Preparation and Characterization of Insulin-Loaded Acrylic Hydrogels Containing Absorption Enhancers

Takahiro UCHIDA,* Yuka TOIDA, Sadako SAKAKIBARA, Yohko MIYANAGA, Hiromi TANAKA, Mayumi NISHIKATA, Keiko TAZUYA, Noriko YASUDA, and Kenji MATSUYAMA

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11–68, Koshien 9-Bancho, Nishinomiya 663–8179, Japan. Received March 27, 2001; accepted June 30, 2001

The objectives of this study were to prepare insulin-loaded acrylic hydrogel formulations containing various absorption enhancers, to perform *in vitro* and *in vivo* characterization of these formulations, and to evaluate the factors which affecting insulin availability on rectal delivery of insulin using this hydrogel system. The acrylic block copolymer of methacrylic acid and methacrylate, Eudispert®, was used to make the hydrogel formulations. As absorption enhancers, 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD), lauric acid (C12), or the sodium salt of C12 (C12Na), were incorporated into the hydrogels. In an *in vitro* release test, the release rate of insulin from the hydrogels decreased as the polymer concentration of the hydrogel increased. The addition of C12Na to the hydrogel further increased the insulin release rate, which was greater at higher concentrations of the enhancer. A portion of the C12Na was found to remain bound to the acrylic polymer in dissolution medium. Serum insulin levels were determined at various time points after the administration of insulin solution or insulin-loaded (50 units/kg body weight) Eudispert® hydrogels containing 5% (w/w) of C12, C12Na, or DM-β-CyD to *in situ* loops in various regions of the rat intestine. The most effective enhancement of insulin release was observed with formulations containing C12Na. The bioavailability of insulin from the hydrogels was lower than that from the insulin solutions. Hydrogel formulations containing 7% or 10% Eudispert® remained in the rectum for 5 h after rectal administration. However, the 5% (w/w) C12Na solution stained with Evan's-blue had diffused out and the dye had reached the upper intestinal tract within 2 h. Finally, the rectal administration of insulin-loaded hydrogels, containing 4%, 7%, or 10% (w/w) Eudispert® and 5% (w/w) of enhancer (C12, C12Na, or DM-β-CyD) to normal rats was shown to decrease serum glucose concentrations. The greatest effect was found with insulin-loaded 7% (Eudispert®) hydrogel containing C12Na which having cosiderable large insulin release rate and bioadhesive characteristics.

Key words hydrogel formulation; insulin, sodium salt of lauric acid; serum glucose level

Recently, good peptide depot systems, such as those for luteinizing hormone–releasing hormone (LH-RH) agonists,[1,2] have appeared on the market. However, injections may cause local side-effects and allergic reactions and result in physical discomfort or psychological distress. Although peptide delivery *via* topical administration routes (*e.g.*, nasal[3] and transdermal[4]) appears attractive, the gastrointestinal route may still be useful if the absorption characteristics of the targetted protein or peptides are suitable.

Hydrogel formulations are now used in a variety of fields, including medicine, where they may be used for topical,[5] oral,[6] and rectal[7] delivery. However, in the application of hydrogels, both the drug release rate *in vitro* and the *in vivo* gastric transit profile may affect *in vivo* usefulness, and it is not always clear whether we have the best gel formulation for the targetted purpose.

In the present study, insulin was chosen as the model peptide. Insulin is generally administered by injection in the treatment of diabetes mellitus. Even in patients suffering from non-insulin-dependent diabetes mellitus, insulin is sometimes administered therapeutically at an early stage of treatment.[8] Gastrointestinal delivery of insulin is the most convenient for chronic administration, but the poor absorption and low stability of insulin in the gastrointestinal tract when administered orally make this problematic. The liver is the principal target organ of insulin and removes half of the insulin presented to it in a single transhepatic circulation. The absorption of insulin in the small intestine (*i.e.*, duodenum and jejunum) is reported to be low, while absorption in the large intestine (*i.e.*, colon or rectum) is much higher.[9]

Recently, a new approach using a carrier-mediated transport system in rat gastrointestinal tract was reported by Alsenz *et al.*,[10] who examined the mechanism of uptake of cobalamin-conjugated peptides in the gastrointestinal tract. Rectal administration is attractive, since although administered drug will diffuse up to the upper intestine, the first-pass effect in the lower intestine can be avoided for many basic drugs and esterase activity is appreciably less in the lower than in the upper intestine. We have previously investigated a rectal gel preparation comprising block copolymers of methacrylic acid and methacrylate.[11,12] These rectal gel preparations showed good retention in the lower regions of the large intestine in animals compared with control suppositories. We decided to apply the above hydrogel system to rectal insulin delivery. Absorption enhancers, such as the sodium salt of lauric acid (C12, C12Na), were added to the hydrogel vehicles to improve the permeability of insulin through the gastrointestinal membrane. The optimized hydrogels were administered to normal rats, and the pharmacological effects (decrease of plasma glucose level) were evaluated. A number of critical factors for rectal insulin delivery *via* hydrogel systems were also examined.

Experimental

Materials Eudispert® (*hv* grade) was donated by the Higuchi Company (Tokyo). Bovine insulin was obtained from Sigma Co. Ltd., (St. Louis, MO, U.S.A.), C12 and C12Na were obtained from Nacalai Tesque Co. (Kyoto, Japan), and 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD) from Nihon Syokuhinn Kako Co. (Tokyo, Japan). Other reagents were all of special

* To whom correspondence should be addressed. e-mail: takahiro@mwu.mukogawa-u.ac.jp

grade.

Preparation of Insulin-Loaded Eudispert® Hydrogels Containing Enhancers Bovine insulin was dissolved in citrate buffer, pH 3.0. The absorption enhancers and Eudispert® were slowly added to the solution which was gently agitated. Then 1 N NaOH solution was added to the suspension, and the system was neutralized to form a hydrogel. The concentrations of bovine insulin and the absorption enhancers in the gel were fixed at 0.15% and 5.0% (w/w), respectively. The Eudispert® concentration was fixed at 7.0% (w/w) unless specified otherwise. The pH of the prepared hydrogel was adjusted to pH 7.4 with citric acid buffer or NaOH solution.

Release Test of Insulin-Loaded Eudispert® Hydrogels Containing Enhancers The release test of the hydrogels was performed as described in the Japanese Pharmacopeia JPXIII using the rotation basket method. A medium of pH 7.4 phosphoric acid buffer solution (500 ml) was used and the test was carried out at 37 °C at 50 rpm. A known quantity (0.5 g) of hydrogel preparation was gently injected into the basket using a 1ml syringe, and the test was started immediately. The sampling was carried out at 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, and 180 min. Five milliliters of the sample medium was taken, centrifuged (3000 rpm for 15 min) and the concentration of the bovine insulin in the supernatant was analyzed using HPLC: 100 μl was injected onto a chromatograph (Shimadzu LC-10A, Kyoto, Japan) equipped with a UV detector (Shimadzu SPD-10AV), an integrator (Shimadzu C-R6A) and a reversed-phase column (Cosmosil 5C18-AR, 4.6×150 mm, Nakalai Tesque Co., Ltd., Kyoto). The following mobile phase system was used: A, 0.1% trifluoroacetic acid (TFA) in H_2O; B, 0.1% TFA in acetonitrile. A linear gradient was used: phase B from 30% to 50% (10 min). The flow rate was 1.5 ml/min and the wavelength was set at 220 nm. The C12 released into the medium from the hydrogels were determined using a commercial kit (ACS/ACOD method; NEFA C-test Wako, Co. Ltd.). The concentration of DM-β-CyD was determined by phenol sulfuric acidmethod.[13]

Animal Studies Wistar male rats weighing 200±20 g were used in the study. Cannulation was performed in a vein in the neck the day before the rectal administration and animals were fasted at least 12 h before the experiment.

***In Situ* Closed-Loop Method** The method was essentially the same as that reported by Morishita *et al*.[9] Male Wistar rats (n=3—5) weighing 200±20 g were fasted for 24 h prior to the experiments and were anesthetized by intraperitoneal injection of 50 mg/kg body weight sodium pentobarbital. All rats were restrained in a supine position on a board which was kept at a surface temperature of 37 °C. A small midline incision was made in the abdomen and 5—6 cm loops were made in the duodenum, ileum, colon and rectum. The duodenal loop was made in the first portion of the intestine, the ileal loop at the end of the small intestine, and the colonic loop in the ascending colon. The rectal loop was made adjacent to the anus. Both ends of the loops were cannulated with a glass tube, and the loops were washed with 10 ml of warmed saline.

A 0.5-ml bolus of either insulin solution or an insulin-loaded hydrogel formulation containing 5% (w/w) of enhancers (C12Na, C12, or DM-β-CyD) was administered directly to each loop. The dose of insulin was fixed at 50 units/kg body weight. Blood samples were taken at 15, 30, 60, 120, and 180 min after dosing. Serum was separated by centrifugation at 4000 rpm for 15 min and kept frozen until analysis. The glucose level in the plasma was determined by the glucose oxidase method (Glucose CII-Test Wako Kit; Wako Pure Chemicals Co., Osaka), and the serum insulin level was determined by a commercial kit (Gurazaim Insulin-EIA Test; Wako Pure Chemicals Co., Osaka).

Rectal Administration of Various Formulations. Retention and Dispersion of Hydrogel Formulations To evaluate the release characteristics of the hydrogel formulations, the following test solutions were administered rectally to healthy rats (n=3—5) anesthetized by infusion of sodium pentobarbital: a solution of 5% C12Na, 5% C12Na-loaded Eudispert® hydrogels with 4, 7, or 10% polymer concentration, and Eudispert® 7% hydrogel containing 5% DM-β-CyD. All test solution contained 0.2% (w/w) Evan's blue as a marker dye instead of insulin. The distance from the rectum travelled by the top of the dye front was measured for each test solution.

Pharmacological Effects Rats (n=4—6) were anesthetized with ether prior to rectal administration of the prepared Eudispert® hydrogels using a sterile polypropylene 1-ml syringe. The hydrogel was administered 2—3 cm into the rectum, which was then closed with an adhesive (Alonalfa, Towa Ksei Co., Tokyo). Thereafter, blood samples were collected at predetermined intervals and plasma obtained by centrifugation (4000 rpm for 15 min). The glucose level in the plasma was determined by the method described above.

Statistical Data Analysis Results are expressed as the mean±S.E. Statistical significance was assessed using the Student's t-test.

Results and Discussion

***In Vitro* Release Characteristics of Eudispert® Hydrogels Containing Insulin and/or Various Enhancers** Figure 1 shows the release profile of insulin (A) and C12Na (B) from hydrogels with various polymer concentrations (4, 7, and 10% Eudispert®) containing 5% (w/w) of C12Na. As shown in Fig. 1A, the release rate of insulin from the hydrogel decreased as the polymer concentration increased.

In the three-dimensional network structure which is assumed to be formed in Eudispert® hydrogel, there seem to be covalent bonds, ionic bonds, hydrogen bonds and intermolecular interactions in the gel structure, although we could not evaluate the contribution of each bond or interaction to the overall structure. The three-dimensional network structure controls the diffusion of water or any ionized molecule in the hydrogel.[12] If the polymer concentration is raised, it is likely that the three-dimensional structure will become more rigid. It was therefore conjectured that the diffusion of the insulin or solubilized C12Na molecule from the hydrogel network would be inversely proportional to the polymer concentration. In fact, the apparent viscosity of the 10% (w/w) Eudispert® hydrogel was much greater than that of the 4% hydrogel. In the 4% (w/w) Eudispert® hydrogel, a more rapid release was observed in the initial phase followed by a slow-release phase. The 7% (w/w) Eudispert® hydrogel was the easiest to handle from the point of view of viscosity.

Figure 1B shows the C12Na release profile, which was slower than that of insulin and failed to reach 100%, even after 3 h, by which time the hydrogel was completely degraded and all the insulin was dissolved in the buffer medium. The unreleased fraction of C12Na at 3 h seems to be proportional to the polymer concentration of the hydrogel; this phenomenon suggests a Langmuir-type adsorption of C12Na to acrylic polymer in the hydrogel. We confirmed that this type of adsorption was possible by demonstrating the

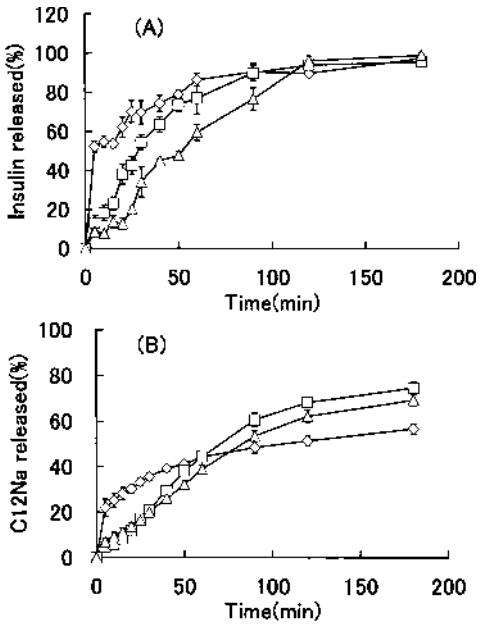

Fig. 1. Release Profiles of (A) Insulin or (B) the C12Na from Various Concentrated Eudispert® Hydrogels Containing 0.15% Insulin or 5% (w/w) C12Na

◇, 4% (w/w) Eudispert® hydrogel; □, 7% (w/w) Eudispert® hydrogel; △, 10% (w/w) Eudispert® hydrogel. Each point represents the mean±S.E. (n=3—5).

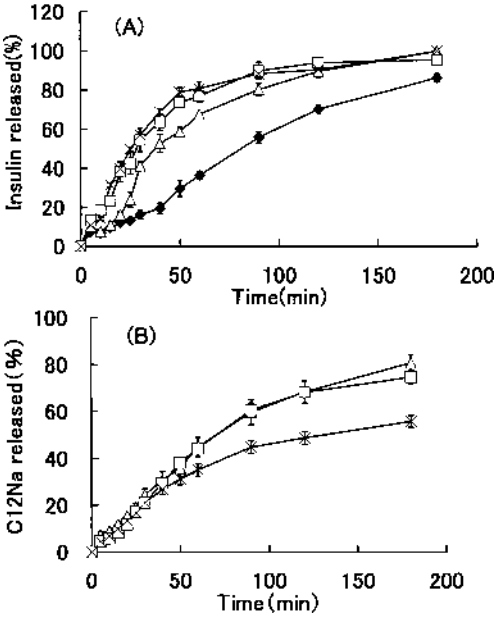

Fig. 2. Release Profiles of (A) Insulin or (B) the C12Na from 7% (w/w) Eudispert® Hydrogel Containing 0.15% Insulin and Various % (w/w) of C12Na

◆, Hydrogel without C12Na; △, hydrogel with 3% (w/w) C12Na; □, hydrogel with 5% (w/w) C12Na; ×, hydrogel with 7% (w/w) C12Na. Each point represents the mean±S.E. (n=3—5).

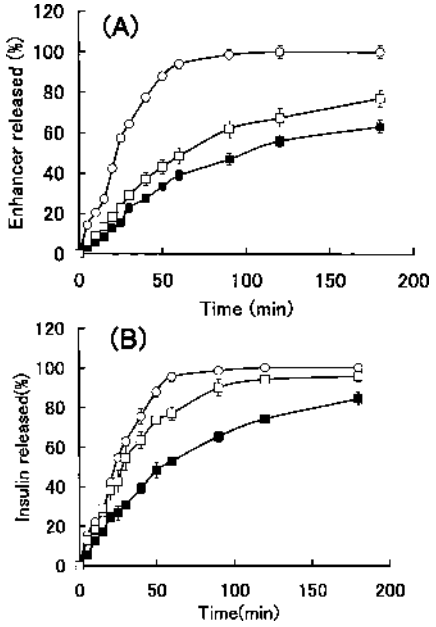

Fig. 3. Release Profiles of (A) Insulin or (B) Enhancers from 7% (w/w) Eudispert® Hydrogels Containing 0.15% Insulin and 5% (w/w) C12Na (□), C12 (■), or DM-β-CyD (○)

Each point represents the mean±S.E. (n=3—5).

binding of the fatty acid to acrylic polymer in a medium containing only fatty acid and acrylic polymer.

The release rate of insulin from the hydrogels increased as the concentration of enhancers in the hydrogels increased. A typical example is shown in Figs. 2A and B. In insulin-loaded hydrogels containing C12Na, the addition of 5% C12Na to 7% (w/w) Eudispert® hydrogel significantly increased the insulin release rate, especially during the initial release phase. The reason for this release rate enhancement could not be determined. One possibility is that this is due to the effect of the C12Na molecule on the three-dimensional network structure described above. Another possibility is the formation of micelles by C12Na, with a consequent increase of the release rate of insulin from the hydrogel matrix. The C12Na release rate from the various gels did not differ up to 50 min. The absolute concentration of C12Na may also affect the release rate of insulin.

Figures 3A and B show the effect of the enhancer on the release rate of enhancer or insulin from 7% (w/w) Eudispert® hydrogel containing 5% (w/w) enhancer. Figure 3B shows that the release rates of bovine insulin from the prepared hydrogels containing 5% of various absorption enhancers were ranked as follows: DM-β-CyD>C12Na≥C12. The insulin release rate from hydrogels containing C12Na and C12 was not significantly different. The release rate of absorption enhancers from hydrogels was ranked as follows: DM-β-CyD≥C12Na>C12, as shown in Fig. 3A. These results suggest that insulin release from hydrogels depends on hydrogel degradation, as the release profiles of the enhancers were almost the same as that of insulin.

***In Vivo* Studies of Insulin-Loaded Eudispert® Hydrogels Containing Various Enhancers. *In Situ* Closed-Loop Method** Figures. 4 and 5 show rat serum insulin levels following administration of either 0.5 ml of insulin solution or insulin-loaded Eudispert® hydrogel formulations containing 5% (w/w) of enhancers (C12Na, C12, or DM-β-CyD) to each of the four gastrointestinal loops. The dose of insulin was fixed at 50 units/kg body weight.

Figure 4 shows serum insulin levels after administration of insulin solutions containing 5% of enhancers. In general, C12Na shows the most effective enhancement. The optimum absorption sites for both C12Na and C12 were ranked as follows: rectum>colon>ileum>duodenum. The reason for the difference in efficacy of enhancement may be a difference in solubility between C12 and C12Na in pH 7.4 buffer since unionised C12 molecules might make a role in enhancement of insulin diffusion through membrane. In the case of DM-β-CyD, the plasma insulin level was almost the same after administration to all absorption sites. Enhancement effect of DM-β-CyD in duodenum was almost same or bigger than the effect of C12Na, but its availability of insulin (absorbed insulin amount) was significantly smaller than that absorbed in rectum loop. This phenomenon means the absorption barrier including hepatic clearance for DM-β-CyD seems to be considerable high, as so in C12Na or C12 in duodenum, upper intestine. Therefore, the most effective portion in the standpoint of enhancement seems to be rectum. As shown in Fig. 3B, the release rate of insulin hydrogel containing DM-β-CyD was larger than that of hydrogels containing C12Na. Nevertheless, enhancement effect of insulin loaded hydrogel containing DM-β-CyD *in situ* was smaller than hydrogel containing C12Na as shown in Fig 5. The discrepancy seems to caused by difference of enhancement efficacy between C12Na and DM-β-CyD in rectum as shown in Fig. 4. Therefore, staying of hydrogel containing insulin with enhancer at rectum for long time is essential for adequate availability, otherwise too fast release of insulin from formulation give rise to dilution of insulin solution and movement of insulin to upper intestine which is not advantageous for insulin absorp-

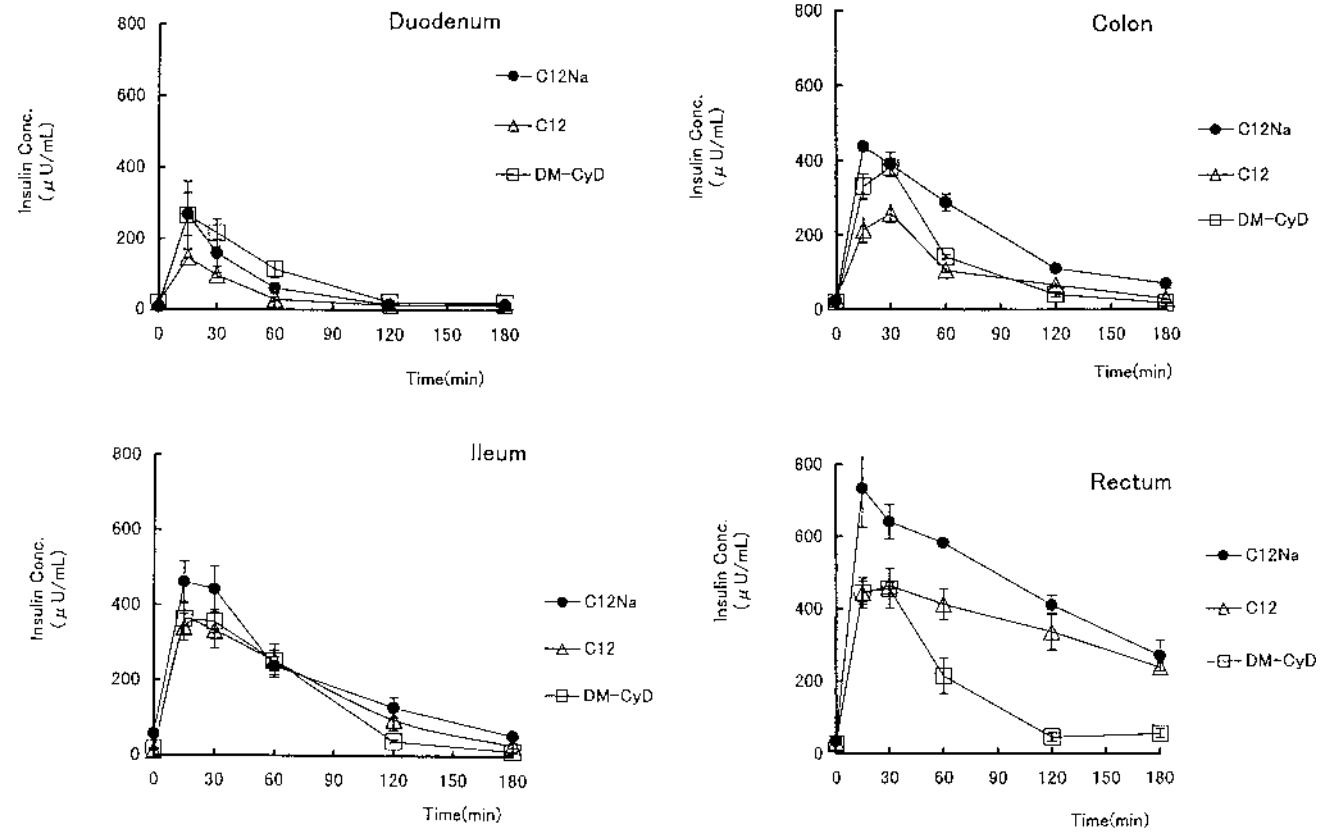

Fig. 4. Rat Plasma Insulin Levels after *in Situ* Loop Administration of 50 Units/kg Body Weight Insulin Solution with 5% (w/w) of Various Absorption Enhancers into the Duodenum, Ileum, Colon, and Rectum

●, C12Na; △, C12; □, DM-*β*-CyD. Error bar represents S.E.M. for 3—5 rats.

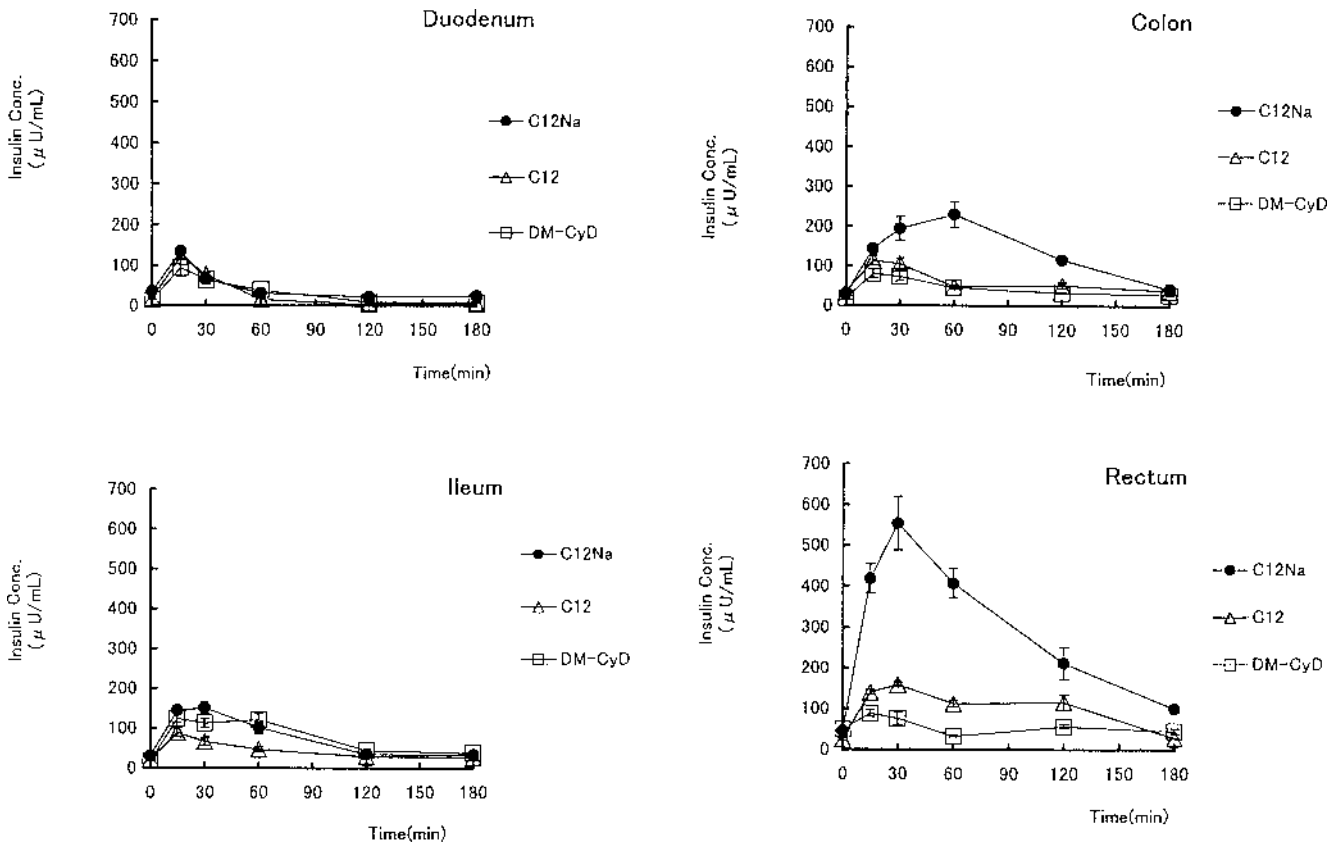

Fig. 5. Plasma Insulin Level after *in Situ* Loop Administration of 50 Units/kg Body Weight Insulin-Loaded Eudispert® Hydrogel with 5% of Various (w/w) Absorption Enhancers into the Duodenum, Ileum, Colon, and Rectum

●, C12Na; △, C12; □, DM-*β*-CyD. Error bar represents S.E.M. for 3—5 rats.

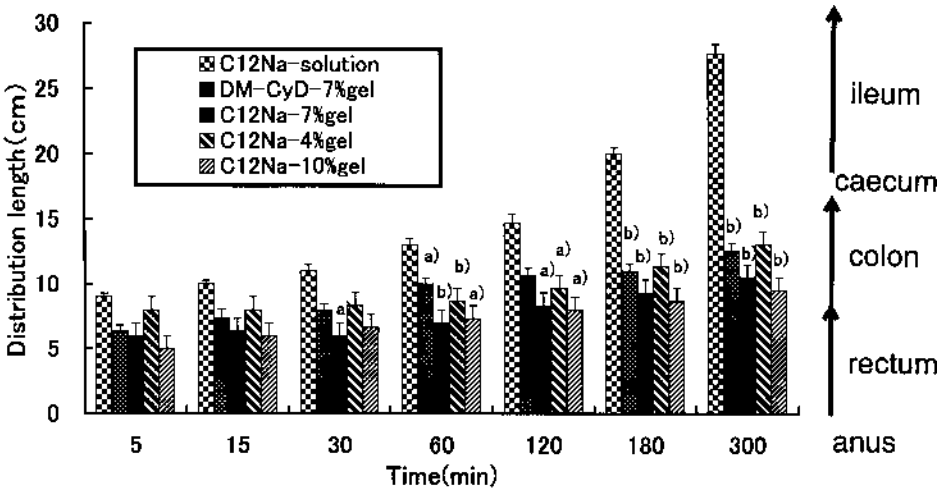

Fig. 6. Distribution of Dye in the Rat Intestine after Rectal Administration of Various Hydrogel Formulations Containing Evan's Blue

a) $p<0.01$, *b*) $p<0.001$, compared with solution of 5% (w/w) C12Na.

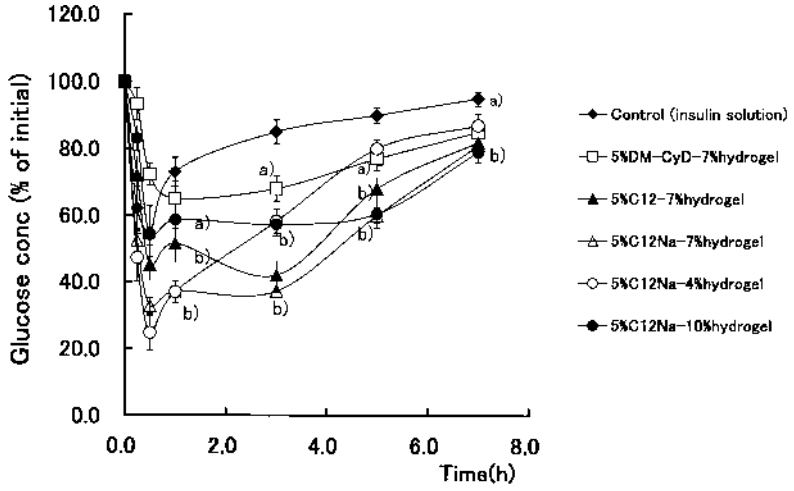

Fig. 7. Change in Plasma Glucose Level after Rectal Administration of 50 Units/kg Body Weight Insulin, Either as a Solution or *via* a Hydrogel with Various Polymer Concentrations and 5% (w/w) of Various Enhancers

The glucose concentration was expressed as a percentage value of the concentration at time zero. For example, 5%C12Na–7%hydrogel represents the hydrogel formulation containing 5% C12Na and 7% (w/w) Eudispert®. The error bar represents S.E.M. for 4—6 rats. *a*) $p<0.01$, *b*) $p<0.001$ compared with control (insulin solution).

tion. In general, in the lower intestine, protease activity and the first-pass effect for peptide drugs seemed to be small. Therefore, as reported in the previous paper,[10] the lower intestine may be a good target site for insulin delivery.

The process of insulin release from the hydrogel formulations further diminished the absorption of insulin at each absorption site, as shown in Fig. 5. The best absorption was obtained in the rectal region with the hydrogel containing C12Na. The plasma insulin level following the administration of hydrogels to the rectal loop was higher than that following administration of insulin solution to the loops in the upper intestinal regions (ileum or duodenum). This phenomenon suggests that the rectal region has the greatest ability to absorb insulin.

Retention and Dispersion of Hydrogel Formulations When the retention characteristics of the various formulations were examined after rectal administration (5% C12Na solution, 5% C12Na-loaded Eudispert® hydrogels with 4, 7, or 10% polymer concentration, and Eudispert® 7% hydrogel containing 5% of DM-β-CyD, all containing Evan's-blue dye), the greatest dispersion of the dye, as measured by the distance traveled from the anus by the dye front, was achieved with the 5% (w/w) C12Na solution, as shown in Fig. 6. With this formulation, the dye front reached the upper portion of intestinal tract, traveling a distance of approximately 14 cm, in about 2 h. This suggests that insulin would also diffuse as far as the upper intestine, where it would have a greater possibility of enzymatic degradation or hepatic clearance. Too rapid release of insulin from the formulation would not necessarily be advantageous with respect to insulin bioavailability.

Good rectal retention of the hydrogel formulations was demonstrated, which increased as polymer concentration increased. For the purposes of the present study, the first 8 cm from the anus was defined as the rectal region. Almost 98% of the hydrogels containing 7% or 10% Eudispert® remained in the rectum for 5 h after administration, even though the 10% hydrogel was very viscous. Even hydrogel containing 4% Eudispert®, which was less viscous, showed better retention than the C12Na solution. These retention characteristics

would be advantageous for insulin absorption from C12Na-containing hydrogels administered rectally, due to the absorption-enhancing effects of C12Na. Insulin or absorption enhancer released in the upper intestine will be diluted by intestinal fluids, and absorbed insulin is likely to be metabolized by first-pass effect. Therefore, the retention characteristics of the insulin-loaded hydrogels offer a real advantage, as the concentrations of insulin and C12Na are not diluted in the rectum.

Pharmacological Effects Insulin-loaded 7% Eudispert® hydrogels containing 5% (w/w) C12Na, C12, or DM-β-CyD were administered rectally to healthy rats (n=4—6) at an insulin dose of 50 units/kg body weight. An insulin solution was administered to another group of rats as a control. The results are shown in Fig. 7. All the hydrogel formulations tested caused a greater decrease in the concentration of serum glucose than did the insulin solution, the most effective being the hydrogel containing 7% Eudispert® and 5% C12Na. (It may still be possible to increase this effect, by further modification of the formulation.) This result was in line with the results of the animal studies described above. Glucose levels were depressed for longer with the 10% Eudispert® hydrogel containing 5% (w/w) C12Na, but retention of the formulation in the human rectum for too long might give a sensation of persistence. The 4% Eudispert® hydrogel containing 5% C12Na showed a larger initial decrease in glucose levels due to the rapid release rate of insulin from the hydrogel, as shown in Fig. 1.

Conclusion

The optimization of the hydrogel system for rectal delivery of insulin requires consideration of the effects of the system components on various factors such as release rate *in vitro* and *in vivo* absorption characteristics. Fatty acids such as caproic acid have been incorporated into commercial ampicillin suppositories and their enhancement effect demonstrated by Kajii[14] and Lindmark.[15]

The greatest pharmacological effect was found with insulin-loaded 7% (Eudispert®) hydrogel containing C12Na which having cosiderable large insulin release rate and bioadhesive characteristics. The strategy used in the present study might be useful for the development of a peptide delivery system *via* the gastrointestinal tract.

Acknowledgments We thank Miss Kikumi Murakami, Megumi Akagi, Mai Okada, and Yosie Boo for their experimental assistance.

References

1) Ogawa Y., Yamamoto M., Okada H., Yashiki H., Shimamoto T., *Chem. Pharm. Bull*., **36**, 1095—1103 (1988).
2) Heya T., Okada H., Tanigawara Y., Ogawa Y., Toguchi H., *Int. J. Pharm*., **69**, 69—75 (1991).
3) *a*) Nagai T., Machida Y., "CRC Bioadhesive Drug Delivery System," ed. by Lenaerts V., Gurney R., CRC Press, Boca Raton, FL, U.S.A., 1990, pp. 179—278; *b*) Nakamura K., Maitani Y., Lowman A. M., Takayama K., Peppas N. A., Nagai T., *J. Control Release*, **58**, 329—335 (1996).
4) Brand R. M., Hamel F. G., *Int. J. Pharm.*, **183**, 117—123 (1999)
5) Kantaria S, Rees G.D., Lawrence T., *J. Control Release*, **60**, 355—365 (1999).
6) Gliko-Kabir I., Yagen B., Baluom M., Rubinstein A., *J. Control Release*, **63**, 129—134 (2000).
7) Uekama K., Kondo T., Nakamura K., Irie T., Arakawa K., Shibuya M., Tanaka J., *J. Pharm. Sci*., **84**, 15—20 (1995).
8) Rizza R. A., *N. Engl. J. Med.*, 3**03**, 1313—1320 (1980).
9) Morishita M., Morishita I., Takayama K., Machida Y., Nagai T., *Biol Pharm. Bull*., **16**, 68—72 (1993).
10) Alsenz J., Russell-Jones G. J., Westwood S., Levet-Trafit B., de Smidt P. C., *Pharm. Res*., **17**, 825—32 (2000).
11) Umejima H., Kikuchi A., Kim N.-S., Uchida T., Goto S., *J. Pharm. Sci.*, **84**, 199—202 (1995).
12) Uchida T., Toida Y., Miyanaga Y., Machida K., Wada K., Ohki T., Matsuyama K., *Chem. Pharm. Bull*., **48**, 1828—1830 (2000).
13) Dubois M., Gilles K. A., Hamilton J. K., ebers P. A., *Anal. Chem*., **28**, 350—356 (1956).
14) Kajii K., Horie T., Hayashi M., Awazu S., *J. Pharm. Sci.*, **77**, 390—392 (1988).
15) Lindmark T., Kimura T., Artursson P., *J. Pharmacol. Exp. Ther*., **295**, 594—600 (1998).