Absorption of Insulin Using Water-in-Oil-in-Water Emulsion from an Enteral Loop in Rats

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The present work was undertaken to prepare water-in-oil-in-water (W/O/W) emulsions as a carrier for insulin via the enteral route. The emulsions were prepared by a two-step procedure using a homogenizer. To avoid insulin escape from the inner aqueous phase, 3, 5 or 10% gelatin was added in the inner phase. The oily phase was composed of 5% lecithin, 20% Span 80 and 75% soybean oil. The purified water containing 3% Tween 80 was used for the outer aqueous phase. In addition, these emulsions were filtered with a membrane filter (0.45 μm) to obtain smaller emulsion particles. The stability of the emulsions was evaluated by a turbidity measurement method and photomicrographic observation. By the addition of gelatin to the inner aqueous phase and storage at 4°C, the stability of the emulsions could be improved. The hypoglycemic effects of insulin after administration of emulsion to the stomach, the duodenum, the jejunum, the ileum and the colon were examined using an in situ loop method in rats. A significant hypoglycemic effect was observed at the ileum and colon loops after administration of the filtered emulsions containing 5% gelatin in the inner phase. These findings suggest that the W/O/W multiple emulsions stabilized by gelatin can improve ileal and colonic absorption of insulin.

Key words insulin; water-in-oil-in-water (W/O/W) multiple emulsion; intestinal absorption; hypoglycemic effect

The bioavailability of insulin after oral administration is limited by enzymatic degradation in the gastrointestinal (GI) tract and low absorption at the mucosal site because of its high molecular weight and low lipophilic properties. To overcome these disadvantages, there have been many strategies in its attempted formulation. Approaches to increase insulin’s bioavailability include chemical modification,1–3 coadministration of protease inhibitors3–5 and/or penetration enhancers,6–8 and incorporation of insulin into specialized drug carrier systems.4–11 However, further studies are required to attain more efficient and reproducible delivery of insulin via the oral route.

Recently, much attention has been paid to water-in-oil-in-water (W/O/W) emulsions for application in drug delivery systems. The emulsions have been examined as a dermatological cream,12 an ophthalmic13 or enteral14–17 dosage form, a prolonged drug release system18,19 and a formulation for cancer treatment.19,20 Improvement of the main problem, i.e., poor stability, has also been reported.21–23 The emulsions are vesicular systems as dispersed oil droplets containing even smaller aqueous droplets. The emulsion systems protect the substances encapsulated in the inner phase.12,24 Since the enzymatic barrier is by far the most important of the multitude of barriers limiting the absorption of protein drugs from the GI tract,25 this type of emulsion might be a useful carrier to protect insulin against proteolysis. However, there have been few applications of this type of emulsion as a carrier for insulin via the enteral route. To date, only Engel el al.14 and Shichiri et al.15,16 have attempted to enclose insulin in a W/O/W emulsion to improve intestinal absorption. In addition, information as to where the emulsion mostly enhances the biologic effect of insulin in the GI tract is limited.

In the present study, W/O/W insulin emulsions were prepared using gelatin as a stabilizing agent in the inner aqueous phase. The oily phase composition was simulated to imitate that of lipid microspheres26,27 used in medical and pharmaceutical practices. We evaluated the usefulness of the emulsion as an enteral carrier of insulin and determined the best region for insulin absorption by comparing the biologic effects of the emulsion after administration to various sites in the rat intestine.

MATERIALS AND METHODS

Materials Crystalline porcine insulin (27.3 U/mg) was kindly supplied by Shimizu Pharmaceutical Co., Ltd. (Shizuoka, Japan). Gelatin, a glucose B-Test kit (the glucose oxidase method), and the lipophilic surfactant, sorbitan monoolesate (Span 80) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The hydrophilic surfactant, polyoxyethylene sorbitan monoolesate (Tween 80) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Soybean oil was obtained from Miyazawa Yakuhin Co., Ltd. (Tokyo, Japan). Egg yolk phospholipids (phosphatidylcholine and phosphatidylethanolamine) were purchased from Nippon Oil & Fats Co., Ltd. (Tokyo, Japan). Anhydrous D-glucose was obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade and commercially available.

Seamless cellulose tubing and membrane filters (Ekiscordic 13CR, 0.45 μm in pore size) were purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan) and Gelman Science Japan, Ltd. (Tokyo, Japan), respectively.

Preparation of W/O/W Emulsion W/O/W emulsions were prepared by a two-step emulsification procedure28 using a homogenizer (Ace Homogenizer, Nihonseiki Kaisha Ltd., Tokyo, Japan). Figure 1 summarizes the procedure. Briefly, the weighed amounts of insulin were dissolved in 200 μl of 0.1 N HCl, and then phosphate buffered saline (PBS) was added to the solution. The pH value of the solutions was adjusted to pH 7.4 by the addition of 0.1 N NaOH as needed. Then, 0, 3, 5 or 10% © 1995 Pharmaceutical Society of Japan
gelatin was dissolved in the solution. The oily phase was composed of 5% lecithin (phosphatidylcholine : phosphatidylethanolamine = 7 : 3), 20% Span 80 and 75% soybean oil. The purified water containing 3% Tween 80 was used for the outer aqueous phase. The weight percentage of each phase was employed as inner aqueous phase: oily phase: outer aqueous phase = 1 : 4 : 5. These emulsions were filtered with a membrane filter (0.45 μm) to make smaller and uniform size droplets.

**Stability of W/O/W Emulsion** The stability of the emulsions was evaluated by a turbidity measurement method for determination of the phase separation described by Pearce and Kinsella. Photomicrographic observation was also performed to evaluate the change in particle size of multiple droplets. In the turbidity measurement method, the emulsion was packed into a syringe (1 ml volume) immediately after preparation and was stored standing vertically at room temperature or 4 °C. Samples taken from the under side of the syringe were diluted with purified water and the turbidity was measured at 600 nm. For the photomicrographic observation, filtered and nonfiltered emulsions which contained 5% gelatin in the inner phase were used as the test samples. The change in particle size was monitored immediately after preparation and storage at room temperature or 4 °C for 10 d. The photomicrographs of the emulsion were taken using a light microscope (Optiphot microscope, Nikon, Tokyo, Japan).

**Percentage of Marker Entrapped in the Inner Aqueous Phase** A dialysis method employing cellulose tubing is generally used for determination of the percentage of drug entrapped in the inner phase. However, a preliminary study indicated that insulin was highly adsorbed on the cellulose tubing during the dialysis test. On the other hand, glucose, which migrated from the inner aqueous phase to the outer aqueous phase due to the breaking of the inner aqueous phase globules, could readily be separated from the W/O/W emulsion by means of dialysis. In this study, therefore, dialysis tests using d-glucose as a marker substance were performed as previously described.

Four grams of the freshly prepared W/O/W emulsion containing d-glucose in the inner phase was placed in seamless cellulose tubing and dialyzed in 120 ml of outer aqueous phase for 6 h at 4 °C. After the dialysis, 100 μl of the sample was taken and the d-glucose concentration was measured by using a glucose B-Test kit.

**In Situ Absorption Experiments** Male Wistar rats, each weighing 180—220 g, were fasted for 24 h prior to the experiments and were anesthetized by an i.p. injection of 50 mg/kg sodium pentobarbital. The 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 the stomach loop was made by ligation at the pars cardiaca ventriculi and the pylorus. A 6—7 cm loop of the duodenum, the jejunum, the ileum or the colon was identified and ligated at both ends. The duodenum loop was made at the first portion of the intestine, which was the closest to the stomach, and the bile duct was ligated. The next portion, 5 cm away from the ligament of Treitz, was utilized as the jejunum loop. The ileum loop was made at the end of the small intestine, just proximal to the ileo-cecal junction. The colon loop was made at the ascending colon. The rats were fixed for 1 h after the operation. The emulsion (1.0 g) was administered directly into the loops. The dose of insulin was fixed at 50 U/kg body weight. Various types of insulin-free emulsions were used as controls. Approximately 5 min before administration, a 0.2 ml aliquot of blood sample was taken from the jugular vein. Subsequent blood samples were taken at 15, 30, 60, 120, 180 and 240 min after dosing. Serum was separated by centrifugation at 3000 rpm for 2 min and kept frozen until analysis. The serum glucose level was determined by using a glucose B-Test kit.

The efficacy of the enteral route of insulin administration relative to i.v. was estimated according to the method described by Morishita et al. Briefly, insulin solution was administered intravenously via the jugular vein. The insulin i.v. doses were 0.08, 0.25, 0.5, 1.0 and 3.0 U/kg body weight. Blood samples were collected from the jugular vein on the opposite side to the injection before and at 0.25, 0.5, 1.0, 2.0 and 4.0 h after dosing. In the intestinal administration experiment, the intrinsic blood glucose levels generally rise due to surgical stress. Thus, the same operation as in the intestinal administration experiment was performed on the rats in the i.v. experiment. The cumulative percentage of change was calculated by summing the areas below the mean control levels using the trapezoidal method from the percentage of change vs. time curves for 0—4 h. The experimental procedures described above were performed according to the rules set by the Committee on Ethics in the Care and Use of Laboratory Animals at Hoshi University.

**Statistical Analysis** Each value was expressed as the mean ± S.D. of the mean. For group comparisons, one-way layout ANOVA with duplication was applied. Significant differences of the mean values were evaluated by Student's unpaired t-test. A p value of less than 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Photomicrographic Observation of W/O/W Emulsions** Figure 2 shows the structure of W/O/W emulsions observed
immediately after preparation. Each droplet contained many smaller aqueous droplets. The diameter of the droplets decreased with increasing gelatin concentration in the inner aqueous phase. It was suggested that aggregation of the aqueous droplets in the oil phase was restrained by the addition of gelatin to the inner phase.

**Stability** During storage at room temperature, all types of emulsions separated into two phases, i.e., the cream layer and water phase (Fig. 3), but only a small amount of oil was separated from the cream layer at 6–7 d after preparation. In contrast, the phase separation was not observed when gelatin was added into the inner aqueous phase of emulsions and they were stored at 4°C.

Immediately after preparation, the mean diameter of the filtered emulsion was clearly smaller and uniform compared with the nonfiltered emulsion (Fig. 4). Non-filtered emulsions tended to aggregate and combine. Therefore, larger droplets were formed after 10 d of storage at room temperature. Similarly, even when these emulsions were stored at 4°C, the droplets became larger after 10 d of storage. On the other hand, the particle size of filtered emulsions could be kept constant over storage for 10 d both at room temperature and at 4°C. Improvement of the stability of the W/O/W emulsions by gelatin has already been reported. The experimental evidence and the results obtained in this study suggest that W/O/W multiple emulsions can be stabilized by the combination of gelatin addition to the inner phase and the preparation of smaller droplets.

**Glucose Entrapped Efficiency** Table 1 shows the percentage of glucose entrapped in the inner aqueous phase of the emulsions. More than 60% of glucose initially incorporated into the emulsion was kept in the nonfiltered emulsion. However, the entrapped amount of glucose obtained from the filtered emulsion markedly decreased. The degree of drug leakage from the internal phase of multiple emulsions was greatest with systems of low particle size due to their large surface area. Furthermore, glucose may have leaked from the disrupted emulsion in the filtration process.

**Changes in Blood Glucose Level after Administration of**
**Filtered emulsion**

0 day

10 day (room temp.)

10 day (4°C)

**Nonfiltered emulsion**

0 day

10 day (room temp.)

10 day (4°C)

Fig. 4. Photomicrographs of W/O/W Emulsions Immediately after Preparation (0d) and Stored at Room Temperature or 4°C for 10d

<table>
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<tr>
<th>Table 1. Comparison of Entrapped Amount of Glucose in the Inner Aqueous Phase of W/O/W Emulsion</th>
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<td>Preparation</td>
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<td>Nonfiltered emulsion</td>
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Each value represents the mean ± S.D. of 3 determinations.

**W/O/W Emulsion Prepared with Various Gelatin Concentrations** To examine the biological effect of these emulsions, nonfiltered or filtered emulsions were administered to the ileum loop. Also, the effect of various gelatin concentrations in the inner phase and droplet size of the emulsion on serum glucose levels were compared. The emulsions prepared with 10% gelatin or without gelatin caused a slight decrease in serum glucose level without a significant difference compared to the control (Fig. 5). The sizing effect was not seen in these emulsions. The droplets in the gelatin-free emulsion system seemed to coalesce easily and their size increased, as compared with the emulsion which contained gelatin in the inner aqueous phase. In the case of the emulsion prepared with 10% gelatin, the system had high viscosity. The phase separation was observed during the filtration. The instability of the emulsions may lead to a lower hypoglycemic effect.

When the emulsions containing 3 or 5% gelatin were administered, the blood glucose levels decreased, and a greater effect was obtained from the smaller droplet size emulsion. The most significant hypoglycemic effect was observed after administration of the filtered emulsion which contained 5% gelatin in the inner aqueous phase. The lipid vehicles have been considered to promote the lymphatic absorption of orally administered drugs. Lipid microspheres, composed of olive and soybean oil, egg lecithin and glycerol have been found to promote the absorption of orally administered drug into the thoracic lymph in the rat. The W/O/W emulsions could also transfer carboxyfluorescein to the lymph. Although the transportation process is unclear at present, some of the stable small droplets could escape from the lipase hydrolysis and may either diffuse across tight junctions intercellularly, or be taken up by endocytosis into the enterocyte as suggested by Ritschel. Then, insulin could be transported by the lymphatic system.

Another possible explanation for the enhancement of the hypoglycemic effect by the filtered emulsion is its greater surface area. Intestinal absorption of cyclosporine in the olive oil emulsion was enhanced by the reduction in droplet size. Both bile salts and pancreatic and intestinal lipase seem to act on the emulsion. Lipase activity on the hydrolysis of the oil will increase with the greater surface area of the emulsion having a small droplet size. As a result, mixed micelles of bile acids and fatty acid or monoglycerides could be formed. A marked absorption enhancement effect by such mixed micelles has been reported. Thus, the mixed micelles may enhance the absorption of free insulin, which came from the disrupted emulsion by the filtration treatment or the coalescence of droplets, and by the hydrolysis of the emulsions by lipase.

**Site-Dependent Hypoglycemic Effect of W/O/W Emulsion** Since the intestinal tract is biologically and physiologically diversified, absorption of the emulsion is expected to differ in the various intestinal regions. In this study, no significant hypoglycemic effect was seen after administration of the emulsion in the upper region of the
GI tract (Fig. 6). The mucous layer covering the intestinal surface may be an absorption barrier for the droplets. The mucous gel thickness was highest in the stomach and tended to decrease toward the lower intestine. Furthermore, the instability of the emulsions in an acid environment was suggested. Therefore, these disadvantageous factors may lead to the lower hypoglycemic effect of the emulsion in the upper part of the GI tract. In addition, the concentration of bile salts is higher in the jejunum than in the ileum, but digestive enzymes are also abundant in the upper small intestine. Even if mixed micelles were formed and enhanced the insulin absorption, insulin degradation by enzymes might be more rapid than the absorption enhancement effects. On the other hand, a
significant hypoglycemic effect was also seen in the colon where the concentration of bile salts was very low. Thus, mechanisms apart from the enhancement absorption by mixed micelles should be taken into account.

In the small intestine, lymphatic vessels called lacteal are located centrally in each villi. In the large intestine the lymphatic vessels are fewer in number and smaller in size. Furthermore, the Peyer's patches, the major site of pinocytosis, are particularly rich in the ileum close to the ileo-cecal junction. These morphological differences might cause a higher hypoglycemic effect in the ileum than in the colon.

**Hypoglycemic Efficacy Relative to i.v.** To calculate the value of percentage efficacy relative to i.v., the relationship between i.v. insulin dose and efficacy, expressed as the cumulative percentage of change in serum glucose levels, was obtained from the results of the i.v. study. The dose/response curve gave the following equation:

\[
\text{cumulative } \% \text{ change } = -209.0 \times \log \text{ dose} - 185.2; \quad r=0.989; \quad p<0.01
\]

Table 2 shows the relative efficacies observed in each administered region. Clearly, insulin was predominantly absorbed from the ileum and the colon regions while the absorbed amount remained low.

Administration of insulin solution (50 U/kg) did not decrease the glucose level in any intestinal region. By using this formulation, a significant hypoglycemic effect was obtained without any additives such as protease inhibitors and/or absorption enhancers. Since the multiple emulsion can easily incorporate protease inhibitors and/or absorption enhancers in each phase according to their solubility, it is expected to increase the relative hypoglycemic efficacy of insulin with formulation improvement.

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**REFERENCES**