°C and freeze–dried (Lyph–lock 6 apparatus, Labconco) at 0°C for at least 48 h.

Morphological and particle size analysis

An Olympus BH2–UMA optical microscope equipped with a Cue–2 image analyzer (Olympus, Tokyo, Japan) was used to study the morphology of the microspheres. The shape and surface texture of the microspheres were examined by scanning electron microscopy (SEM) (JEOL JSM–840®, 10 kV, Tokyo, Japan). The samples were mounted on metal stubs, using double-sided adhesive tape, gold-coated under vacuum and then examined.

The granulometric size distribution was determined in washing media by laser diffractometry (Fraunhofer model) using a Coulter LS130 particle analyser (Beckman Coulter Inc., Fullerton, CA), with a size range from 0.1 to 1000 µm. The particle size was expressed as the volume mean diameter (µm) ± standard deviation (S.D.) values of the mean. Measurements were made in triplicate for each batch. The polydispersity was determined by the SPAN factor [28] expressed as $Span = [D(v,90)–D(v,10)]/D(v,50)$, where D(v,90), D(v,10) and D(v,50) are volume size diameters at 90, 10 and 50% of the cumulative volume, respectively. A high value of SPAN indicates a wide distribution in size and a high polydispersity.

Encapsulation efficiency

To determine the encapsulation efficiency of insulin, 5 mg of lyophilized insulin–loaded microspheres were accurately weighed and incubated in 5 ml of EDTA 0.1 M prepared in phosphate buffer at pH 7.4 (USP XXVIII), under magnetic stirring (100 rpm/2 h). Samples were withdrawn after incubation and analysed by high performance liquid chromatography (HPLC).The encapsulation efficiency of insulin was calculated from the difference between the theoretical initial amount of protein and the total insulin recovered from the microspheres. Assays were performed in triplicate.

In vitro release studies

A multiple stirring point plate was used for in vitro release studies. Simulated gastric fluid (pH 1.2) and intestinal fluid (pH 6.8) without enzymes were used as

dissolution media and the rate of stirring was 100 rpm. Insulin–loaded microspheres (20 mg) were placed in 20 ml of hydrochloric acid buffer at pH 1.2 (USP XXVIII). After 2h, the microspheres were transferred to 20 ml of phosphate buffer at pH 6.8 (USP XXVIII) and incubated for two hours. Two millilitres of sample were taken at appropriate intervals from both media and the insulin content was determined by HPLC. Assays were performed in triplicate.

Insulin analysis

Samples withdrawn from the acidic medium were centrifuged (13400 rpm/10 min) and the supernatant was used for insulin analysis. Samples obtained from the phosphate media were mixed with NaOH 0.2 M to increase the pH above 7.0 followed by the addition of ethanol (50/50 v/v) to precipitate alginate. After mixing, the samples were centrifuged (13400 rpm/10 min) and the supernatant was analyzed to determine the insulin content.

The concentration of insulin was determined by using a HPLC system (model HP1100 series, Hewlett Packard, Germany) equipped with an autosampler (Agilent 1100 series, Germany). A reversed–phase X–Terra C–18 column, 5 μm, 4.6 x 250 mm (Waters, USA), with a Purospher® STAR RP–18 precolumn, 5 μm, 4 x 4 mm (Merck, Germany) was employed. Mobile phase A consisted of 0.04% trifluoroacetic acid (TFA) in water, while the mobile phase B was 0.04% TFA in acetonitrile. A linear gradient of 30–40% B over 5 min was used with a flow rate of 1.2 ml/min. Injections of 20 μl were made with an autosampler. The UV detector was set at 210 nm and HPLC analysis was carried out at 27°C. A retention time of 4.5 min was obtained.

Statistical analysis

Each value was expressed as the mean ±S.D. Statistical differences were analysed by using a one–way analysis of variance (ANOVA) followed by a Bonferroni post test. For a value of P less than 0.05 the difference was considered significant.

RESULTS AND DISCUSSION

Characterization of microspheres

Alginate (ALG) microspheres showed a mean diameter value of 69 μm and a SPAN factor of 0.8 (Table 1).

Table 1. Influence of the addition of dextran sulfate on the mean diameter, SPAN factor and insulin content of alginate microspheres

Type of micro-spheres	Mean diameter (μm)	SPAN factor	Alginate solution viscosity (mPa·s)	Content (%, w/w)
ALG	68.7 ± 20.8	0.82	2894.3 ± 115.9	4.72 ± 0.12
DS	71.3 ± 22.9	0.87	2901.4 ± 113.6	4.51 ± 0.07

Dextran sulfate was blended into an alginate matrix (DS microspheres), at a concentration of 0.5% (w/v), in order to improve the properties of the microspheres. No significant changes of the mean diameter or size distribution were observed compared to plain alginate microspheres. A unimodal size distribution was observed for both formulations.

The size distribution of the microspheres obtained by emulsification/internal gelation was correlated with the size distribution of the emulsion droplets. If one parameter such as apparatus design, the viscosity of the two immiscible phases or the speed of mixing changes, the final average droplet size will proportionally change [29]. Dextran sulfate had no influence on the viscosity of the original alginate solution before the emulsification step, which may explain the absence of interference on the size of the microspheres.

As illustrated in Figure 1, the microspheres were discrete and spherical and no aggregation was observed for ALG and DS microspheres. Tubular and star–like structures were visible on ALG microspheres and attributed to the presence of insulin. Nevertheless,

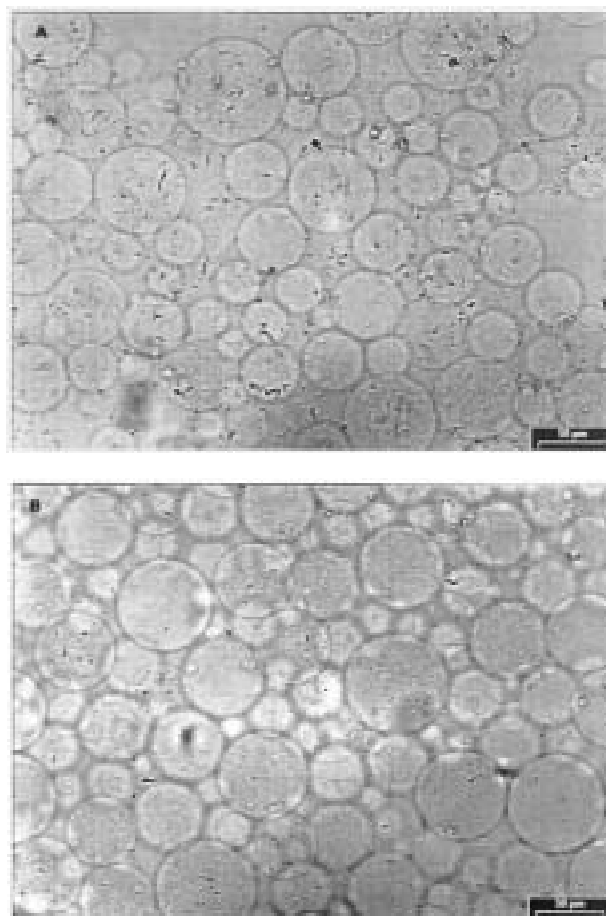

Figure 1. Optical microphotograph of insulin–loaded alginate microspheres containing (A) no adjuvant and (B) dextran sulfate (magnification 200 X)

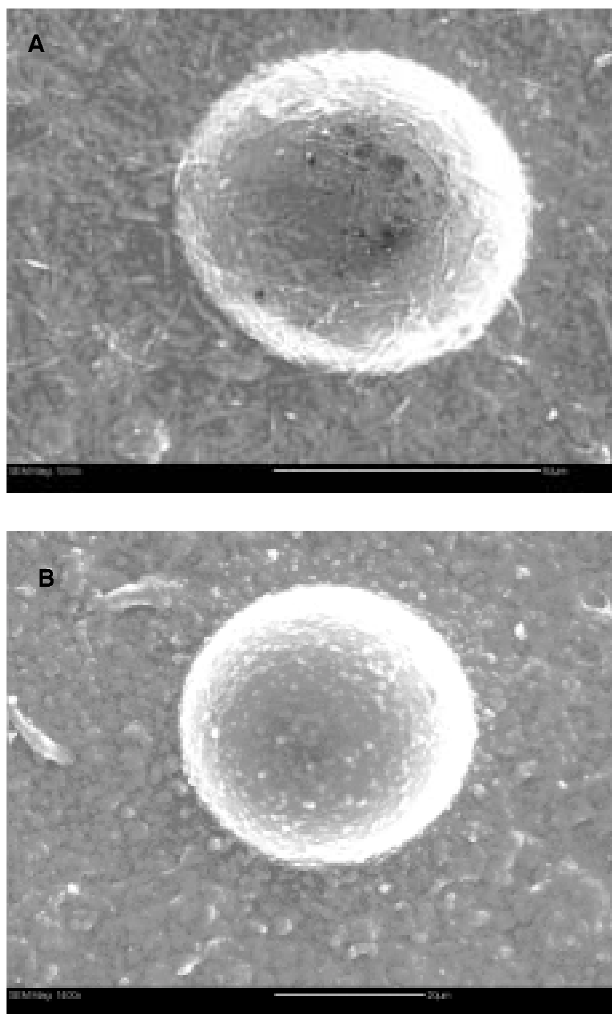

Figure 2. Surface morphology of insulin–loaded alginate microspheres containing (A) no adjuvant and (B) dextran sulfate according to SEM micrograph (magnification 1000 X)

the presence of insulin was not clear when dextran sulfate was added to the alginate matrix.

The surface structure of the formulations was also examined by SEM (Figure 2). The insulin structure was very visible on the surface of ALG microspheres but, in the case of DS microspheres, insulin was not visible on the surface, confirming optical microscopy results.

The insulin content on the alginate microspheres was 4.7% (w/w) corresponding to approximately 135 IU per 100 mg of microspheres and an insulin encapsulation efficiency of nearly 83%. The incorporation of dextran sulfate on the alginate microspheres decreased the insulin content to 4.5% (w/w), but due to an increase of the total theoretical mass, this value corresponded to an increase of the insulin encapsulation efficiency to nearly 100%.

Although the insulin encapsulation efficiency was very high for DS microspheres, no insulin was visible by optical microscope observation. Probably, the protein was located inside the microspheres and not at its surface. The increase on insulin encapsulation efficiency promoted by dextran sulfate could be explained by a higher network density causing physical retention of the insulin inside the microspheres or to an electrostatic interaction between the protein and the polyanion that could favour the retention of insulin in the microspheres.

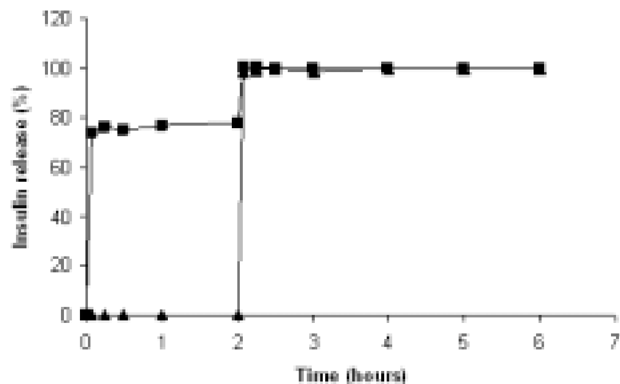

Figure 3. In vitro release profile of insulin–loaded alginate microspheres containing (■) no adjuvant and (▲) dextran sulfate incubated in simulated gastric fluid for 2 hours and then in simulated intestinal fluid. The results are the mean of three experiments ±S.D.

In vitro release studies

The insulin encapsulated in microspheres should not release in acidic media, due to the protein susceptibility to enzymatic attack. Microspheres should protect the encapsulated protein from the harmful gastric environment by retaining the protein inside the polymer matrix during acid incubation.

The microspheres were evaluated under gastrointestinal simulated conditions without enzymes. When incubated at pH 1.2, ALG microspheres showed an initial burst effect of insulin. At the end of the 2 h–period of acid incubation, a total insulin release of nearly 80% was obtained (Figure 3).

The effect of pH on the Ca–alginate hydrogel network structure has been neglected in previous studies. The incubation of microspheres at pH 1.2 causes an ion–exchange the between calcium and proton ions and the calcium alginate gel is converted to the unionized form of alginic acid [30]. After this acid treatment, even if the calcium ions no longer contribute to the stabilisation of the microspheres, the particles maintain their macroscopic structure without any visible changes in the morphology [31,32]. However, a reduction in the gel strength may occur, which can favour drug release by diffusion [33]. On the other hand, high insulin losses found at pH 1.2 can be attributed to the end of an interaction existing during the recovery between insulin and alginate. The recovery is performed at pH 4.5 and, at this pH value, the negatively charged alginate interacts with the positively charged insulin. When the microspheres are transferred to pH 1.2, this

interaction is lost with the partial precipitation of alginate in the form of alginic acid.

Blending of the dextran sulfate with the alginate matrix, completely disabled insulin release during the 2 h–incubation in acidic medium. This capacity may be explained by electrostatic interactions formed between the different molecules. The addition of another polyanion to the alginate also increases the polymer concentration, which may result in a higher network density.

Cellulose acetate phthalate, a derivatized cellulosic polymer, is commonly used as an enteric coating and was added as a third crosslinkable polymer to a calcium alginate–pectinate matrix [34]. A low drug release was obtained at pH 1.5 that, however, was not completely prevented by the incorporation of CAP.

Eudragit has been one of the mostly used adjuvants to reinforce alginate microspheres due to the fact of being an enteric polymer. However, the polymeric reinforcement of alginate beads with Eudragit RS100 did not retard the release rate of melatonin in gastric fluid [35]. In another study, the drug release from microspheres containing dipyridamole was unaffected by enteric Eudragit L100 55 incorporated into the alginate gel [36]. It was hypothesized that since the microspheres were small in size, the amount of incorporated polymer was in sufficient to form a strong barrier gel in the matrix to delay drug release.

In our opinion the effect observed on insulin release at pH 1.2, due to the presence of DS on the matrix, may be mainly attributed to electrostatic interactions. Insulin is a protein that due to its composition in aminoacids, has an isoelectric point of 5.3–5.4, which means that it shows a positive charge at pH 1.2. The physical mixture of alginate and dextran sulfate provides both pH–sensitive (carboxylic) and permanently charged (sulfate) groups [37]. Calcium alginate matrices are depleted of calcium ions and converted to insoluble alginic acid at pH values below its pKa value. The presence of sulfate groups, permanent negatively charged, may be responsible for an interaction with insulin at acidic pH, preventing its release.

After changing to intestinal pH, a fast and complete dissolution of the microspheres occurred for both formulations, permitting the total release of insulin in few minutes. By increasing the pH value to 6.8, the alginic acid formed during acid incubation was converted to a soluble salt of sodium alginate. This caused the matrix to swell and disintegrate, enabling the complete release of insulin.

The reinforcement of ALG microspheres with dextran sulfate had no effect on insulin release at intestinal pH and a fast release of insulin was also obtained. In fact, the increase of pH to a value higher than insulin pH causes a charge alteration for the insulin molecule and, probably, the reversion of the interaction with the negatively charged groups of dextran sulfate and, consequently, insulin is released.

Influence of adjuvant concentration

In order to achieve more retardation of the drug release from alginate microspheres at intestinal pH, the dextran sulfate concentration was increased from 0.5 to 1%. The increase in polyanion concentration caused an increase of the mean diameter, possibly due to a higher tendency for agglomeration (Table 2), but the size distribution did not change.

Table 2. Influence of the concentration of dextran sulfate on the mean diameter, SPAN factor and insulin content of alginate microspheres

Concentration of dextran sulfate (%, w/v)	Mean diameter (μm)	SPAN factor	Content (%, w/w)
0.5	71.3 ± 22.9	0.87	4.51 ± 0.07
1	85 ± 24.4	.4 0.78	4.39 ± 0.08

Increase of the adjuvant concentration caused a slight decrease of the insulin content explained by an increase in the total polymer mass. It was not possible to determine the insulin encapsulation efficiency since the protein content was higher than the theoretical content. This may have occurred due to partial loss of the adjuvant blended with the alginate matrix, causing a decrease in the total theoretical mass of the microspheres.

Alginate microspheres containing 1% dextran sulfate showed a similar release profile compared to microspheres reinforced with 0.5% dextran sulfate. Insulin release was prevented at gastric pH (Figure 4)

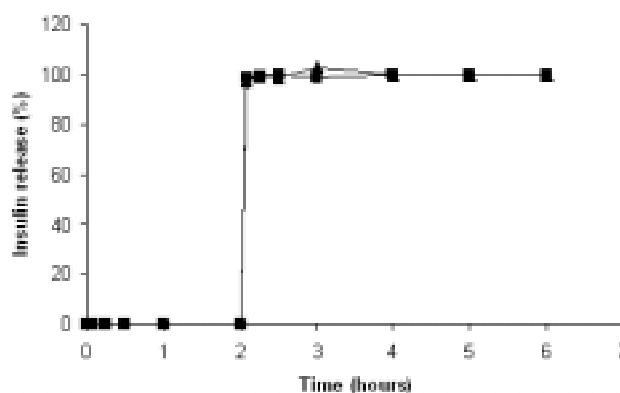

Figure 4. In vitro release profile of insulin–loaded alginate microspheres containing 0.5% (■) and 1% (▲) of dextran sulfate incubated in simulated gastric fluid for 2 hours and then in simulated intestinal fluid. The results are the mean of three experiments ±S.D.

and complete insulin release was obtained just after changing to intestinal pH.

Increase of the polyanion concentration could result in a higher network density that could decrease the permeability of the microspheres at intestinal pH. However, such an effect was not observed since no suppression of the initial release of insulin was obtained at intestinal pH. These results support the theory that dextran sulfate prevents insulin release in acidic medium more probably due to an electrostatic interaction with the protein than to an increase in the polymer network density of the alginate microspheres.

CONCLUSION

The goal of this study was to develop microspheres protecting insulin from gastric passage by reinforcement of the calcium alginate matrix with another polyanion. It was considered an objective to maintain the optimal characteristics of size distribution and encapsulation efficiency for the reinforced microspheres.

Plain alginate microspheres showed a fast and high release of insulin in simulated gastric medium as a result of protonation of the alginate and conversion to alginic acid at low pH. In order to enhance the protein retention capacity under gastric conditions, dextran sulfate was blended with alginate. The addition of this polyanionic adjuvant completely prevented insulin release at pH 1.2 without affecting the size distribution of the microspheres and the encapsulation efficiency was increased. Increasing the dextran sulfate concentration did not compromise the retention of insulin at gastric pH, however it did not contribute to increase insulin retention at intestinal pH. These results indicate that dextran sulfate reinforced microspheres are good candidates for the oral delivery of insulin. The formulation could be extensively applicable for the oral delivery of pharmaceutical peptides and proteins.

ACKNOWLEDGEMENTS

This research was financially supported by a grant from the "Fundaçao para a Ciência e Tecnologia" (FCT) of Portugal (SFRH/BD/5085/2001). The authors would like to thank Eng. Vítor Redondo and Dr. Margarida Figueiredo from the "Instituto Pedro Nunes" (Coimbra, Portugal) for the size distribution determination and Professor A. Rocha Gonçalves from the "Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra" for the HPLC equipment.

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IZVOD

KONTROLISANO OTPUŠTANJE INSULINA IZ ALGINATNIH ČESTICA SA DEKSTRAN SULFATOM

(Naučni rad)

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U prethodnim istraživanjima insulin je efikasno inkapsuliran u alginatne čestice, korišćenjem emulsifikaciong procesa formiranja mikrokapsula. Tako dobijene kapsule su imale visok stepen otpuštanja ali i negativne mikoefekte u gastrointersticijalnom (GI) traktu. U ovom radu je metoda inkapsulacije poboljšana inkorporiranjem dextan sulfata (DS) u alginatni matriks. Veličina dobijenih mikrokapsula je ostala nepromenjena u prisustvu DS dok se efikasnost inkapsulacije poboljšala za 100%. Ovaj efekat se može tumačiti interakcijom negativno naelektrisanih funkcionalnih grupa na DS sa insulinom pri niskim (realno fiziološkim uslovima) pH vrednostima. Pri neutralnom okruženju dolazi do momentalnog rastvaranja alginatnih čestica, što takođe važi i u slučaju povećavanja koncentracije DS.

Ključne reči: Alginat, Dekstran sulfat, Gastrointersticijalni trakt, Insulin, Geliranje, Mikročestice.