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Characterization and *in vivo* evaluation of ketotifen-loaded chitosan microspheres

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ABSTRACT

Ketotifen (KT)-loaded chitosan microspheres (MS) were prepared for controlled release of the antihistaminic drug, and their use as delivery systems in the intraperitoneal cavity of rats was investigated. Microspheres were prepared by a spray-drying method followed by treating with glutaraldehyde solutions in methanol as cross-linker. Results showed that very small spherical microspheres (1.0–1.3 μm) with a high load of KT (92 ± 6 μg KT/mg) were obtained. KT loading decreased with cross-linking (52 ± 2–46 ± 7 μg KT/mg). Interactions between KT and chitosan avoided total KT release from cross-linked MS. After intraperitoneal (i.p.) administration, microsphere aggregations were adhered to muscle subjacent to the tegument and to adipose tissue, and there were no evident sings of rejection; KT was detected in blood stream (0.37–0.25 μg/mL) at 24 h, which was longer than the i.p. administration of the drug in solution (39.4 μg/mL at 2.4 h).

1. Introduction

Chitosan is a linear polysaccharide composed by units of glucosamine and *N*-acetyl-glucosamine linked by (1 → 4) β-glycosidic bonds. It is obtained by the alkaline deacetylation of chitin (Muzzarelli & Muzzarelli, 2005). Chitosan is a weak base and it is insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution (pH < 6.5), which can convert the glucosamine units into a soluble form with protonated amine groups (Sinha et al., 2004). The non-toxic, biodegradable and biocompatible properties of chitosan provide potential for many applications (Arvanitoyannis, 1999). Due to its polyelectrolyte nature, chitosan can be used as absorbent for treatment of textile industry effluents (Morais, de Almeida, Pereira, & Fonseca, 2008) as well as for heavy metal ions uptaking from wastewater (Vieira & Beppu, 2006). It has been also used as template for the preparation of mesoporous metal oxides spheres (Pinheiro et al., 2009). Chitosan has been frequently proposed for applications in pharmaceutical and biomedical fields (Muzzarelli, 2009) because of their biocompatibility and biodegradability. Its polycationic character, along with the presence of reactive functional groups, has given chitosan particular possibilities for utilization in controlled-release technologies (Felt, Buri, & Gurny, 1998). Over the years, a variety of natural and synthetic polymers have been explored for the preparation of microparticles, of which chitosan has been extensively investigated (Davidenko et al., 2009; Muzzarelli & Muzzarelli, 2005). Because of its bioadhesive properties, chitosan has received a substantial attention as novel bioadhesive drug delivery systems (Varum, McConnell, Sousa, Veiga, & Basit, 2008), which is aimed at improving the bioavailability of drugs by prolonging the residence time at the site of absorption. It makes suitable for delivery of drug via nasal (Corrigan, Healy, & Corrigan, 2006; Grenha, Seijo, & Remuñán-López, 2005; Learoyd, Burrows, & French, 2008), or gastrointestinal routes (Felt et al., 1998; Sinha et al., 2004; Gupta & Jabrail, 2007).

Ketotifen fumarate (KT), 4-(methyl-4-piperidylidene)-4*H*-benzo[4,5]cyclophepta[1,2]thiophen-10(9*H*)-one hydrogen fumarate, has been widely use as an antiallergic and antianaphylactic agent in adults and children in the treatment of bronchial asthma and allergic diseases (Grant, Goa, Fitton, & Sorkin, 1990). It is an antihistamine that inhibits release of inflammatory mediators derived from mast cells (MC). By this way, ketotifen can prevent local tissue damage and multiorgan dysfunction due to vasoactive and proinflammatory mediators derived from MC after intestinal ischemia/reperfusion (Kalia, Brown, Wood, & Pockley, 2005); it can also preclude the mesenteric alterations and splanchnic inflammatory changes related to acute portal hypertension in rats (Sánchez-Patán et al., 2008). Ketotifen has also investigated in multidrug resistance in human breast cancer cells and doxorubicin toxicity in mice (Zang & Berger, 2003). Several delivery systems have been designed to modulate ketotifen delivery by different routes of administration (Chiang, Chen, Liu, & Wang, 1998; Elsayed, Abdallah, Naggar, & Khalafallah, 2006; Inoue, Ogawa, Okada, & Sugibayashi,

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2005; Karlgard, Wong, Jones, & Moresoli, 2003). Due to its use in the treatment of bronchial asthma, particularly of an allergic origin, dry powder inhalation formulations of liposomally entrapped drug have been prepared (Joshi & Misra, 2001) for direct ketotifen delivery in the respiratory tract.

The purpose of this study was to prepare ketotifen-loaded chitosan microspheres by spray-drying technology. The morphology and size characterization of microspheres as well as *in vitro* drug release experiments were studied. *In vivo* evaluation of the systems has been carried out by intraperitoneal administration in rats to evaluate plasma levels of the drug and possible tissue affectation.

2. Materials and methods

2.1. Materials

Chitosan (Aldrich, Barcelona, Spain; deacetylation grade ~85%; Brookfield viscosity 200.000 cps), glutaraldehyde (25 v%), acetic acid, methanol, acetonitrile, chloroform, potassium monohydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) (Panreac, Barcelona, Spain), heparin (Analema, Vigo, Spain), isoflurane (Isoba Vet, Schering Plough Animal Health, Harefields, England), were used as received. Milli-Q® water (Millipore, Madrid, Spain) was used. Ketotifen hydrogen fumarate was kindly supplied by Novartis (Madrid, Spain).

2.2. Methods

2.2.1. Preparation of microspheres

Preparation of microspheres was carried out by the spray-drying process (Mini Spray-dryer B-191, Büchi, Flawil, Switzerland). To obtain microspheres without drug, chitosan (0.25 wt.%) was dissolved in an aqueous solution of acetic acid (5 v%). Microspheres with ketotifen (KT) were prepared from 0.225 wt.% chitosan (90 wt.% of solute) and 0.025 wt.% of KT (10 wt.% of solute) dissolved in an aqueous solution of acetic acid (5 v%). Assay conditions were: Inlet air temperature: 150 °C, spray flow 5 mL/min, and compressed spray air flow (represented as the volume of air input) 700 NL/h. Un-loaded and KT-loaded chitosan microspheres were stabilized by cross-linking with solutions of glutaraldehyde (Glut) (2, 5 and 10 v%) in methanol. The resulting chitosan microspheres were placed in a vacuum oven (Bioblock Scientifics, Illkirch, Strasbourg) for 24 h at 100 mbar of pressure and 30 °C, and then, they were stored in a desiccator under vacuum condition.

2.2.2. Particle size and appearance

The size and appearance of microspheres were studied by scanning electron microscopy (SEM) (Jeol JSM-6400 Electron Microscope, resolution 36 mm from Centro de Microscopía y Citometría, UCM). From SEM micrographs, more than 500 particles were measured using micrographs enlargements. The number-average diameter (D_n), the weight-average diameter (D_w) and the polydispersity index (U) (Guerrero et al., 2008) were calculated. The particle distribution is considered to be monodisperse when U is between 1.0 and 1.1.

2.2.3. Estimation of drug content

In previous studies, stability of KT in different solvent media (phosphate buffer 1 mM, pH 7.4, and chloroform) was determined by HPLC and UV/V spectra (Guerrero et al., 2008).

In order to determine the amount of KT included in chitosan microspheres, 10 mg of KT-loaded microspheres were added to 2 mL of a solution containing chloroform:phosphate buffer 1 mM, pH 7.4 (1:1), and stirred in a vortex for 5 min. After centrifugation (3500*g*, 10 min), 0.4 mL of the aqueous phase, as well as 0.4 mL of the organic phase, were collected to determine the amount of the drug. KT was recovered from these samples by acetonitrile according to a modification of the method proposed by Yagi (Yagi, Taniuchi, Hamaa, Sudo, & Sekikawa, 2002): 1 mL of acetonitrile was added to 0.4 mL of the aqueous and the organic phases; after vigorous shaking for 5 min and centrifugation (3500*g*, 10 min), the supernatant was collected and then evaporated. The samples were reconstituted with 0.4 mL of an aqueous solution of acetonitrile (50 v%) and the KT concentration determined by HPLC (Spectra-Physics SP8800 HPLC pump, SP 100 UV absorbance detector and SP4400 commuting integrator) in accordance with a method developed by our group (Guerrero et al., 2008). The KT retention time was 6.0 ± 0.2 min.

2.2.4. In vitro drug release studies

For drug release studies, 30 mg of KT-loaded microspheres was added to 50 mL of phosphate buffer (1 mM; pH 7.4), which was placed in a vessel covered with Parafilm® at 37 °C and at a constant shake (200 rpm) in an orbital incubator (Ecotron® Inforts AG CH-4103). At desired times, samples (100 μL) were withdrawn from the solution in order to follow the change in KT concentration by UV/V spectroscopy at 297 nm. For calibration, KT standards of 1–100 μg/mL in phosphate buffer (1 mM, pH 7.4) were used. The volume removed from the vessel was replaced with phosphate buffer. Sink conditions were maintained (Alkhamis, Obeidat, & Najib, 2001). The experiments were carried out in triplicate.

2.2.5. In vivo ketotifen administration

Male Wistar rats, weighing 241 ± 5 g, were obtained from the Animalario of the Universidad Complutense de Madrid (Spain) (DC 86/609/CEE; OM 13/X/1989, RD 1201/2005). Experiments were approved by the Animal Care Committee of the Universidad Complutense. The animals were housed in cages under environmentally controlled conditions of light (12:12 light:dark cycle), temperature (22 ± 2 °C) and were fed standard rat food and water ad libitum. The surgical material used in the experiment was previously autoclaved. Just before injection, the dissolution solvent was put under ultraviolet light (Ecogen Lamp, Viber Lourmat, Intensity 7 mW/cm^2) at 254 nm for 4 s because of the germicidal action of this wavelength.

Different groups of animals were established. The animals that were intraperitonealy (i.p.) administered the drug were divided into two groups. Group 1: Animals injected with KT-loaded microspheres. Microspheres were dispersed in 1 mL of saline solution (0.9% NaCl). The animals were anaesthetized with an equipment of isoflurane [Burtons, Series 5 T.C.V. Kent, United Kingdom] and then the microsphere dispersion was i.p. injected in the rat using a sterile syringe with a 1.2 × 40 mm nozzle (Microlance 3). Group 1A (n = 6): 45 mg of KT-loaded microspheres cross-linked with 2%-Glut (KT content = 2.36 mg). Group 1B (n = 6): 47 mg of KT-loaded microspheres cross-linked with 5%-Glut (KT content = 2.41 mg). Group 1C (n = 6): 53 mg KT-loaded microspheres cross-linked with 10%-Glut (KT content = 2.47 mg). Group 2 (n = 6): Animals i.p. injected with 1 mL of an aqueous solution of KT of 2 mg/mL. Furthermore, three control groups of animals (n = 3 per group) were i.p. injected with each one of the three types of chitosan microspheres without drug.

2.2.6. Determination of ketotifen in plasma

At predetermined times after injection of KT-loaded microspheres and the KT solution, animals were anaesthetized with isoflurane. Blood (0.2 mL) was collected by puncturing the jugular vein in heparinized (15 U = 3 μL) polypropylene tubes. The heparinized blood was centrifuged at 11,000*g* for 10 min immediately after collection so as to obtain plasma. Plasma samples were then

stored at −20 °C. In animals administered KT-loaded microspheres, blood samples were taken 6–24 and 30 h after injection and at 24 h intervals thereafter. In animals administered a KT solution, blood samples were taken 20–40 and 84 min, and then every hour up to 4.4 h after injection.

KT was precipitated from plasma samples by acetonitrile according to a modification (Guerrero et al., 2008) of the method proposed by Yagi (Yagi et al., 2002). KT concentration in the sample was determined by the HPLC system (Guerrero et al., 2008). The KT retention time was 6.3 ± 0.2 min.

2.2.7. Histological studies

Animals were sacrificed in CO_2 atmosphere 17 days after the intraperitoneal injection of the microspheres. An incision was made on the peritoneal region of the rat to examine tissues. Tissues where the presence of microspheres was detected were removed. Histological studies were carried out. A piece of the removed tissue, fixed with formol (10% v/v) was immersed in paraffin. Cuts (10 μm) were carried out with a paraffin microtome (Minot type). Samples were dyed using the hematoxylin–eosin and trichromic methods (Humason, 1979).

2.2.8. Statistical analysis

Statistical comparisons were performed with unpaired Student's *t*-test. A value of $p < .05$ was considered significant.

3. Results and discussion

Preparation of KT-loaded microspheres was carried out using the spray-drying technology. The preparation and characteristics of the microspheres can be affected by several parameters of the technique such as inlet temperature, spray rate of feed and air flow rate. Polymer concentration and solvent as well as assay conditions were chosen based on previously published data on preparation and optimization of chitosan microspheres by spray-drying (Corrigan et al., 2006; He, Davis, & Illum, 1999; Huang, Chiang, & Yeh, 2003; Mi, Wong, Shyn, & Chang, 1999). In this study, 0.25 wt.% chitosan solution (5 v% acetic acid; pH 2.8) was chosen in order to obtain a feed dissolution of suitable viscosity, which avoids the obstruction of the nozzle. An acidic solution with a significant percentage by volume of acetic acid was used to dissolved chitosan because of ketotifen was also dissolved in that solution. Ketotifen is sparingly soluble in water, ketotifen solubility (Alkhamis et al., 2001) increases with the decrease in pH (Al-Nimry, Assaf, Jalal, & Najib, 1997) due to ketotifen is a weak base. Spray-drying is a solvent evaporation process. Since the boiling point of water is 100 °C at standard condition, and that of acetic acid is 118 °C, the inlet temperature used (150 °C) in this study was higher than those of the solvents. Furthermore, after microspheres were collecting, they were maintained under vacuum conditions in order to obtain microparticles with the lowest moisture content.

SEM studies indicated that un-loaded microspheres were of small size and their surface was smooth without hollows or deformations. The size distribution of microspheres was: 37% of 0.2–0.6 μm, 41% of 1–2 μm and 22% of 2.3–3 μm. The average diameter (mean ± SD) of the microspheres was 1.3 ± 0.1 μm, and the polydispersity index was 1.7 (Table 1).

Non cross-linked chitosan microspheres cannot be kept suspended in water because of swelling and dissolution (He et al., 1999). In order to prepare stabilized chitosan microspheres, glutaraldehyde (2, 5 and 10 v%) was used to solidify the particles. Different cross-linking agents have been used to obtain chitosan microspheres with good sphericity and compact structure (Sinha et al., 2004; Wei et al., 2008). When microspheres were prepared by spray-dryer, cross-linkers were usually added before the pulverization (He et al., 1999; Liu, Desai, Tang, & Chen, 2006). On the contrary, in this study cross-linking was carried out after obtaining microspheres by spray-drying. Glutaraldehyde-cross-linking occurs through a Schiff's base reaction between aldehyde ends of the cross-linking agent and the amine moieties of chitosan to form aldimine functions (Vieira & Beppu, 2006).

Table 1
Particle size and polydispersity index (U) of microspheres (MS).

Chitosan MS	Un-loaded MS		KT-loaded MS	
	D_n (μm)	U	D_n (μm)	U
Uncross-linker	1.3*	1.7	1.0*	1.7
2%-Glut	1.2	1.6	1.0	1.9
5%-Glut	1.2	1.7	1.1	1.8
10%-Glut	1.2	1.6	1.3	1.6

D_n: number-average diameter.
* $p < .05$.

Cross-linked and uncross-linked chitosan microspheres, with or without KT, were small size and their surface was smooth. The microsphere surface was smoother as the cross-linking degree increased (Fig. 1), independently of the presence or absence of KT. These results are in agreement to those obtained by Gupta (Gupta and Jabrail, 2007), in which microspheres cross-linked with glutaraldehyde have shown smooth surface morphology in comparison to uncross-linked chitosan microspheres. The size distribution of un-loaded and cross-linked microspheres was: (A) microspheres cross-linked with 2%-Glut: 27% of 0.3–0.6 μm, 54% of 1–1.6 μm, 19% of 1.9–2.8 μm; (B) microspheres cross-linked with 5%-Glut: 16% of 0.2–0.4 μm, 61% of 0.7–1.5 and 23% of 1.8–3 μm; (C) microspheres cross-linked with 10%-Glut:17% of 0.2–0.4 μm, 68% of 0.8–1.6 μm, 15% of 2–3 μm. For KT-loaded chitosan microspheres, the size distribution was: (A) uncross-linked microspheres: 34% of 0.3–0.7 μm, 59% of 1–1.7 μm and 7% of 2–3 μm; (B) microspheres cross-linked with 2%-Glut: 28% of 0.2–0.4 μm, 52% of 0.8–1.2 μm and 20% of 1.6–3 μm; (C) microspheres cross-linked with 5%-Glut: 22% of 0.2–0.4 μm, 62% of 0.8–1.6 μm and 16% of 2–3 μm; (D) microspheres cross-linked with 10%-Glut: 10% of 0.2–0.4 μm, 70% of 0.8–1.6 μm and 20% of 2–2.5 μm. The average diameter (mean ± SD) was between 1.3 ± 0.1 μm and 1.2 ± 0.1 μm for unloaded microspheres, and between 1.0 ± 0.1 μm and 1.3 ± 0.1 μm for KT-loaded microspheres, and their polydispersity index between 1.6 and 1.9 (Table 1). There were significant differences of the average diameter between uncross-linked loaded- and unloaded microspheres. Hence, the presence of the drug in the feed mixture seemed to influence the size of KT-loaded microparticles. However, there were not significant differences of the average diameter regarding to the cross-linking degree (Table 1). Chitosan microparticles prepared by Huang and coworkers (Huang et al., 2003) using a spray-drier showed a particle size of 2.12 μm and their external surface appeared smooth. He and coworkers (He et al., 1999), using a spray-drying procedure, have obtained uncross-linking chitosan microspheres, whose size in the swollen state (in isopropanol) was of 4–5 μm, and cross-linked microparticles with a swollen size of 2–10 μm. In general, the appearance of microspheres prepared in our study were similar to those obtained in equivalent experimental designs mentioned before; however, the average diameter was smaller.

The percentage of entrapment efficiency (EE) of KT in uncross-linked chitosan microspheres was 83 ± 6%. This high EE was similar to that reported for the encapsulation of drugs that were soluble in the same solvent as polymers using the spray-drying technique (Blanco et al., 2003). Furthermore, chitosan and ketotifen were dissolved in an acidic solution before spray-drying, a Schiff's base reaction can occur between the ketone group of the drug and the amine primary group of the polysaccharide to form ketimine functions.

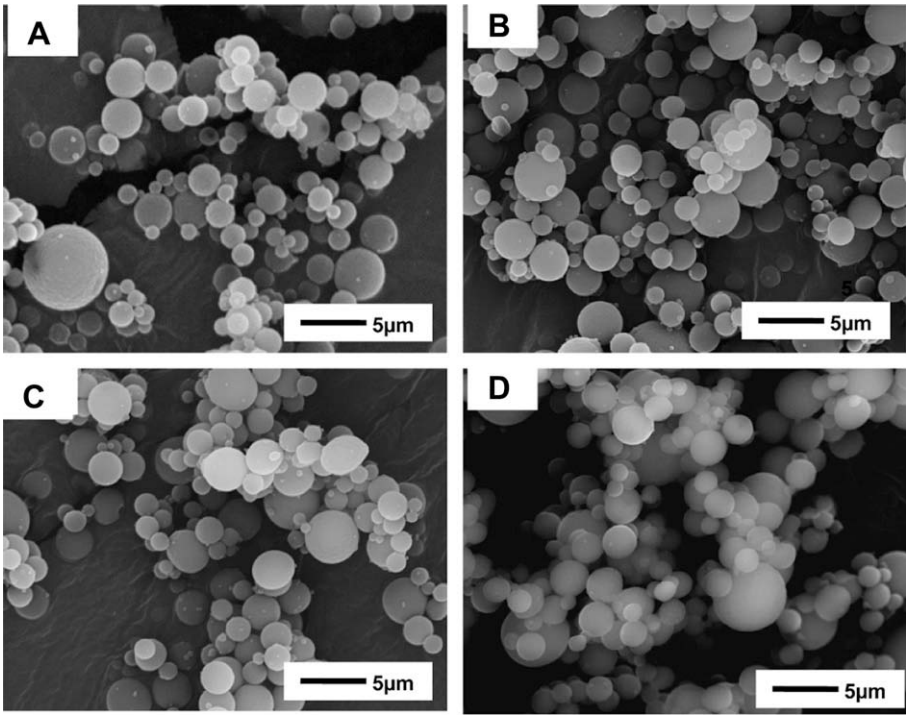

Fig. 1. Scanning electron micrographs (SEM) of ketotifen-loaded chitosan microspheres: uncross-linked (A), cross-linked 2%-Glut (B), cross-linked 5%-Glut (C) and cross-linked 10%-Glut (D).

After cross-linking with glutaraldehyde, the percentage of EE of KT decreased: 47 ± 2% for microspheres cross-linked with 2%-Glut; 46 ± 7% for microspheres cross-linked with 5%-Glut; and 42 ± 7% for microspheres cross-linked with 10%-Glut. The amount of KT loaded in microspheres was between 92 ± 6 and 46 ± 7 μg KT/mg (Table 2). Therefore, a greater degree of cross-linking caused a lesser percentage of EE. It might be speculated that the aldimine formation by glutaraldehyde is prevailing on the ketimine (quite reasonable considering the high reactivity of glutaraldehyde and the bulky nature of ketotifen), thus displacing the loaded drug. Furthermore, the cross-linking procedure of microspheres was carried out in a methanolic solution, which can contributed to remove part of the loaded KT, since KT is slightly soluble in methanol (3.4 mg/mL). Significant differences between the percentages of EE with regarding the amount of glutaraldehyde used for cross-linking were not observed (Table 2). KT has been included in other delivery systems with different EE: 97.92 ± 0.54% in liposomes (Joshi & Misra, 2001), 43.98 ± 0.96% in ethosomes (Elsayed et al., 2006), and 74 ± 7% and 81 ± 6% for PLA and PLGA microspheres, respectively (Guerrero et al., 2008).

In drug delivery experiments, the maximum cumulative release of KT from uncross-linked microspheres (Fig. 2) took place at 2 h, time at which 74% of the loaded KT was released. From this time the gelification of the medium made very difficult the measure of the released drug. Therefore, due to its hydrophilic properties, glutaraldehyde was used as cross-linking reagent in order to better the control of drug release. Regarding the amount of glutaraldehyde (2, 5 and 10 v%) used to prepare stabilized chitosan microspheres; similar concentrations were employed by other researchers to obtain a suitable drug release rate and to limit the toxicological effect of the cross-linking agent (Mi, Tan, Liang, & Sung, 2002). The maximum percentage of KT released from chitosan microspheres cross-linked with 2%-Glut was 68.2% (35.5 μg KT/mg microspheres) at 50 h (Fig. 2B). When microspheres were cross-linked with 5%-Glut, the cumulative release of KT at 50 h was 54% (Fig. 2C), and the maximum release (58.4%), obtained at 100 h, indicated 29.7 μg KT/mg microspheres. Microspheres cross-linked with 10%-Glut showed the largest release of the drug; 74.4% (34.5 μg KT/mg microspheres) of the loaded KT was released at 50 h (Fig. 2D).

Chitosan microspheres, independently of their cross-linking degree with glutaraldehyde, did not release the total amount of loaded KT, and there was not a linear relationship between the percentage of KT released and the amount of glutaraldehyde used for cross-linking. In fact, the maximum percentage of released KT took place from the most cross-linked microspheres. Thus, the extent of the cross-linking and, as a consequence, the equilibrium swelling degree of the microspheres (Gupta & Jabrail, 2006), contributes to KT release. As larger the cross-linking smaller the number of available amine groups of chitosan to interact with carbonyl groups of KT, which can explain the largest percentage of drug release from the most cross-linked microspheres. Furthermore, the swelling of chitosan microspheres in the aqueous medium made possible ketimine hydrolysis and the release of KT, factor more significant at lower cross-linked microspheres.

Three well-defined stages can be observed in the release of KT from chitosan microspheres, independently of their cross-linking degree (Table 3). A burst effect was observed during the first 5 min of drug release. The amount of KT released decreased as the percentage of cross-linker increased, although the percentage of KT released at this stage was quite similar from 5%-Glut to 10%-Glut cross-linked microspheres. The drug placed on the most

Table 2
Amount of ketotifen (KT) loaded in chitosan microspheres (MS) and percentage of entrapment efficiency.

Cross-linker	Ketotifen load (μg KT/mg MS)	Entrapment efficiency (%)
Uncross-linker	92 ± 6*	83 ± 6*
2%-Glut	52 ± 2	47 ± 2
5%-Glut	51 ± 8	46 ± 7
10%-Glut	46 ± 7	42 ± 7

* $p < .05$.

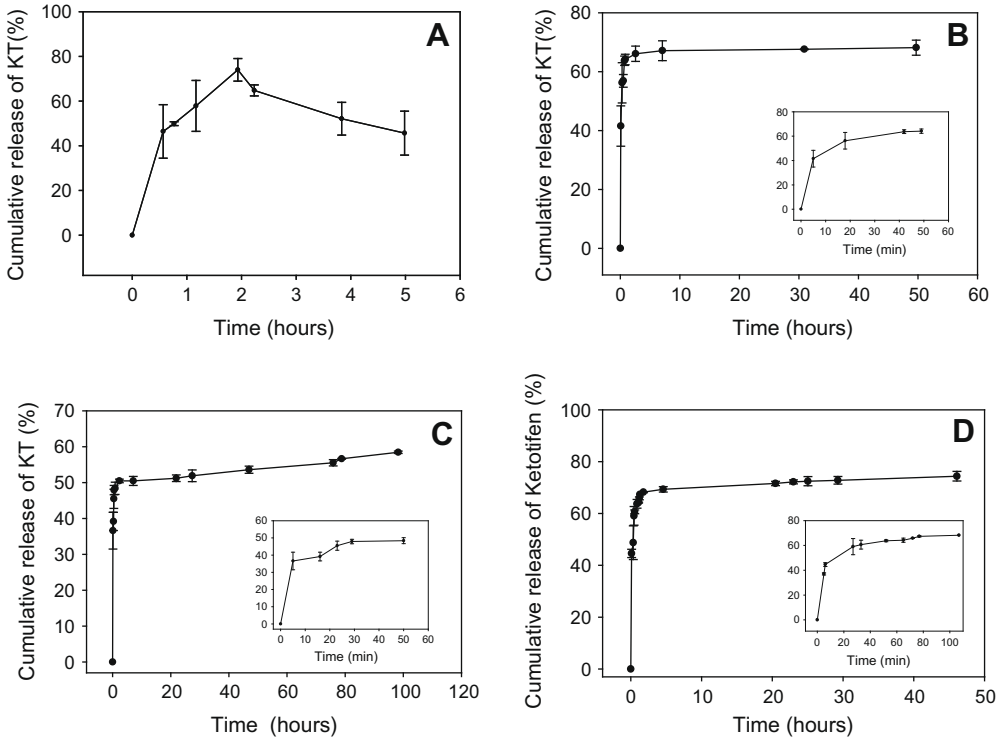

Fig. 2. *In vitro* release kinetic of ketotifen (KT) from KT-loaded chitosan microspheres uncross-linked (A), cross-linked with 2%-Glut (B), 5%-Glut (C) and 10%-Glut (D). Inset: KT release during the first hours. Each point shows average values ± standard deviation (n = 3).

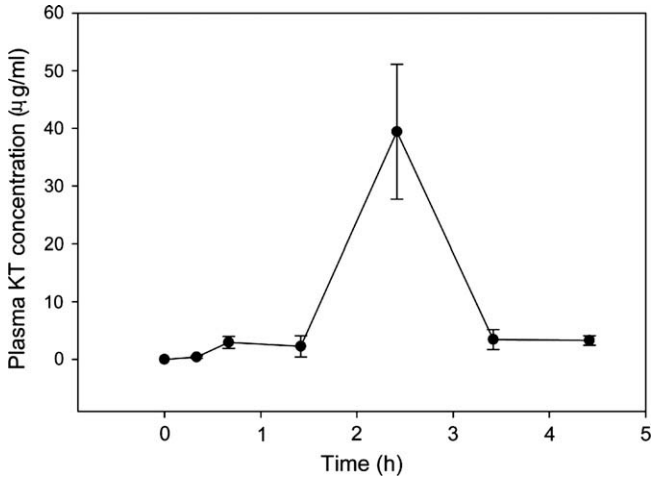

Fig. 3. Plasma concentration of ketotifen (KT) after intraperitoneal injection of KT solution (dose 10 mg KT/kg body weight).

superficial part of the microspheres was released quickly, where the contribution of the smaller microspheres was bigger. This burst phase has been also described for the release of different drugs from chitosan microspheres (Blanco, Gómez, Olmo, Muñiz, & Teijón, 2000; Corrigan et al., 2006; Huang et al., 2003). The burst effect observed in drug release from microparticulate systems is not itself an advantage or disadvantage of the formulation, it depends on the type of drug entrapped and also on the type of application of the microspheres (Trapani et al., 2003). Since KT is an antihistaminic drug, a quick release of the drug could be an advantage in the control of the delivery of histamine from cells at first state of the treatment, and then a slow release of the drug allows maintaining the antihistaminic effect, the microspheres acting as reservoir to prevent the enzymatic metabolism of the drug.

Following the burst effect, two stages of different KT release rate were observed (Table 3). The first release rate was determined between 5 and 50 min, and the second release rate from 50 min at the end. For every period, a straight line was obtained by using a least square fit and the release rate was determined from the slope of this line. From 5 to 50 min, the release rate of the drug was between 11.6 ± 0.6 and 16.9 ± 2.5 μg/h per milligram of microspheres (Table 3), and it decreased as the cross-linking degree of the microspheres increased. Thus, the pore size seems to determine the KT release rate at this stage, which implicates a significant amount of released drug. On the contrary, during the second stage of release, from 2 h up to the end of the release process, the release rate of KT increased with the percentage of glutaraldehyde (Table 3). A high percentage of KT has been released from the microspheres during the first stage; this released drug is the one that has established fewer interactions with the polymeric matrix. However, the residuary KT is establishing interactions with the amine groups of the polysaccharide, and as freer amine groups present in the polymer more interactions with KT take place. Thus, the release rate of KT was slower from the lower cross-linking microspheres during the second stage of drug release. Total release of loaded KT did not take place; a significant amount, between 40% and 25%, of the loaded KT was maintained in the inner part of the microspheres. Thus, by simple swelling of the chitosan microspheres in phosphate buffer, total release of KT was not originated. This effect is similar to that observed in studies in which a 45% of α-lipoic acid was remaining in chitosan microspheres because of bonds between chitosan matrix and α-lipoic acid, producing an incomplete release (Weerakody, Fagan, & Kosaraju, 2008).

KT-loaded chitosan microspheres were intraperitonealy administrated to rats in order to evaluate their biocompatibility and the plasma levels of the drug in blood stream. In this study, 10 mg KT/kg body weight was the dose used. Doses of ketotifen between 1 and 50 mg/kg have been studied in different experimental designs with animals (Drugs.com; Fujii, Tanaka, Harada, Hirai, & Kamei,

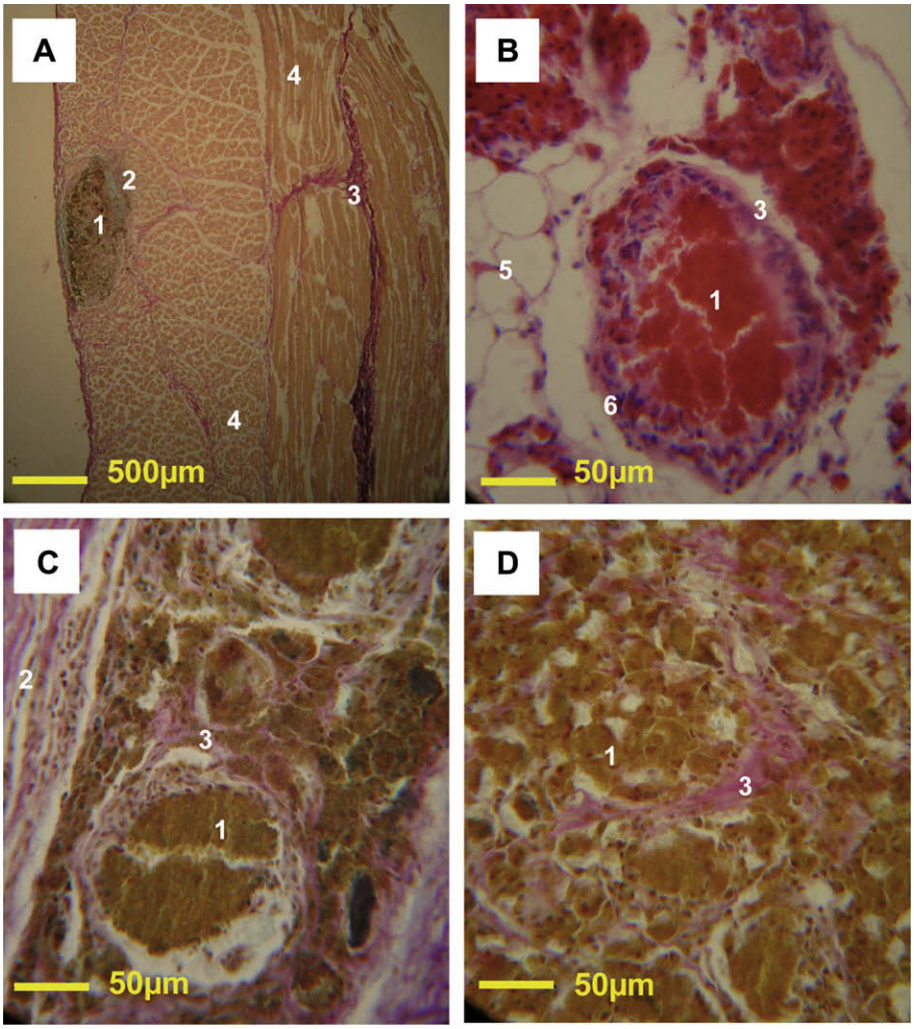

Fig. 4. Photomicrographs of microspheres and the surrounding tissue after 17 days of intraperitoneal injection of ketotifen-loaded chitosan microspheres cross-linked with 2%-Glut (A and B), 5%-Glut (C) and 10%-Glut (D). Microspheres (1), connective tissue (2), collagenous fibers (3), muscle tissue (4), adipose tissue (5), lymphocytes (6).

Table 3
Percentage of ketotifen (KT) released due to the burst effect and release rates of the drug from ketotifen-loaded chitosan microspheres (MS).

Cross-linker	Burst effect (% KT) 0–5 min	First release rate 5 min–50 min		Second release rate from 50 min	
		(μg/h per mg MS)	r^2	(ng/h per mg MS)	r^2
2%-Glut	41% 21.6 ± 3.6 μg KT/mg MS	16.86 ± 2.55	0.96	40.43 ± 8.30	0.91
5%-Glut	36% 18.6 ± 2.6 μg KT/mg MS	12.86 ± 1.01	0.91	50.50 ± 3.28	0.97
10%-Glut	37% 17.2 ± 0.4 μg KT/mg MS	11.57 ± 0.57	0.95	88.83 ± 1.50	0.92

2003; Yagi et al., 2002). The pathophysiology of prehepatic portal hypertension has been studied using the experimental model of partial portal vein-ligated (PVL) rat (Sánchez-Patán et al., 2008). One of the characteristics of the PVL rats is an increased infiltration of the intestinal mucosa and submucosa by mast cells whose inflammatory mediators could produce vasodilatation and angiogenesis. Thus, the administration of KT in the intraperitoneal cavity could exert its antihistaminic activity decreasing the level of inflammatory mediators, as it has been observed after the administration of KT-loaded PLGA 50/50 microspheres in PVL rats (Guerrero et al., 2008; Sánchez-Patán et al., 2008).

After administration of KT-loaded chitosan microspheres, the drug only was detected in the blood stream at 24 h. KT plasma concentration was 0.370 ± 0.007, 0.260 ± 0.017 and 0.250 ± 0.004 μg/mL after administration of KT-loaded chitosan microspheres cross-linked with 2, 5 and 10 v% glutaraldehyde, respectively; the largest value was obtained with microspheres of lower cross-linking degree. This behavior was in accordance with the *in vitro* results and corresponds to the initial quick release of KT. In general, *in vivo* drug release is slower than *in vitro* because the amount of aqueous medium for hydration of the polymeric systems is smaller (Blanco et al., 2000). Thus, the plasma concentration of KT after 24 h of the injection of drug-loaded microspheres in rats can be considered the result of the quick release of the drug during the first stages of release. The plasma concentration of KT obtained during the second stage of release from the microspheres probably was so low that it could not be quantified in the blood stream. When KT was intraperitonealy administered at the same dose in solution (Fig. 3) the maximum drug concentration (39.4 μg/mL) was observed at 2.4 h, and the drug was not detected

at 4 h. Thus, the administration of KT entrapped in chitosan microspheres allowed a controlled release of the drug in the intraperitoneal region.

The examination of the peritoneal region showed that unloaded and KT-loaded microspheres were adhered to the muscle subjacent to the tegument in all cases (Fig. 4). This fact can be related to the mucoadhesive properties of chitosan (Varum et al., 2008). Chitosan is believed to enhance drug absorption through tight junctions via the paracellular route by direct interaction of the cationic polymer molecule with the negatively charged cell membrane. By this way, the inflammation produced in intestinal mucosa after experimental prehepatic portal hypertension could be reduced if an i.p. administration of KT-loaded chitosan microspheres took placed in that region. Fig. 4A shows a group of chitosan microspheres cross-linked with 2%-Glut placed in the muscle tissue subjacent to the tegument. Small groupings of these microspheres were surrounded by connective tissue. Collagen and elastic fibers can be seeing between groupings of microspheres. When chitosan microspheres cross-linked with 5%-Glut (Fig. 4C) and 10%-Glut were i.p. injected, groups of chitosan microspheres, equivalent to the above described, were also observed in the muscle tissue subjacent to the tegument. This fact can be considered in accordance with the normal body reaction to a biocompatible material, which consists in walling it off in an avascular, collagenous bag. The implantation of biocompatible and biodegradable microspheres induces the activation of humoral and cellular mechanisms to produce inflammatory and healing responses of the material (Sastre et al., 2007). The acute and chronic inflammatory response depend on the polymer composition of the biodegradable microspheres, and polymorphonuclear leukocytes, monocytes and lymphocytes are the cell types associated with this event. Thus, the presence of some lymphocytes (Fig. 4C) close to some microspheres can be considered part of the normal process of response of the host after the injection of these drug delivery systems. Furthermore, chitosan microspheres were also observed in the adipose tissue close to liver, bladder or intestine. Fig. 4B shows KT-loaded chitosan microspheres cross-linked with 2%-Glut in the adipose tissue close to liver; collagen fibers are surrounding microsphere groupings, and a small number of lymphocytes can be observed.

The degree in inflammatory reaction observed in our experiments was low, and it did not depend on degree of cross-linking. Mi and coworkers (Mi et al., 2002) have studied biocompatibility of glutaraldehyde cross-linked chitosan microspheres after intramuscular injection; those microspheres were cross-linked with 0.44 M of glutaraldehyde, which is 1.7 times more concentration than the largest concentration (10%-Glut is 0.25 M of glutaraldehyde) used in our studies. They observed an inflammatory reaction, with the presence of lymphocytes and giant cells surrounding the tissue implanted with the glutaraldehyde-cross-linked chitosan microspheres at first-week postoperatively; this inflammatory reaction decreased with time, and at 20-week postoperatively it was almost all eliminated. Results obtained by Wei (Wei et al., 2008) showed that the Schiff base C=N bond involved with glutaraldehyde during the cross-linking reaction could turn into a stable C-N form *in vivo*, which is difficult to transform back into the aldehyde group. Such biodegradation would reduce the side effects and confirm the potential used of glutaraldehyde as a cross-linking agent in the clinical application. Many studies have been reported in the literature to evaluate the safety of glutaraldehyde and this has been proven that it is non-carcinogenic and safe (NTP, 1999). To date, although the native glutaraldehyde is somewhat toxic, several blood substitutes cross-linked with glutaraldehyde are available in the market or in the clinical phase III evaluation (Hemopure, Hemolink and PolyHemes) and non obvious toxicity has been found (Sprung, Kindscher, & Wahr, 2002). Glutaraldehyde-cross-linked chitosan microspheres have been shown to have ability for long-acting delivery of drug (Jameela & Jayakrishnan, 1995), however, both the cytotoxicity of the cross-linker used, and the degradation rate of the cross-linked microspheres should be taken into considerations in the clinical application (Mi et al., 2002). Although there are different opinions about the suitability of glutaraldehyde, there is not evidence of toxicity with the concentration range studied in our researches.

4. Conclusion

Encapsulation of KT using chitosan as encapsulant material was successfully carried out by spray-drying process. Both un-loaded and drug-loaded microspheres exhibited spherical geometry and smooth surface, and small number-average diameter. High percentage of entrapped efficiency was obtained from chitosan microspheres, which decreased after cross-linking with glutaraldehyde. However, the cross-linking allowed a controlled release of the drug from chitosan microspheres during a significant period of time (50–100 h); and a burst effect was observed within 5 min. Interactions with chitosan avoided the total release of KT. The intraperitoneal administration of KT-loaded microspheres allowed detecting the drug in the blood stream at 24 h, and plasma levels of KT were lower than those obtained from KT solution at the same dose. Histological studies showed that cross-linked microspheres were non-toxic. Hence, these microspheres may be considered as suitable candidate for delivery of ketotifen in the intraperitoneal cavity to reduce the inflammatory process originated in partial portal vein ligation (PVL) rats and in general in portal hypertension.

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