Drug Development and Industrial Pharmacy, 27(6), 567–576 (2001)

RESEARCH PAPER

Bioadhesive Characteristics of Chitosan Microspheres to the Mucosa of Rat Small Intestine

Junko Shimoda, Hiraku Onishi,*
and Yoshiharu Machida

Department of Clinical Pharmacy, Hoshi University, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

ABSTRACT

Chitosan (Chi) microspheres were examined in vitro and in vivo in terms of their bioadhesive characteristics to the mucosa of rat small intestine. Chi was labeled with fluorescein isothiocyanate (FITC), and the microspheres (FTC-MS) were prepared by the dry-in-oil method using the obtained fluorescein thiocarbamyl–chitosan (FTC-Chi). FTC-MS with a mean diameter of 27 μm and size distribution of a few micrometers to several tens of micrometers was used for the bioadhesion experiment. FTC-MS exhibited a tendency to adhere to each part of the small intestine to a greater extent than dissolved FTC-Chi, and the ratio of adhering FTC-MS increased as the amount of added FTC-MS decreased. FTC-MS showed slower transit following intraduodenal injection than oral administration. Following the intraduodenal injection of FTC-MS, more than half remained in the upper or middle part of the small intestine for over 8 h. Further, insulin-containing chitosan microspheres with a mean diameter of 20 μm and size distribution of 5 μm to 45 μm were checked in situ for drug absorption, but intraduodenal or intraileal application hardly gave any decrease in plasma glucose level at a very high dose. The present chitosan microsphere system showed good adhesion to the intestinal mucosa, but scarcely facilitated absorption of insulin.

Key Words: *Bioadhesive characteristics; Chitosan microsphere; Insulin-containing chitosan microsphere; Rat small intestine.*

* Corresponding author. Fax: 0081-3-3787-0036; E-mail: onishi@hoshi.ac.jp

INTRODUCTION

The amount and rate of drug absorption after oral administration are often influenced by their gastrointestinal (GI) transit rate (1). When the region of drug absorption is limited to a specific area of the GI tract, it is important for the enhancement of bioavailability to localize the drug in that area for extended periods of time. Recently, bioadhesive polymers have been utilized in terms of improvement or modification of drug absorption after oral administration (1–3). When they interact with mucous membranes, they are considered localized directly at the site of absorption. The bioadhesive dosage form has been suggested to increase the drug residence time around the adhesive region and to facilitate the contact of drugs with biological membranes.

The effects of bioadhesive polymers on GI absorption have been reported (1–3). Polycarbophil exhibits good adhesion to intestinal mucosa and enhances absorption of peptide drugs (3). The latter property seems to be based greatly on the suppression of enzymatic degradation. Also, chitosan has been found to be adhesive to the intestinal or nasal mucosa, and some dosage forms prepared using chitosan as an additive have shown a positive influence on drug absorption (2,4,5).

Such adhesive characteristics have been reported to be dependent on type of chitosan and media conditions (4). Chitosan with high molecular weight seems to exhibit good adhesion to mucosa, and the adhesive force of chitosan to mucosa is inferior under acidic conditions (4).

Recently, the efficacy of chitosan to enhance drug absorption through the mucous membrane has been reported; especially, chitosan operated well to enhance absorption of the peptide drugs through the intestinal or nasal mucous membrane (6–8). The bioadhesion was achieved better in microparticulate forms than in solution form (6,9). The strong bioadhesion of the microparticulate system increases the residence time of a drug and deepens the penetration of the dosage form into the mucous layer, which is considered to be one of the reasons for enhancement of drug absorption (6,7,9). Further, chitosan enhances the drug absorption by altering the integrity of the tight junction of epithelial cells, that is, widening the tight junction related to paracellular transport based on its polycationic properties, which is also considered another reason for the ability of chitosan to increase the drug absorption (10,11). Plus, chitosan and *N*-trimethylated chitosan, enabling permeation enhancement of the peptide drug, operate as drug permeation enhancers more safely than other enhancers, such as calcium chelators or surfactants (11). Thus, a chitosan microparticulate system is attractive with regard to improving drug absorption.

However, the bioadhesive features of chitosan microspheres to the mucosa of the small intestine have not necessarily been characterized in detail. Therefore, in this study, for the purpose of more detailed characterization of chitosan microspheres, the in vitro adhesion properties were examined for dissolved chitosan and chitosan microspheres, the transition profiles after oral or intraduodenal administration were checked in vivo, and then insulin-loaded chitosan microparticles were tested in situ for efficacy.

EXPERIMENTAL

Materials

Chitosan (Daichitosan H) with a deacetylation degree of 82% and molecular weight of 500,000–800,000 (designated Chi) was supplied by Dainichi Seika Color and Chemicals Manufacturing Company, Limited (Tokyo, Japan). Fluorescein isothiocyanate (FITC) and fluorescein were purchased from Sigma Chemical Company. Sorbitan sesquioleate (SO-15) was obtained from Nikko Chemicals (Tokyo, Japan). Soybean oil was purchased from Wako Pure Chemical Industries, Limited (Tokyo, Japan). All other chemicals were reagent grade.

Chitosan was labeled with FITC as follows. Chitosan (500 mg) was dissolved in 500 ml of aqueous HCl solution, pH 3, and then the solution pH was returned to pH 6.5 by addition of 1 N aqueous NaOH solution. FITC (15 mg) was dissolved in 30 ml of water, and the FITC solution was added to the chitosan solution. The mixture was stirred for 24 h at room temperature in the dark. Then, after adjusting to pH 9 with 1 N aqueous NaOH solution, the precipitate was collected by centrifugation at 3000 rpm for 5 min. The product was dissolved in 1 N aqueous HCl solution. The product was precipitated again by addition of 1 N aqueous NaOH solution and collected by centrifugation at 3000 rpm for 5 min. The precipitate was washed with a mixture of acetone:water (3:1 v/v) and dried in vacuo to yield fluorescein thiocarbamyl–chitosan (FTC-Chi) (Fig. 1). A specified amount of FTC-Chi was dissolved in 0.1 M acetate buffer, pH 4.5, and the fluorescence intensity of the FTC-Chi solution was measured to determine the content of FTC. Using FITC solution in the same acetate buffer as a standard, the FTC content of FTC-Chi was calculated to be 0.66 % (w/w).

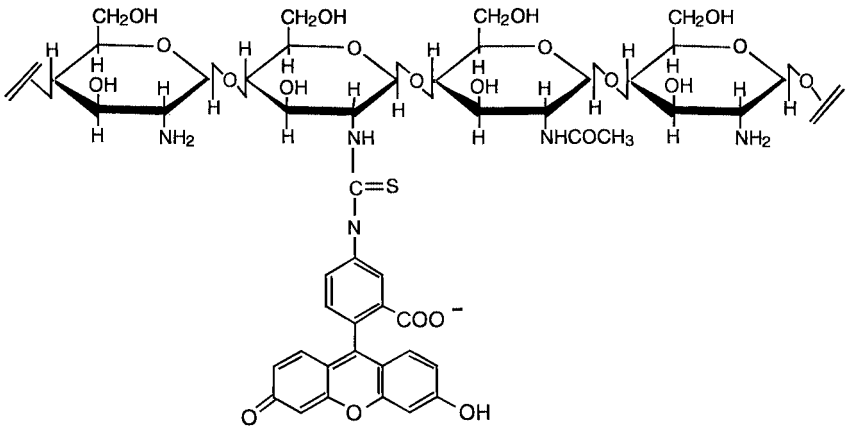

Figure 1. Chemical structure of fluorescein thiocarbamyl–chitosan (FTC-Chi).

Microsphere Preparation

Fluorescein Thiocarbamyl–Chitosan Microspheres

FTC-Chi was dissolved in 2% v/v aqueous acetic acid at concentrations of 0.2%, 0.5%, and 1.0% w/v. Each solution (30 ml) was dropped consecutively into soybean oil (300 ml) containing SO-15 at 1% w/v with stirring at 500 rpm using a magnetic stirrer at 50°C. Acetic acid and water were evaporated at 50°C for 24 h by aspiration using a Handy Aspirator model WP-33 (Yamato Scientific Co., Ltd., Tokyo, Japan), and drying was continued using a vacuum pump for 5 h. The product was then washed sequentially with ether, ethanol, 0.2% w/v aqueous ammonia solution, ethanol, and ether. Then, after drying in air, FTC-Chi microspheres (FTC-MS) were obtained. The particle size of FTC-MS was examined by measuring the Green diameter of 200 particles selected at random. The FTC-MS prepared using 0.2%, 0.5%, and 1.0% w/v chitosan solutions were named FTC-MS-S, FTC-MS-M, and FTC-MS-L, respectively.

Insulin-Containing Chitosan Microspheres

The microspheres containing insulin, designated Chi-Ins, were prepared in the same manner as FTC-MS. Briefly, 10 mg of insulin was dissolved in 30 ml of 0.5% w/v chitosan solution in 2% v/v acetic acid, and the solution was dropped into soybean oil (300 ml) containing 1% w/v SO-15 with stirring at 500 rpm at 50°C. Acetic acid and water were evaporated at 50°C for 24 h by aspiration using the Handy Aspirator, and the drying was continued at room temperature using a vacuum pump for 5 h. The product was washed sequentially with ether, ethanol, 0.2% w/v aqueous ammonia solution, ethanol, and ether. Chi-Ins were then obtained by drying in air. The particle size of Chi-Ins was examined in the same way as described for FTC-MS, that is, by measuring the Green diameter of 200 particles selected at random.

The insulin content of Chi-Ins was determined as follows: First, 10 mg Chi-Ins were dispersed in 5 ml of 0.1 M acetate buffer, pH 4.5, and were dissolved by mashing with a glass homogenizer using a Teflon pestle. The solution was filtered using a membrane filter with a pore diameter of 0.45 μm. The concentration of insulin in the filtrate was determined by high-performance liquid chromatography (HPLC), which was carried out using a Shimadzu (Kyoto, Japan) LC-6A equipped with a Nucleosil $5C_{18}$ column (4.6 × 150 mm) and a Shimadzu SPD-2A UV detector set at 220 nm. The mobile phase was a mixture of 0.1% v/v trifluoroacetic acid and acetonitrile (69:31 v/v), and the flow rate was 1.2 ml/min (12).

In Vitro Bioadhesion of Fluorescein Thiocarbamyl–Chitosan Microspheres to the Small Intestine Mucosa

Wistar rats weighing 240–270 g underwent fasting for 24 h and were anesthetized with ether. Then, the small intestine was excised, and the animal was immediately sacrificed by excessive ether anesthesia. From the excised intestine, 2-cm lengths of the duodenum, jejunum, and ileum were taken and turned inside out, and a cylindrical glass bar 4 mm in diameter was passed through the tissue tube. The tissue attached to the glass bar was

immersed in 1/15 M phosphate buffer (8 ml), pH 6.5, containing a specified amount of FTC-MS with stirring at 200 rpm in the cylindrical bottle (1.8 cm inner diameter, 4 cm high) so that the top of the tissue was kept 5 mm below the solvent surface. Aliquots (100 μl) were withdrawn from the medium at 0, 15, 30, 60, and 120 min after immersion. Then, the whole tissue was simply washed with 100 μl of 1/15 M phosphate buffer, pH 6.5, and immersed in the same manner into fresh 1/15 M phosphate buffer (8 ml), pH 6.5, with stirring at 200 rpm. After 5 min, an aliquot (100 μl) was withdrawn. To all the withdrawn samples, 5 ml of 0.1 M acetate buffer, pH 4.5, was added, and each mixture was mashed using a glass homogenizer with a Teflon pestle to dissolve the FTC-MS. After centrifugation at 3000 rpm for 20 min, the supernatant was analyzed fluorometrically (Ex. 490 nm; Em. 520 nm) to determine the amount of FTC-MS in the samples (i.e., the nonadhesive amount). The adhesive amount was calculated by subtracting the nonadhesive amount from the added one ($n = 3$).

The bioadhesive properties of dissolved FTC-Chi to the intestinal mucosa were checked in the same manner for comparison with those of FTC-MS. Further, fluorescein was examined as a control in the same manner. Briefly, Wistar rats weighing 240–270 g were treated in the same way as described above, and 2-cm lengths of the duodenum, jejunum, and ileum were taken. They were turned inside out, and a glass bar 4 mm in diameter was passed through the tissue tube. The tissue attached to the glass bar was set in 1/15 M phosphate buffer (8 ml), pH 6.5, containing 2 mg of polymer or fluorescein with stirring at 200 rpm at the same conditions as described for FTC-MS. The sampling and measurement were executed in the same manner as described above.

Transit of Fluorescein Thiocarbamyl–Chitosan Microspheres After Oral and Intraduodenal Administration

In this experiment, FTC-MS-M was used as the FTC-MS. Male Wistar rats (240–270 g) underwent fasting for 24 h, and they were anesthetized by intraperitoneal administration of pentobarbital at 60 mg/kg. A suspension of 50 mg FTC-MS in 1 ml of saline was made, and the whole suspension was orally administered using an oral sound. The rats were placed on their backs on a warm plate set at 40°C throughout the experiment. At 2, 5, and 8 h after administration, the stomach and small intestine were excised, and the animal was immediately sacrificed by excessive ether anesthesia.

The small intestine was divided into five parts of equal length (12–15 cm). The stomach was cut open, and each part of the small intestine was turned inside out. All FTC-MS located in each part were collected by scratching the mucosa with a spatula. To the collected samples was added 5 ml of 0.1 M acetate buffer, pH 4.5, and the mixtures were mashed using a glass homogenizer with a Teflon pestle to dissolve the FTC-MS. After cetrifugation at 3000 rpm for 20 min, the supernatant was analyzed fluorometrically (Ex. 490 nm; Em. 520 nm) to determine the amount of FTC-MS. This scratching method permitted the recovery of the microspheres from the intestinal mucosa at a high recovery ratio (more than 80% w/w) (data not shown), which was probably because FTC-MS could be dissolved well in 0.1 M acetate buffer at pH 4.5. Therefore, the results were evaluated without correction.

Intraduodenal administration was performed as follows. Male Wistar rats (240–270 g) underwent fasting for 24 h and were anesthetized by intraperitoneal administration of pentobarbital at 60 mg/kg. The abdominal skin and membrane were cut open, and 50 mg of FTC-MS suspended in 1 ml of saline were administered into the intestine 3 cm distant from the pylorus. Then, the part below the rift for the administration was ligatured to prevent leakage. The incisions of the abdominal membrane and skin were then sutured. The rats were placed on their backs on a warm plate set at 40°C throughout the experiment. At 2, 5, and 8 h after administration, the small intestine was excised, and the rat was immediately sacrificed by excessive ether anesthesia. The small intestine was treated in the same manner as described above for oral administration. FTC-MS located in each part were also collected, and the adhering amount was measured and evaluated in the same way as described above for oral administration.

In Vitro Release and In Situ Absorption of Insulin for Chitosan Microspheres Containing Insulin

The insulin release characteristics from Chi-Ins were examined as follows: In 5 ml of the JP 13 second fluid, 10 mg of Chi-Ins were dispersed. The mixture was incubated at 37°C at 60 strokes/min. Aliquots (300 μl) were withdrawn from the upper layer after incubation for 0, 0.25, 0.5, 1, 2, 3, 4, and 8 h; immediately after each sampling, 300 μl of the JP 13 second fluid were added to the

incubation medium to maintain a constant volume. Each sample was filtered using a membrane filter with a pore diameter of 0.45 μm. The concentration of insulin in the filtrate was determined by HPLC in the same manner as described above for the drug content study of Chi-Ins.

The duodenal loop administration was executed as follows: Male Wistar rats (240–270 g) underwent fasting for 24 h, then were anesthetized by intraperitoneal administration of pentobarbital at 60 mg/kg. The abdominal skin and membrane were cut open, and the intestinal position at 13 cm below the pylorus was ligatured. The incisions of the abdominal membrane and skin were simply sutured.

At 1 h after the operation, the abdominal skin and membrane were reopened, and Chi-Ins suspended in 1 ml of saline was administered at 50 units/kg (U/kg) from the intestinal position 3 cm below the pylorus into the duodenal part. Then, the part just below the rift for the administration was ligatured to prevent leakage. After these operations, the incisions of the abdominal membrane and skin were sutured. The rats were placed on their backs on a warm plate set at 40°C throughout the experiment. Immediately before and at 0.5, 1, 2, and 4 h after administration, the blood samples (200 μl) were withdrawn from the jugular vein. Plasma was obtained by centrifugation of the blood at 3000 rpm for 5 min, and the plasma glucose level was determined using a Glucose-B Test Wako (Wako Pure Chemical Industries, Ltd., Japan) based on the glucose oxidase method. After final withdrawal of blood samples, the animals were sacrificed by excessive ether anesthesia.

Ileal loop administration of Chi-Ins was performed as follows: Male Wistar rats (240–270 g) underwent fasting for 24 h, then were anesthetized by intraperitoneal administration of pentobarbital at 60 mg/kg. The abdominal skin and membrane were cut open, and the ileocecal region was ligatured. The incisions of the abdominal membrane and skin were simply sutured. At 1 h after the operation, the abdominal skin and membrane were reopened, and Chi-Ins suspended in 1 ml of saline was administered at 100 U/kg from the intestinal position 10 cm above the ileocecal region into the ileal part. Then, the part just below the rift for the administration was ligatured to prevent leakage. After these operations, the incisions of abdominal membrane and skin were sutured. Other procedures and conditions such as warming, blood sampling, and determination of the plasma glucose level were carried out in the same way as described for duodenal loop administration. The animals were sacrificed as described above for duodenal administration.

RESULTS AND DISCUSSION

Particle Characteristics of Fluorescein Thiocarbamyl–Chitosan Microspheres

The dry-in-oil method gave spherical particles, that is, microspheres. The size was dependent on the concentration of chitosan solution added to the oily phase. The particle diameters of FTC-MS-S, FTC-MS-M, and FTC-MS-L were distributed in the ranges of a few micrometers to 50 μm, a few micrometers to 60 μm, and 20–70 μm, respectively. The mean diameters of FTC-MS-S, FTC-MS-M, and FTC-MS-L were 11, 27, and 42 μm, respectively. Most diameters were distributed near the mean particle diameter for all kinds of FTC-MS. These suggested that the increases in the chitosan concentration should cause increases in particle size. Such size increases were considered to be because the chitosan content increased in each emulsion droplet and because the increase in viscosity due to the increase in chitosan concentration results in the larger size of emulsion droplets.

In Vitro Bioadhesion to the Mucosa of Small Intestine

Bioadhesion of Fluorescein Thiocarbamyl–Chitosan Microspheres

The effect of the amount of FTC-MS on the adhesive ratio was examined using FTC-MS with a mean particle diameter of 27 μm (FTC-MS-M). The adhesive ratio, which was calculated as the ratio of adhering FTC-MS to the total amount used, increased with decreasing amount of FTC-MS added (Fig. 2). The amount of adhering FTC-MS also reached a plateau at 30 min to 1 h after the start of the test. The adhesive amount at a plateau was approximately equivalent regardless of amount added. These observations suggested that there should be saturation in the adherence. The saturated amounts were calculated as 0.6, 0.5, and 0.4 mg for 2-cm lengths of the duodenum, jejunum, and ileum, respectively. These observations also suggested that it should be necessary to administer the microspheres with a higher drug content to achieve the effective localization of loaded drugs by bioadhesion. Since the mucous layer has been reported to be several tens of micrometers thick (13–15), a simple calculation with diameter (5 mm) and length (2 cm) of used intestinal tissue samples gave a mucus volume of about 10 mm^3 per 2-cm tube. The amount of adhering microspheres at the saturation, 0.4–0.6 mg, was calculated to correspond to several percent of the mucous layer

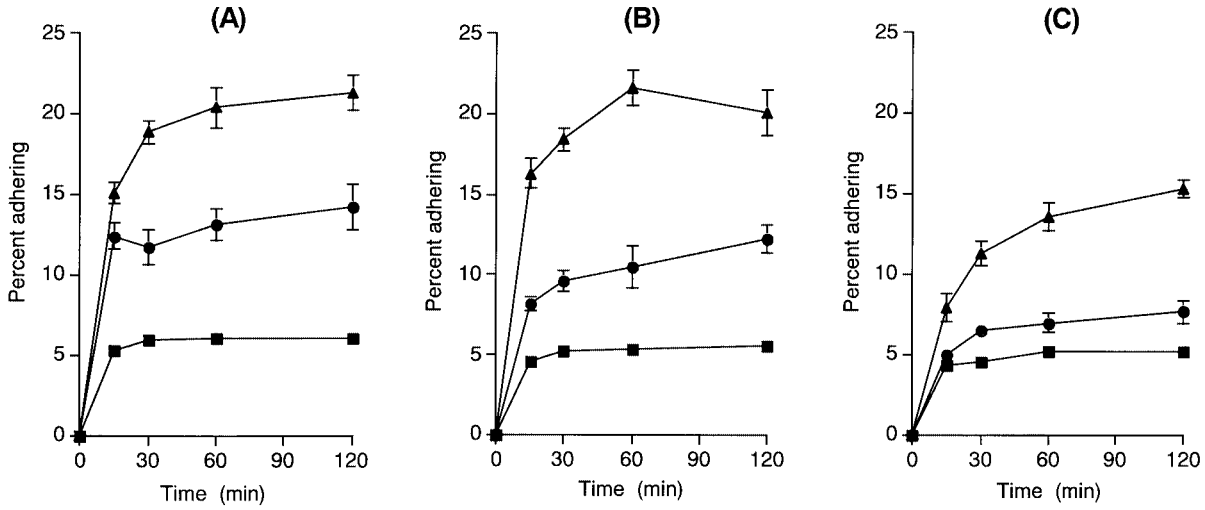

Figure 2. Effect of added amount of FTC-MS on adhering ratio to (A) duodenal, (B) jejunal, and (C) ileal parts: ▲, 2 mg FTC-MS; ●, 5 mg FTC-MS; ■, 10 mg FTC-MS. Each point represents the mean ± SD (n = 3).

volume. Considering that the mucous layer is heterogeneous in thickness and that microspheres with a size similar to the mucous layer thickness were used, the saturated amount in the in vitro adhesion may be reasonable.

The effects of particle size of FTC-MS on bioadhesion to each part of the small intestine were investigated using 10 mg of FTC-MS in the same manner. The results are shown in Fig. 3. The amount of adhering FTC-MS reached a plateau at 30 min to 1 h after the start of the test for each sample, and the amount at the plateau was not dependent on the particle size. These observations suggested that the saturated level of adherence should not be affected by particle size. The amount of adhering FTC-MS tended to be larger in duodenum than jejunum and to be greater in the jejunum than the ileum, although the differences between these regions were not marked.

After the in vitro adhesion test for 2 h, each small intestine tube was washed using 100 μl of 1/15 M phos-

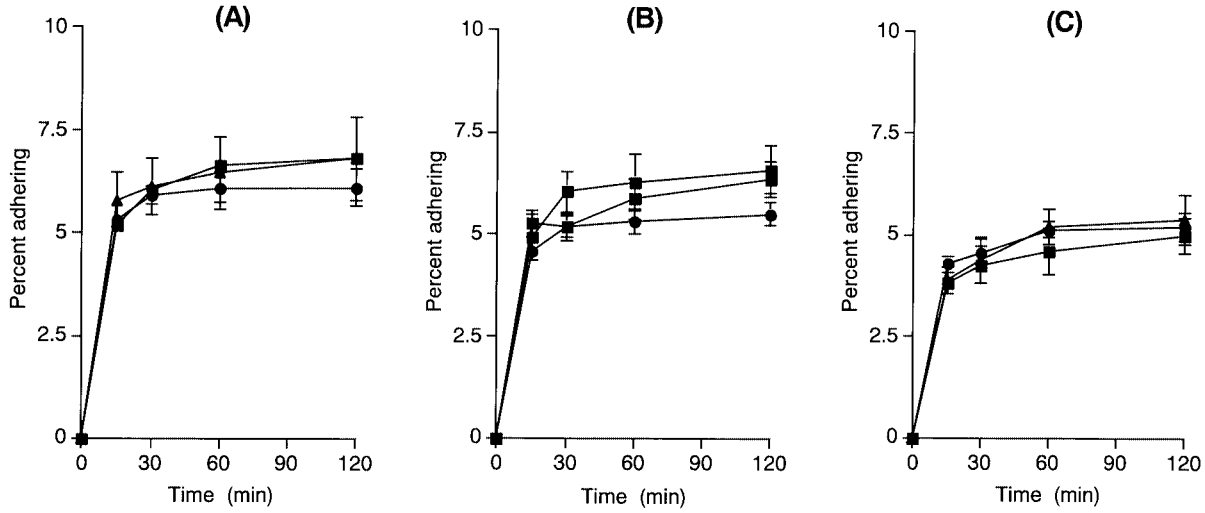

Figure 3. Effect of particle size on adhering ratio of FTC-MS to (A) duodenal, (B) jejunal, and (C) ileal parts. Initially, 10 mg FTC-CM was added to each tested medium: ▲, FTC-MS-S; ●, FTC-MS-M; ■, FTC-MS-L. Each point represents the mean ± SD (n = 3).

phate buffer, pH 6.5, put into the fresh same buffer, and stirred for 5 min in the same way. Most of the FTC-MS, hardly appearing in the bathing solution, remained on the intestinal mucosa. This suggested that the attached FTC-MS should adhere fairly tightly to the mucosa.

Bioadhesion of Dissolved Chitosan

The adhesive properties of FTC-Chi and fluorescein were examined in the dissolved form. Fluorescein exhibited very little adhesion to any part of the intestine examined (Fig. 4). Fluorescein itself is considered to have little affinity to the mucosa of the small intestine. As compared with FTC-CM, FTC-Chi showed lower bioadhesion in the form of solution. As to the adhesive ratio, duodenum was greater than jejunum, which was greater than ileum. Although chitosan is thought to be bioadhesive, its adhesive potency was found to be low in the dissolved form.

Soane et al. (9) reported that chitosan was cleared faster in the dissolved form than in the microparticulate form. The microspheres could absorb water from the mucous layer and allow polymer penetration to the mucin network and subsequent formation of adhesive bonds, while dissolved chitosan, having been already hydrated, could not cause the dehydration (water absorption) important for the polymer penetration. However, the situation of the present experiment was different from that of Soane et al. (9) because FTC-MS was considered somewhat hydrated in suspension.

Others (6) reported that the nasal absorption of insulin was enhanced better in the suspension of insulin-loaded chitosan nanoparticles than in the mixed solution of chitosan and insulin. This reason was thought to be because the chitosan nanoparticles showed stronger contact with the absorption layer, leading to the increased concentration of the absorption site (6).

The strong mucoadhesion and deep penetration of chitosan-coated nanoparticles to the mucosa were also proposed as a mechanism of drug absorption enhancement in the intestinal peptide absorption study by Kawashima et al. (7). They suggested that the solid form of chitosan might be favorable for mucoadhesion, probably due to close contact and deep penetration to the mucosa. Although FTC-MS is much larger in size than such nanoparticles, FTC-MS might also show stronger adhesion than dissolved FTC-Chi.

Thus, it was indicated that chitosan showed better adhesion to the intestinal mucosa in the form of microspheres than solution. Further, once chitosan microspheres adhered to the intestinal mucosa, they were not easily detached.

Transit of Fluorescein Thiocarbamyl–Chitosan Microspheres After Oral or Intraduodenal Administration

Figure 5 shows the time courses of the distribution of FTC-MS in the tracts, including the stomach and small intestine, after oral or intraduodenal administration. Fol-

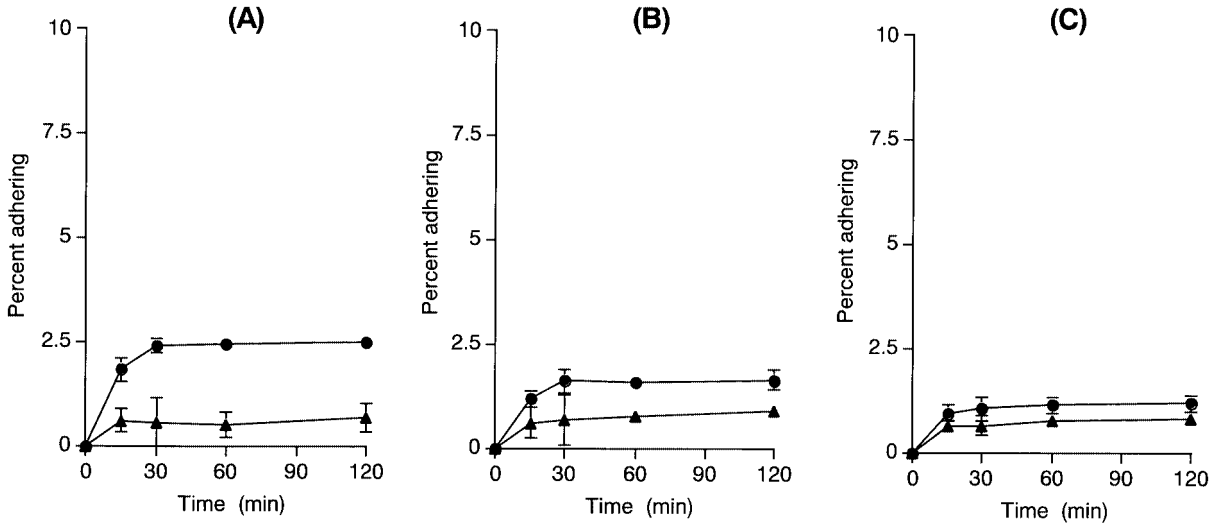

Figure 4. In vitro adhering ratio of dissolved fluorescent materials to (A) duodenal, (B) jejunal, and (C) ileal parts: ▲, 2 mg fluorescein; ●, 2 mg FTC-Chi. Each point represents the mean ± SD (n = 3).

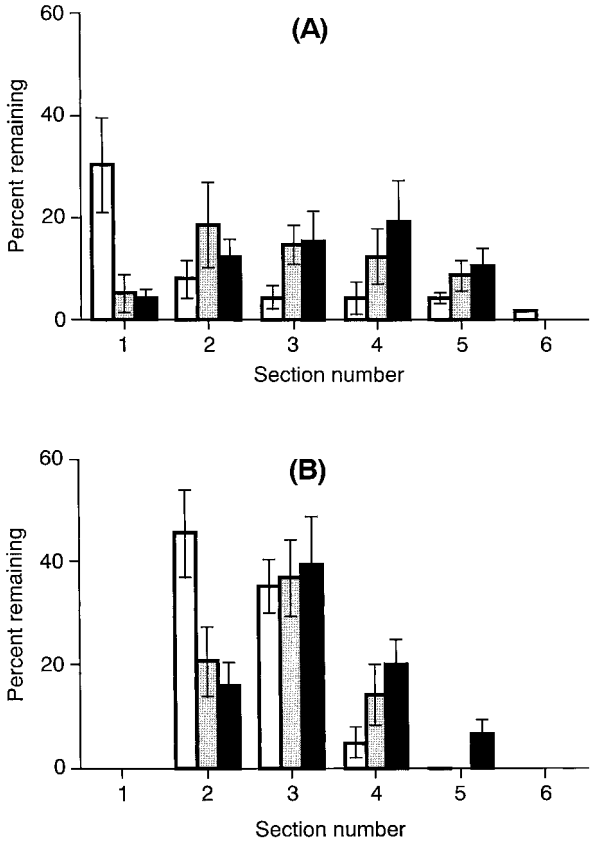

Figure 5. Distribution time courses of FTC-MS in (A) the tract of the stomach and small intestine after oral administration and (B) the tract of the small intestine after intraduodenal administration. A suspension of 50 mg of FTC-MS in saline (1 ml) was administered per rat. The small intestine was divided into the top (2) through bottom (6) as follows: 1, stomach; 2–6, small intestinal part (2, top part; 6, bottom part; each 12–15 cm long). □, 2 h after administration; ▒, 5 h after administration; ■, 8 h after administration. Each point represents the mean ± SD ($n = 3$).

lowing oral administration, more than 30% of the dose was observed in the stomach after 2 h, but less than 10% was located there after 5 h. Most FTC-MS orally administered passed from stomach within 5 h after administration and spread widely throughout all parts of the small intestine. At 8 h after administration, a considerable amount of FTC-MS was transferred to the lower part of the small intestine. On the other hand, following intraduodenal administration, approximately 80% of FTC-MS administered was observed in the area consisting of the duodenum (section 2) and jejunum (section 3) after 2 h; approximately 60% remained there even after 8 h.

The jejunum to which FTC-MS adhered was shortly soaked in normal saline, and this was repeated five times. After the soaking operation, 72% w/w of the FTC-MS adhering to the jejunum at 2 h after oral administration still remained, and 62% w/w of the FTC-MS adhering to the jejunum at 5 h after oral administration was still maintained ($n = 3$). On the other hand, after the same operation, 88% of the FTC-MS adhering to the jejunum at 2 h after intraduodenal administration still remained, and 85% of the FTC-MS adhering to the jejunum at 2 h after intraduodenal administration was still maintained ($n = 3$). Since the pH of the stomach and jejunum were 4 and 6, respectively, FTC-MS administered orally were considered to be swollen and dissolved at a low pH, which would cause the orally administered FTC-MS to detach more easily.

In Situ Absorption of Insulin for Chitosan Microspheres Loaded with Insulin

Chi-Ins showed a mean particle diameter of 20 μm and a particle diameter distribution ranging from 5 to 45 μm. The insulin content was 5.5% w/w. Chemical transformations of insulin, especially deamidation, have been reported to occur in acidic media (16–18), although deamidated insulins appear to retain potency to a fair extent. When compared with the experimental conditions for stability reported in the literature (16), Chi-Ins was considered to be produced in weaker acid and for a shorter time. Therefore, chemical transformation was not expected to be extensive.

The results of HPLC analysis indicated that insulin was incorporated in the microspheres at a content of 5.5% w/w; namely, more than 80% w/w of insulin used was calculated as incorporated with its dignity into the microspheres. However, the determination of insulin by HPLC might need to be verified further by examination of the potency of incorporated insulin because another reported HPLC analysis indicated that there was a deamidated insulin appearing very close to the original one in the chromatogram (17). In this study, only the HPLC method was used for determination of insulin.

The release of insulin from microspheres is shown in Fig. 6A. Of the loaded drug, 60% was released at 30 min, and the amount released reached more than 70% w/w at 2 h, after which the remaining insulin was released slowly. Figure 6B shows the time course of the plasma glucose levels following administration of Chi-Ins (50 U/kg) to the duodenal loop and following administration of Chi-Ins (100 U/kg) to the ileal loop. The decreases in plasma glucose levels were little observed after administration of Chi-Ins (50 U/kg) into the duodenal loop. Only a slight

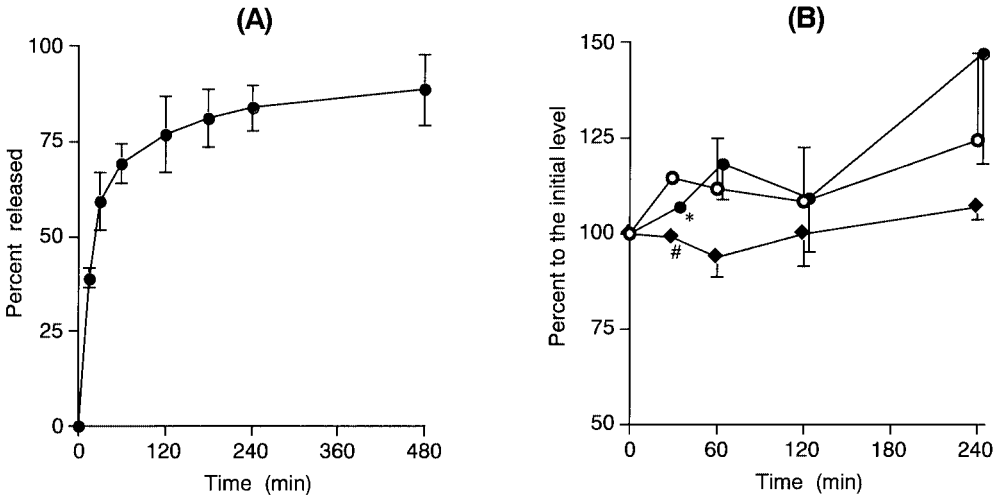

Figure 6. In vitro release of insulin from Chi-Ins in JP 13 2nd fluid (A) and blood glucose level after in situ loop administration of Chi-Ins (B). In Fig. 6B, ○ control, ● intraduodenal administration (50 U/kg), ◆ intraileal administration (100 U/kg); $*p < .01$ versus control, $\#p < .001$ versus control based on Student t test. Each point represents the mean ± SD ($n = 3$).

transient decrease in plasma glucose level was initially observed at each administration compared with the control. Ileal administration tended to cause suppression of the plasma glucose level through all the observations. The plasma glucose level has been reported to be slightly decreased following administration of insulin solution (50 U/kg) in the washed ileal loop (19). The administration of insulin solution (50 U/kg) in the nonwashed ileal loop resulted in no decrease in plasma glucose level (19). Therefore, adhering microspheres might prevent insulin to a small extent from degradation by proteolytic enzymes in the mucosal membrane.

Reportedly, chitosan-coated nanoparticles or liposomes acted well for enhancement of peptide absorption (5–7). Namely, these dosage forms show the strong mucoadhesion and deep penetration to the mucous layer, which can increase the residence time and close contact of the peptide at the absorption. As a result, the peptide absorption is supposed to be enhanced.

However, overall, the enhancing effects of the present microspheres, which possessed a diameter of a few dozen micrometers, were not proved. The difference in particle size was supposed to influence the peptide absorption; namely, the larger particles, Chi-Ins, might not provide contact enough to cause the peptide absorption, and more insulin might be released from the mucous layer to the lumen of the intestine because the penetration was not complete due to the larger size. Further, a fair amount of insulin might be released from Chi-Ins before achievement of close contact with the mucosa, resulting in little absorption. Thus, as to the present microspheres, further modification such as the coexistence of inhibitors against proteolytic enzymes will have to be added to facilitate absorption of insulin.

CONCLUSION

The present study demonstrated in vitro and in vivo bioadhesive characteristics of chitosan microspheres with a particle diameter ranging from several micrometers to several tens of micrometers in the rat small intestine. It is suggested that encapsulation or coating using chitosan should increase the residence of included drugs in the upper and middle regions of the small intestine. However, the chitosan microspheres obtained in the conditions of this study did not show an ability to enhance peptide absorption.

ACKNOWLEDGMENT

We would like to thank Ms. Emi Funatsu for assistance in this experiment.

REFERENCES

1. Longer, M.A.; Chíng, H.S.; Robinson, J.R. Bioadhesive Polymers as Platforms for Oral Controlled Drug Delivery

III: Oral Delivery of Chlorothiazide Using a Bioadhesive Polymer. J. Pharm. Sci. **1985**, *74*, 406–411.
2. Luesen, H.L.; Lehr, C.-M.; Rentel, C.-O.; Noach, A.B.J.; de Boer, A.G.; Verhoel, J.C.; Junginger, H.E. Bioadhesive Polymers for the Peroral Delivery of Peptide Drugs. J. Controlled Release **1994**, *29*, 329–338.
3. Lehr, C.-M.; Bouwstra, J.A.; Kok, W.; de Boer, A.G.; Tukker, J.J.; Verhoef, J.C.; Breimer, D.D.; Junginger, H.E. Effect of the Mucoadhesive Polymer Polycarbophil on the Intestinal Absorption of a Peptide Drug in the Rat. J. Pharm. Pharmacol. **1992**, *44*, 402–407.
4. Lehr, C.-M.; Bouwstra, J.A.; Schacht, E.H.; Junginger, H.E. In Vitro Evaluation of Mucoadhesive Properties of Chitosan and Some Other Natural Polymers. Int. J. Pharm. **1992**, *78*, 43–48.
5. Takeuchi, H.; Yamamoto, H.; Niwa, T.; Hino, T.; Kawashima, Y. Enteral Absorption of Insulin in Rats from Mucoadhesive Chitosan-Coated Liposomes. Pharm. Res. **1996**, *13*, 896–901.
6. F.-Urrusuno, R.; Calvo, P.; R.-Lopez, C.; V.-Jato, J.L.; Alonso, M.J. Enhancement of Nasal Absorption of Insulin Using Chitosan Nanoparticles. Pharm. Res. **1999**, *16*, 1576–1581.
7. Kawashima, Y.; Yamamoto, H.; Takeuchi, H.; Kuno, Y. Mucoadhesive DL-Lactide/Glycolide Copolymer Nanospheres Coated with Chitosan to Improve Oral Delivery of Elcatonin. Pharm. Dev. Technol. **2000**, *5*, 77–85.
8. E.-Shafy, M.A.; Kellaway, I.W.; Taylor, G.W.; Dickinson, P.A. Improved Nasal Bioavailability of FITC-Dextran (Mw 4300) from Mucoadhesive Microspheres in Rabbits. J. Drug Targ. **2000**, *7*, 355–361.
9. Soane, R.J.; Frier, M.; Perkins, A.C.; Jones, N.S.; Davis, S.S.; Illum, L. Evaluation of the Clearance Characteristics of Bioadhesive Systems in Humans. Int. J. Pharm. **1999**, *178*, 55–65.
10. Kotze, A.F.; Lueβen, H.L.; de Boer, A.G.; Verhoef, J.C.; Junginger, H.E. Chitosan for Enhanced Intestinal Permeability: Prospects for Derivatives Soluble in Neutral and Basic Environments. Eur. J. Pharm. Sci. **1998**, *7*, 145–151.
11. Thanou, M.; Florea, B.I.; Langemeyer, M.W.E.; Verhoef, J.C.; Junginger, H.E. *N*-Trimethylated Chitosan Chloride (TMC) Improves the Intestinal Permeation of the Peptide Drug Buserelin In Vitro (Caco-2 Cells) and In Vivo (Rats), Pharm. Res. **2000**, *17*, 27–31.
12. Nakazawa, H.; Nagase, M. Reversed-Phase High-Performance Liquid Chromatography of Peptides. Yakugaku Zasshi **1986**, *106*, 398–405.
13. Park, H.; Robinson, J.R. Physico-chemical Properties of Water Insoluble Polymers Important to Mucin/Epithelial Adhesion. J. Controlled Release **1985**, *2*, 47–57.
14. Lehr, C.-M.; Poelma, F.G.J.; Junginger, H.E.; Tukker, J.J. An Estimate of Turnover Time of Intestinal Mucus Gel Layer in the Rat In Situ Loop. Int. J. Pharm. **1991**, *70*, 235–240.
15. Rubinstein, A.; Tirosh, B. Mucus Gel Thickness and Turnover in the Gastrointestinal Tract of the Rat: Response to Cholinergic Stimulus and Implication for Mucoadhesion. Pharm. Res. **1994**, *11*, 794–799.
16. Sundby, F. Separation and Characterization of Acid-Induced Insulin Transformation Products by Paper Electrophoresis in 7 M Urea. J. Biol. Chem. **1962**, *237*, 3406–3411.
17. Brange, J.; Langkjar, L.; Havelund, S.; Volund, A. Chemical Stability of Insulin. 1. Hydrolytic Degradation During Storage of Pharmaceutical Preparations. Pharm. Res. **1992**, *9*, 715–726.
18. Brange, J.; Langkjar, L. Chemical Stability of Insulin. 3. Influence of Excipients, Formulation, and pH. Acta Pharm. Nord. **1992**, *4*, 149–158.
19. Morishita, M.; Morishita, I.; Takayama, K.; Machida, Y.; Nagai, T. Site-Dependent Effect Aprotinin, Sodium Caprate, Na_2EDTA and Sodium Glycocholate on Intestinal Absorption of Insulin. Biol. Pharm. Bull. **1993**, *16*, 68–72.