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# Enteral insulin delivery by microspheres in 3 different formulations using Eudragit L100 and S100

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## Summary

Three types of insulin microspheres (IMS) were prepared in order to deliver insulin selectively in the intestinal tract using copolymers having pH-dependent solubility, Eudragit L100 (L), Eudragit S100 (S) and a 1:1 mixture of these (LS). In a release study, the insulin release rate from S-IMS was much slower than that from L-IMS at a pH below 7.0. At a pH of 7.5, all the IMS released more than 90% of the insulin within 60 min. Thus, the three types of IMS were expected to release insulin at different sites through the small intestine. To confirm this distribution in the intestinal tract, the amount of insulin in the residual IMS in the stomach and several parts of the small intestine was measured. L-IMS emptied from the stomach seemed to release insulin immediately in the upper parts of the small intestine. In contrast, many of the S-IMS appeared in the lower area of the small intestine (corresponding to the ileum). From these results, insulin released from L-IMS may exist in the jejunum to upper ileum at higher concentration compared with the cases of LS- or S-IMS. The hypoglycemic effect was observed to be the greatest after administration of L-IMS. The biological effect of each IMS was significantly amplified by a protease inhibitor, aprotinin (AP). The most remarkable effect of AP was seen in L-IMS. Our results suggest that L-IMS has the advantage of carrying insulin to the optimum sites for absorption and that AP effectively enhances its absorption.

## Introduction

Insulin requires parenteral administration mainly via the subcutaneous route, the most popular in daily clinical practice. However, in clinical therapy there are also disadvantages associated with this route such as local discomfort, inconvenience of multiple administration and occasional hyperinsulinemia due to overdose. The oral route is considered to be the most acceptable and convenient route of drug administration for chronic therapy. However, insulin delivery by this route is not as efficient as the subcutaneous route, because of low bioavailability from oral administration due to degradation by proteolytic enzymes

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and poor absorption in the gastrointestinal (GI) tract (Zhou and Po, 1991). Lee et al. (1991) reported that the enzymatic barrier was by far the most important of the multitude of barriers limiting the absorption of peptide drugs from the GI tract. On the other hand, the fact that the cellular morphology of intestinal organs changes gradually and that the activities of proteolytic protease gradually decrease from the duodenum to the large intestine may suggest the existence of an optimal site for insulin absorption. Recently, Schilling and Mitra (1990) reported that the optimal region for insulin absorption would be present in the small intestine and that the selective release of insulin to the mid-jejunum would also help to protect insulin from gastric and pancreatic enzymes.

In previous papers, we reported that insulin microspheres (IMS) containing a protease inhibitor could protect insulin against enzymatic degradation in vitro (Morishita et al., 1992a) and could lower serum glucose levels in normal and diabetic rats (Morishita et al., 1992b). In these studies, the microspheres were prepared using Eudragit L100 (L) which is a pH-sensitive copolymer soluble at a pH above 6. These microspheres were expected to release considerable amounts of the drug rapidly to the upper-intestinal regions. To assure insulin delivery to the mid- and lower-intestinal regions in this study, IMS were newly prepared using both Eudragit S100 (S), which is soluble at a pH of 7 and above, and a 1:1 mixture of L and S (LS). In this study we performed release tests on three types of IMS with media of various pH values. To confirm the distribution of IMS in the GI tract, we also measured the amount of insulin in the residual IMS in the GI tract by dissection treatment. In addition, the hypoglycemic effects of oral administration of three types of IMS were compared.

## Materials and Methods

*Materials*

Crystalline bovine insulin (Zn-insulin, 24.4 U/mg) and aprotinin (13.0 TIU) were purchased from Sigma Chemical Co., (St. Louis, MO, U.S.A.). Eudragit L100 and Eudragit S100 were gifts from Higuchi Co., Ltd (Tokyo, Japan). Gelatin and a glucose B-Test kit (the glucose oxidase method) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All other chemicals were obtained from commercial sources and were of analytical reagent grade.

*Preparation of the microspheres*

Three types of IMS were prepared by the method reported previously (Morishita et al., 1991). Briefly, weighed amounts of insulin either with or without aprotinin were dissolved in 300 $\mu$l of 0.1 N HCl. Ethanol and Eudragit were then added to the solution, which was stirred at 1200 rpm. The resultant solution was then poured into liquid paraffin, and the IMS were formed by the addition of a gelatin solution (0.5% w/w) and then further coated with Eudragit. All preparations were sized by sieving and the fraction ranging from 180 to 500 $\mu$m was used for the following experiments.

*Determination of insulin incorporation efficiency of the microspheres*

A 20 mg sample of microspheres was completely dissolved in 10 ml of phosphate buffer (pH 7.5). Insulin incorporation efficiency was then determined as the ratio of the assayed insulin amount to the theoretical amount.

*In vitro insulin release measurements*

A 200 mg sample of IMS was placed in a rotating basket (100 mesh size) and was introduced into a double walled beaker (250 ml) containing 200 ml of phosphate buffer solutions with pH values of 6.0, 6.5, 7.0 or 7.5 at 37°C under constant stirring at 150 rpm. Before these experiments, methylcellulose (0.001% w/v) was added into phosphate buffer solutions to prevent the adsorption of insulin on the surface of glass throughout the experiment (Yamakawa et al., 1990). At appropriate intervals, 2-ml samples were taken from the medium and filtered using a Fine Filter F (10 $\mu$m, Ishikawa Manufactory Co., Ltd, Tochigi, Japan). In addition, 2 ml of fresh fluid was added to the beaker immediately after each sampling to maintain a constant volume.

*Determination of IMS distribution in the GI tract*

Male Wistar rats weighing from 180 to 220 g were allowed to fast at least 16–20 h before the experiments and were allowed water ad libitum. A 20 mg sample of IMS was administered orally by force-feeding 1 ml of water via a rubber tube under non-anesthesia. At 1, 2, 4, 6, 8 and 10 h after administration, the rats were killed, and then the stomach and entire length of the small intestine were isolated. Immediately after isolation, the stomach (section 1) and the small intestine, which was divided into six sections (sections 2–7; length of each section 12–15 cm) were placed in 10 ml of purified water. The residual IMS in each section were collected by filtration, rinsed several times with purified water, and completely dissolved in 10 ml phosphate buffer (pH 7.5). The time required for 50% of the IMS to leave the stomach (mean gastric emptying time) was calculated from the linear portion of the log (residual%) plots against time as described by Takahashi et al. (1985). To investigate the distribution of IMS in the small intestine, ratios expressed as percentage were calculated based on the amount of insulin in the residual IMS found in the small intestine versus the amount of insulin in the IMS emptied from the stomach.

*In vivo absorption experiments*

The animal experimental outline and data observed in rats are summarized in Table 1. Male Wistar rats, having fasted for 16–20 h, were restrained in a supine position and administered IMS (50 U total insulin/kg body weight) with 1 ml of water by force-feeding via rubber tube. Exactly 5 min before insulin dosing, a 0.2 ml aliquot of blood sample was taken from the jugular vein. Subsequent blood samples were taken every 2 h up to 10 h. In order to calculate the efficacy of the oral route of insulin administration relative to i.v., insulin solutions were administered intravenously via the jugular vein. Insulin solutions were prepared by dissolving an appropriate amount of crystalline bovine insulin in 0.1 M phosphate buffer, pH 7.4. The insulin i.v. doses were 0.5, 1.0, 2.0 and 3.0 U/kg body weight. A 0.2 ml aliquot of blood sample was collected from the jugular vein on the side opposite to the injection before and at 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 h after dosing. Serum was separated

TABLE 1

*Animal experimental design and summarized data observed in rats*

| Preparation | No. of rats | Body weight (g) | Initial serum glucose concentration (mg/dl) | Insulin dose (U/kg) |
|---|---|---|---|---|
| i.v. | | | | |
| 0.5 | 3 | 203.3 ± 5.4 | 78.2 ± 5.9 | 0.5 |
| 1.0 | 3 | 190.0 ± 7.1 | 70.0 ± 4.6 | 1.0 |
| 2.0 | 4 | 202.0 ± 3.9 | 79.0 ± 3.4 | 2.0 |
| 3.0 | 4 | 198.8 ± 4.9 | 87.2 ± 3.4 | 3.0 |
| p.o. | | | | |
| L-control | 8 | 197.4 ± 2.2 | 84.8 ± 6.0 | – |
| L-IMS | 10 | 203.5 ± 2.1 | 90.4 ± 4.2 | 50.0 |
| L-IMS containing AP | 8 | 201.5 ± 3.3 | 87.8 ± 2.7 | 50.0 |
| LS-control | 8 | 201.8 ± 4.9 | 91.7 ± 6.5 | – |
| LS-IMS | 10 | 209.0 ± 4.6 | 88.5 ± 2.7 | 50.0 |
| LS-IMS containing AP | 8 | 194.8 ± 4.0 | 75.6 ± 2.9 | 50.0 |
| S-control | 8 | 197.8 ± 4.1 | 76.5 ± 2.5 | – |
| S-IMS | 10 | 211.0 ± 4.2 | 88.4 ± 5.4 | 50.0 |
| S-IMS containing AP | 5 | 196.4 ± 6.2 | 85.8 ± 8.5 | 50.0 |

Each value represents the mean ± S.E.

by centrifugation at 3000 rpm for 2 min and kept frozen until analysis. The absorption of intact biologically active insulin was evaluated by measuring the hypoglycemic effect. The relative efficacy (compared with i.v.) was calculated according to the method described by Morishita et al. (1992b).

*Analytical method*

Insulin concentration was measured by the HPLC method described by Nakazawa and Nagase (1986). The serum glucose level was determined enzymatically using a glucose B-Test kit.

*Statistical analysis*

Each value was expressed as mean ± standard error of the mean. For group comparisons, an analysis of variance (ANOVA) with a one-way layout was applied, followed by the Student's unpaired *t*-test.

## Results and Discussion

*Comparison of insulin incorporation efficiency and particle size distribution*

Insulin incorporation efficiencies obtained from three types of IMS are shown in Table 2. The efficiencies were different among the three types of IMS. The highest and lowest efficiencies were obtained with L-IMS and S-IMS, respectively. The particle size distribution of S-IMS was shifted towards the larger side compared with L-IMS and LS-IMS (Fig. 1). In the IMS preparation process, a gelatin solution (pH 5.7) was used for solidification of the microspheres. Since Eudragit S100 rapidly solidifies at a pH below 7 and dissolves at a pH above 7, the solidifying rate of Eudragit S100 seemed to be more rapid than that of Eudragit L100. Immediately after addition of the gelatin solution, some aggregation of microspheres was observed in S-IMS. In addition, each of these IMS samples was coated further with Eudragit to enhance the ability to protect against pepsin in the intestinal tract. In this coating process, it was considered that some amounts of coating polymer were solely solidified. This may lead to a lower insulin incorporation rate, especially in the case of S-IMS.

*Release of insulin from the microspheres*

The release profiles of insulin from three types of IMS in the media of various pH values are shown in Fig. 2. The release profiles of insulin from IMS were obviously dependent on the pH of the test solution, according to the properties of the Eudragit types used. At a pH of 6.0, more than 70% of the insulin was released from L-IMS, but less than 30% of the insulin was released from LS- and S-IMS during the 180 min test. The release rate from S-IMS was much slower than that from L-IMS at a pH below 7.0. At a pH of 7.5, all IMS showed similar release profiles and released more than 90% of the insulin during a 60 min. L-, LS- and S-IMS could release more than 90% of the insulin during the first 60 min at pH values of 6.5, 7.0 and 7.5, respectively. The pH value of the small intestinal fluid increased progressively from the duodenum (pH 6.9) to the ileum (pH 7.6) (Ritschel et al., 1991). Thus, three types of IMS are expected to release insulin at different sites through the small intestine.

*Distribution of IMS in the GI tract*

Fig. 3 shows the GI distribution of three types of IMS. The mean gastric emptying times obtained from L-, LS- and S-IMS were 1.3, 1.1 and 1.1 h, respectively. Thus, gastric emptying of IMS was very rapid and was similar among the three preparations. 6 h after administration, more than 90% of each IMS had moved into the small intestine.

TABLE 2

*Comparison of insulin incorporation efficiency among three preparations*

| Preparation | Insulin incorporation efficiency (%) |
|---|---|
| L-IMS | 80.2 ± 8.4 |
| LS-IMS | 78.1 ± 7.0 |
| S-IMS | 65.8 ± 5.4 |

Each value represents the mean ± S.E. of 5 determinations.

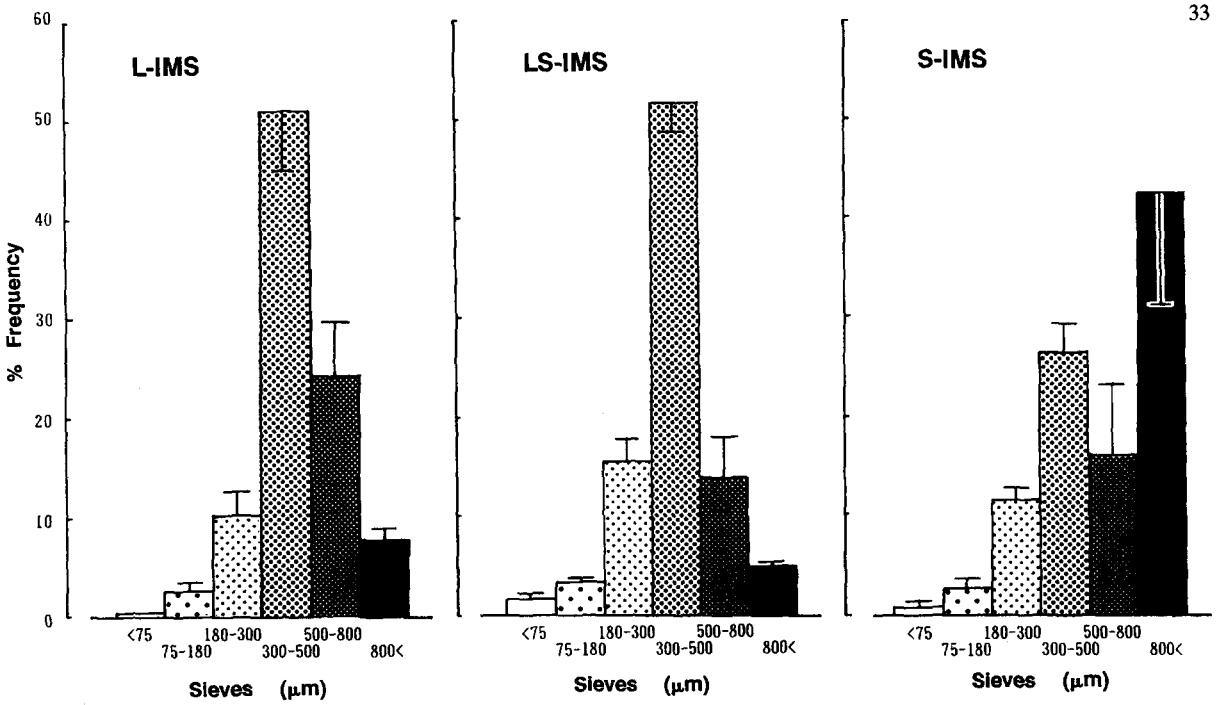


Fig. 1. Percent weight size distribution of L-, LS- and S-IMS. Each column represents the mean ± S.E. of 5 determinations.

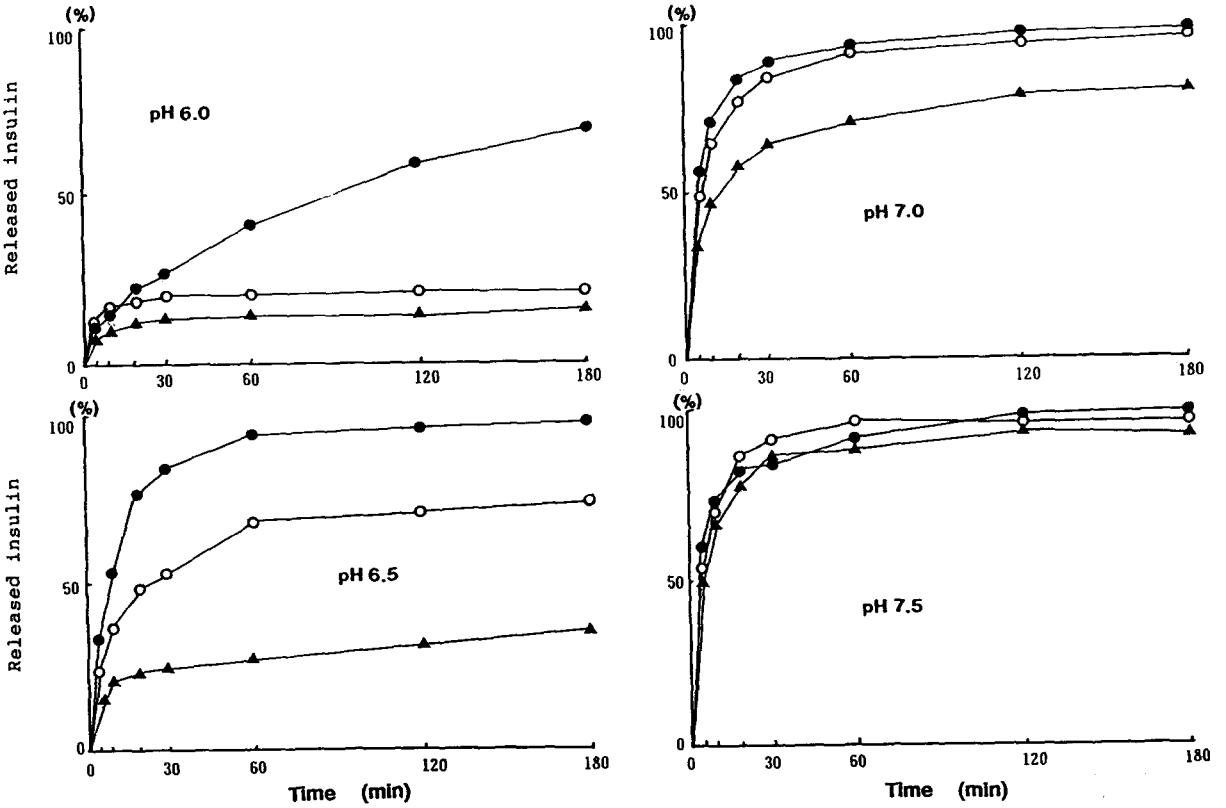


Fig. 2. Release profiles of insulin from L- (●), LS- (○) and S- (▲) IMS in phosphate buffer solutions, pH values 6.0, 6.5, 7.0 and 7.5. Each point represents the mean of 4–6 determinations.

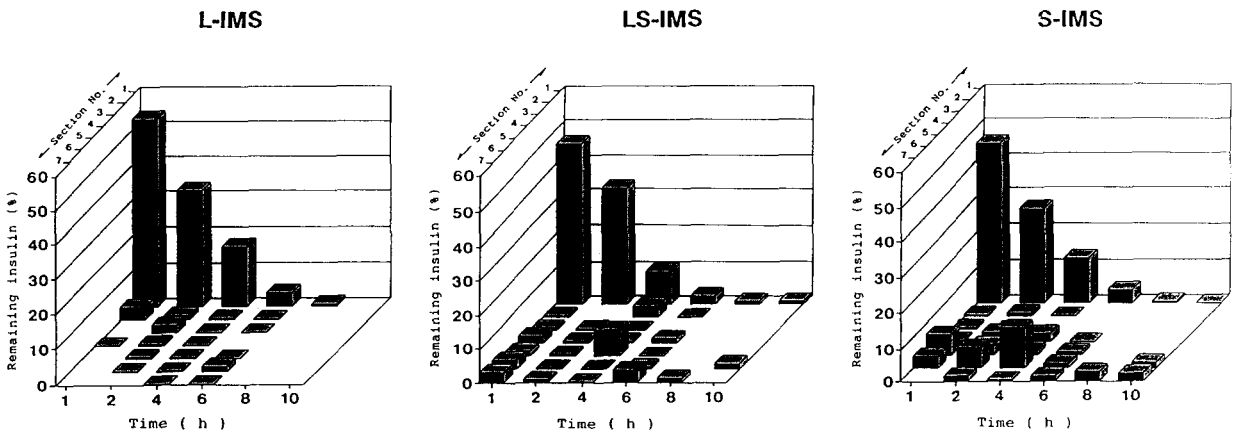


Fig. 3. Distribution of L-, LS- and S-IMS in the intestinal tract of rats. Each column represents the mean of 4–6 determinations.

The ratios (%) which were calculated based on the residual IMS in the small intestine against the IMS emptied from the stomach are shown in Table 3. Generally, the ratios (%) of L-IMS were much lower than those of LS- or S-IMS at each time. A small part of L-IMS appeared in the upper intestine (corresponding to the duodenum and upper jejunum) only during the initial 2 h. Thus, the L-IMS that left the stomach seemed to release insulin immediately in the upper parts of the small intestine. In contrast, approx. 20% of S-IMS that left the stomach remained in the small intestine until 4 h post-administration. Many of these appeared in the lower region of the small intestine. Thus, the S-IMS that emptied from the stomach seemed to release insulin into the lower area of the small intestine (corresponding to the ileum). A part of the S-IMS presumably reached the distal area below the cecum. Although the ratios (%) were lower than those of S-IMS, LS-IMS also appeared in the lower region of the small intestine until 10 h post-administration.

*Hypoglycemic effect of IMS administered orally*

Average serum glucose levels vs time profiles after oral administration of three types of IMS are shown in Fig. 4. A small but significant continuous decrease in levels was observed on administration of L- and LS-IMS. During the initial 6 h, however, the hypoglycemic effect observed with L-IMS was greater than that observed with LS-IMS. On the other hand, administration of S-IMS gave obvious hypoglycemic effects only after 2 h post-administration. As shown in Fig. 2, L-IMS released insulin completely within 60 min at a pH of 6.5 and above. Additionally, approx. 70% of the L-IMS was emptied from the stomach 2 h after administration (Table 3). These results

TABLE 3

*The ratio of the residual IMS in the small intestine against the IMS emptied from the stomach*

| Time (h) | L-IMS (%) | | | LS-IMS (%) | | | S-IMS (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | Upper | Lower intestine | Total | Upper | Lower intestine | Total | Upper | Lower intestine |
| 1 | 8.2 | 8.2 | 0 | 19.0 | 6.1 | 12.9 | 20.7 | 3.0 | 17.7 |
| 2 | 7.3 | 6.1 | 1.2 | 4.2 | 1.8 | 2.4 | 20.2 | 5.8 | 14.4 |
| 4 | 2.3 | 1.2 | 1.1 | 12.9 | 4.2 | 8.7 | 18.7 | 3.1 | 15.6 |
| 6 | 2.5 | 0.4 | 2.1 | 5.8 | 1.5 | 4.3 | 4.0 | 0.3 | 3.8 |
| 8 | 0.3 | 0 | 0.3 | 1.1 | 0 | 1.1 | 2.8 | 0 | 2.8 |
| 10 | 0 | 0 | 0 | 1.6 | 0 | 1.6 | 4.1 | 0 | 4.1 |

Upper intestine, sections 2–4; lower intestine, sections 5–7 (see text). Each value represents the mean of 4–6 determinations.

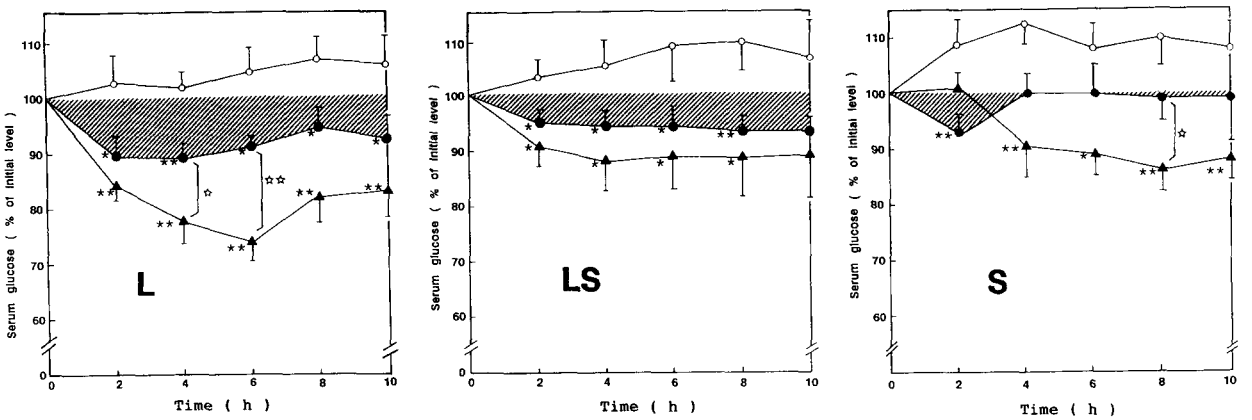


Fig. 4. Hypoglycemic effects of L-, LS- and S-IMS administered to rats. (○) Control (insulin-free microspheres); (●) IMS; (▲) TMS containing AP. Each point represents the mean ± S.E. Comparisons calculated at each period against controls: * $p < 0.05$, ** $p < 0.01$. Comparisons calculated at each period for IMS vs IMS containing AP: ☆ $p < 0.05$, ☆☆ $p < 0.01$.

suggest that the majority of L-IMS emptied from the stomach was dissolved within the initial 2 h and during passage into the upper small intestine. In fact, insulin released from L-IMS flowed into the lower jejunum and the ileum; therefore, much of their effects presumably came from lower intestinal absorption. Recently, using the everted gut sac technique, Schilling and Mitra (1990) found that the apparent permeability of insulin was significantly greater in the jejunum and ileum than in the duodenum. Thus, L-IMS has the advantage of carrying insulin to the optimal site for its absorption.

LS- and S-IMS released insulin gradually at more distal areas than L-IMS. At least a part of S-IMS might release insulin in the colon. The colon has relatively low digestive enzyme activity (Ritschel, 1991); however, the absorption of insulin is extremely limited (Kidron et al., 1982). Although both LS- and S-IMS released insulin almost completely within 60 min at a pH of 7.5 (Fig. 2), their release of insulin in the rat intestine was unexpectedly delayed. It was reported that the water content in the small intestine fell dramatically from the stomach to the ileocecal valve, leading to a significant increase in viscosity of the intestinal contents (Fordtran and Locklear, 1966). The effect of increased viscosity on the release of insulin is not clear, however, it should lead to a reduced release rate in some instances.

The biological effects of L-, LS- and S-IMS were amplified by AP which is a protease inhibitor against trypsin and $\alpha$-chymotrypsin (Fig. 4). The most remarkable effect of AP was seen in L-IMS. It was found that a chymotrypsin inhibitor promoted insulin absorption in the upper jejunum (Yokoo et al., 1988). Further, AP (Ziv et al., 1987) or soybean trypsin inhibitor (Kidron et al., 1982) enhanced insulin absorption in the ileum while no promoting effect of soybean trypsin inhibitor was evident in the ascending colon (Kidron et al., 1982). Actually, L-IMS could release insulin in a relatively short time; however, insulin and AP seemed to exist in the jejunum to ileum

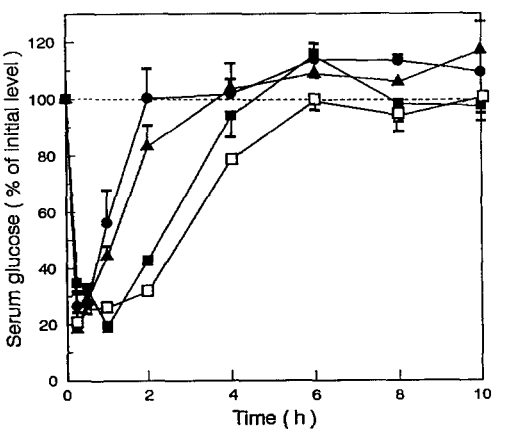


Fig. 5. The mean serum glucose levels after i.v. administration of insulin in doses of 0.5 (●), 1.0 (▲), 2.0 (■) or 3.0 (□) U/kg.

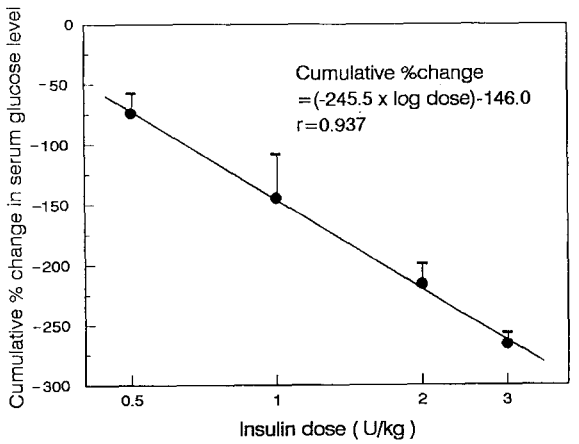


Fig. 6. Relationship between i.v. insulin dose and efficacy, expressed as the cumulative percentage of change in serum glucose level. Each point represents the mean ± S.E.

at a higher concentration compared with the cases of LS- or S-IMS. Thus, L-IMS has the advantage of demonstrating the effect of AP on the absorption of insulin.

Serum glucose levels normalized to a percentage of the initial level after intravenous administration of insulin to normal rats are shown in Fig. 5. The relationship between i.v. insulin dose and efficacy expressed as the cumulative percentage of change in serum glucose levels is shown in Fig. 6. Using this relationship, the relative efficacies of IMS were calculated (Table 4). Although the value remains low, L-IMS produced the highest relative efficacy. AP could obviously enhance the relative efficacies of all types of IMS though the degree of the effect varied among the preparations. The relative efficacies of L-, LS- and S-IMS were enhanced 2.8-, 2.3- and 1.5-times by AP, respectively.

TABLE 4

*Comparison of hypoglycemic effect obtained from various types of IMS administered orally*

| Treatment | [AUC] (% glucose reduced) | Relative efficacy (%) |
|---|---|---|
| L-control | 17.2 ± 12.0 | – |
| L-IMS | 86.8 ± 17.3 | 1.3 ± 0.2 |
| L-IMS containing AP | 180.0 ± 31.5 | 3.6 ± 1.0 |
| LS-control | 23.3 ± 21.5 | – |
| LS-IMS | 68.1 ± 15.5 | 1.0 ± 0.2 |
| LS-IMS containing AP | 104.2 ± 42.5 | 2.3 ± 1.3 |
| S-control | 17.2 ± 17.4 | – |
| S-IMS | 45.9 ± 10.7 | 0.8 ± 0.1 |
| S-IMS containing AP | 80.0 ± 22.2 | 1.2 ± 0.3 |

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