# Intestinal insulin delivery

# using water-in-oil-in-water multiple emulsion

Yoshinori Onuki

# CONTENTS

# List of Publication

1. In vivo effects of highly purified docosahexaenoic acid on rectal insulin absorption: Yoshinori Onuki, Mariko Morishita, Kozo Takayama, Shinji Tokiwa, Yoshiyuki Chiba, Koichi Isowa, Tsuneji Nagai. *Int. J. Pharm.* 198 (2000) 147-156. <presented in Chapter 1 of this dissertation>

2. Improved insulin enteral delivery using water-in-oil-in-water multiple emulsion incorporating highly purified docosahexaenoic acid: Yoshinori Onuki, Mariko Morishita, Hisae Watanabe, Yoshiyuki Chiba, Shinji Tokiwa, Kozo Takayama, Tsuneji Nagai, *S.T.P. Pharma Sci.* 13 (2003) 231-235. <presented in Chapter 1 of this dissertation>

3. Docosahexaenoic acid and eicosapentaenoic acid induce changes in the physical properties of a lipid bilayer model membrane: Yoshinori Onuki, Mariko Morishita, Yoshiyuki Chiba, Shinji Tokiwa, Kozo Takayama, *Chem. Pharm. Bull.* 54 (2006) 68-71. <presented in Chapter 2 of this dissertation>

4. Formulation optimization of water-in-oil-in-water multiple emulsion for intestinal insulin delivery: Yoshinori Onuki, Mariko Morishita, Kozo Takayama, *J. Controlled Release* 97 (2004) 91-99. <presented in Chapter 3 of this dissertation>

# Abbreviations

| | |
|---|---|
| AAC | area above the curve |
| ANOVA | analysis of variance |
| AUC | area under the concentration-time curve |
| $C_{max}$ | maximum concentration |
| DHA | docosahexaenoic acid, (22:6, n-3) |
| DPH | 1,6-diphenyl-1,3,5-hexatriene |
| DPPC | dipalmitoylphosphatidylcholine |
| DSC | differential scanning calorimeter |
| EPA | eicosapentaenoic acid, (20:5, n-3) |
| MG | 2-monoglyceride |
| MVS | multivariate spline interpolation |
| NEFA | nonesterified fatty acid |
| OA | oleic acid, (18:1, n-9) |
| OD | optical density |
| PBS | phosphate-buffered saline, pH 7.4 |
| PF127 | Pluronic F127 |
| PUFAs | polyunsaturated fatty acids |

| | |
|---|---|
| SA | stearic acid, (18:0) |
| TG | triglyceride |
| $T_{max}$ | the time to reach the $C_{max}$ |
| W/O/W | water-in-oil-in-water |

# GENERAL INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by varying or persistent hyperglycemia. Hyperglycemia in itself can lead to dehydration and ketoacidosis, and prolonged hyperglycemia can lead to many serious complications, including cardiovascular disease, chronic renal failure, retinal damage, nerve damage, and gangrene with risk of amputation of the toes, feet and even the entire leg. According to a survey of World Health Organization, today more than 170 million people worldwide suffer from diabetes, and its incidence is on the increase at an alarming rate. Diabetes mellitus is one of the most significant diseases in the developed world, and is the subject of increasing attention.

Insulin, composed of 51 amino acids, is a key hormone regulating the blood glucose level, and it is the mainstay of drug therapy for patients with insulin-dependent diabetes mellitus. There are several types of insulin preparations, such as Regular, Lispro, NPH, Lente and Ultra Lente, and they differ in characteristics, such as time of onset, peak activity, and duration of biological action [1,2]. Although the blood glucose level can be controlled by these insulin preparations, they are all limited to the injection dosage form. The subcutaneous injection of insulin has several disadvantages, including peripheral hyperinsulinemia, hypoglycemia, and unacceptability due to the use of painful and troublesome injections.

To this date, a variety of routes of administration [3,4], including dermal [5,6], nasal [7], pulmonary [8-11] and oral routes [12,13], have been investigated for their applicability, mainly in order to reduce the pain associated with injection, and to improve the pharmacodynamic properties of the applied insulin. In particular, a great deal of work has been focused on the development of oral insulin delivery. Endogenous insulin is secreted by the pancreas into tributaries of the hepatic portal vein, resulting in the direct delivery of insulin to the liver,

which is the principal target organ of insulin and also removes half of the insulin presented to it in a single transhepatic circulation [14]. Oral insulin delivery is thought to be close to the physiological state, since it offers a means of improving the portal levels of insulin, and also curbs the peripheral hyperinsulinemia. However, most peptides, including insulin, are poorly absorbed via the intestinal membrane because of extensive proteolytic degradation by intestinal enzymes and their insufficient membrane permeability due to their high molecular weight and low lipophilicity.

Numerous strategies have been devised to improve the oral bioavailability of peptide drugs. They include approaches regarding the development of multifunctional drug carriers [15-17] and the co-administration of protease inhibitors and/or absorption enhancers [7,18]. A water-in-oil-in-water (W/O/W) multiple emulsion (Fig. 1) is thought to be an efficient drug carrier because it can protect the peptides against proteolysis [19,20]. In addition, the emulsion system can incorporate various types of ingredients in each phase, in accordance with its solubility. Several researchers have reported that the enteral bioavailability of drugs, including peptides, was successfully promoted by using the W/O/W multiple emulsion [19,21,22]. Previous studies in our laboratory have also documented that the intestinal absorptions of peptide drugs, such as insulin and vancomycin, were improved by using this system [23-26], and also found that the unsaturated fatty acids shown in Fig. 2, such as oleic acid (OA, 18:1, n-9), eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3), were effective in improving the insulin mucosal absorption following co-administration [24,25]. They have an advantage for use in pharmaceuticals because they are endogenous compounds present in mammalian cells and probably induce less toxicity. OA alters the membrane permeability by increasing the membrane fluidity, and is often used as a permeation enhancer [27,28]. EPA and DHA are polyunsaturated fatty acids (PUFAs) highly enriched in brain lipids and retinal photoreceptor cell phospholipids [29,30]. It is well

known that the dietary intake of PUFAs is linked to the prevention of diseases, such as cancer [31,32] and heart disease [33-35], and is needed for neurological and brain development [36]. The various actions of PUFAs on biological functions have been paid attention in recent years.

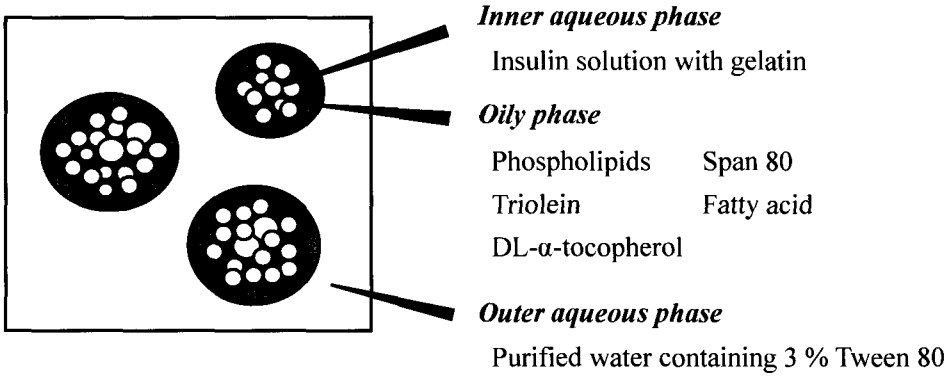


Figure 1. Composition of W/O/W multiple emulsion system

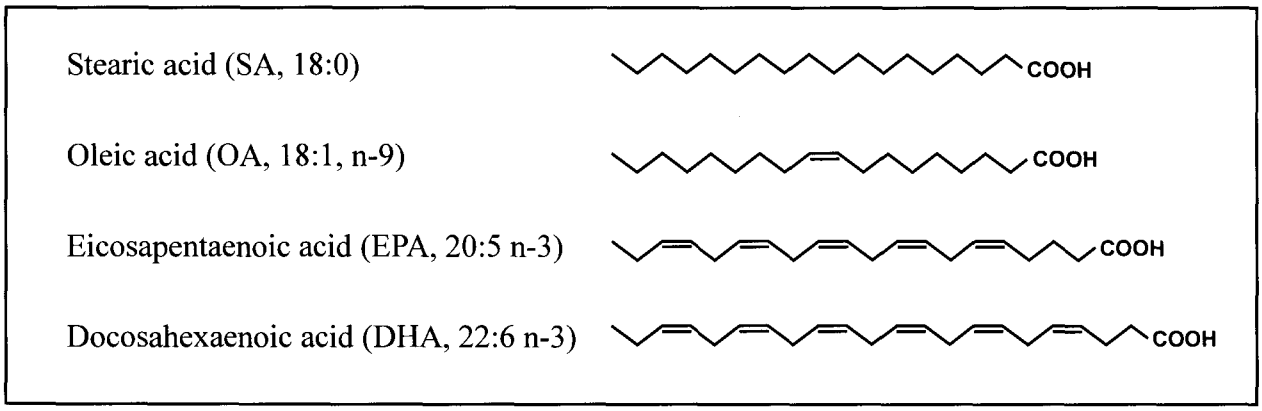


Figure 2. Chemical structure of fatty acids

The aim of this study was to establish a novel insulin enteral delivery system using a W/O/W multiple emulsion. In Chapter 1, the effectiveness and the toxicity of PUFAs as a potential absorption enhancer were investigated in *in situ* and *in vivo* insulin absorption studies. In Chapter 2, in order to examine the action of unsaturated fatty acids on the lipid bilayer, dipalmitoylphosphatidylcholine (DPPC) liposomes were used as a model of the lipid

bilayer and the structural changes in the lipid bilayer induced by fatty acids were assessed by measuring the changes in the phase transition temperature, fluorescence anisotropy, and detergent insolubility. In Chapter 3, according to experimental design, the relationships between causal factors and individual characteristics of emulsion were identified. Moreover, formulation optimization was carried out by means of a novel optimization technique.

# CHAPTER 1

## Enhancement effect of polyunsaturated fatty acids on intestinal insulin absorption

# 1. Introduction

Extensive studies have been conducted in the intestinal absorption of peptides and proteins, especially insulin. They generally exhibit poor absorption from the gastrointestinal tract in the absence of an absorption modifying component, the so-called absorption enhancer [7]. In order to improve oral bioavailability of insulin, so far various kinds of absorption enhancers, such as fatty acids, bile salts, surfactants and chelating agents, have been utilized [18,37-39].

In our laboratory, unsaturated fatty acids have been tested as an absorption enhancer for intestinal insulin absorption, and their marked enhancement effects were observed in the *in situ* loop experiments [24,25]. These unsaturated fatty acids include 18-carbon fatty acids, such as OA, linoleic acid (18:2) and linolenic acid (18:3), and PUFAs, such as DHA and EPA. Particularly, the enhancement effects of PUFAs on insulin absorption in the rectum or colon were more effective than those of 18-carbon unsaturated fatty acids [24].

To further investigate the effectiveness and the toxicity of PUFAs as a potential absorption enhancer for intestinal insulin absorption, *in situ* loop and *in vivo* absorption experiments were accomplished. Firstly, the enhancement effects of various fatty acids incorporated in insulin emulsion on rectal insulin absorption were determined. The site-dependency and the reversibility of the effect of DHA on intestinal insulin absorption were also assessed. Considering the clinical use of insulin preparations, multiple administrations of the insulin emulsion were performed, and the change in the hypoglycemic effect and mucosal damage induced by the emulsions were examined. In order to control the duration of hypoglycemic effect of the formulation, insulin emulsion containing Pluronic F127 (PF127), gel forming block copolymer, was prepared and its effect on the time profiles of the serum glucose level was evaluated.

## 2. Materials and Methods

### 2.1. Materials

Crystalline porcine insulin (27.3 IU/mg) was supplied by Simizu Pharmaceutical Co., Ltd. (Shizuoka, Japan). Lauric acid (purity: 99.0 %), gelatin, glucose B-Test kit, triolein, sorbitan monooleate (Span 80) and DL-α-tocopherol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Capric acid (purity: 99.0 %), glycocholic acid (purity: 97.0 %), SA (purity: 99.0 %) and PF127 were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Polyoxyethylene sorbitan monooleate (Tween 80) and OA (purity: 99.0 %) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Egg yolk phospholipids (phosphatidylcholine and phosphatidylethanolamine) were purchased from Nippon Oil & Fats Co., Ltd. (Tokyo, Japan). DHA (purity: 99.0 %), EPA (purity: 99.0 %), docosahexaenoic acid 2-monoglyceride (DHA-MG, purity: 30.0 %) and docosahexaenoic acid triglyceride (DHA-TG, purity: 30.0 %) were provided by Nippon Suisan Kaisya Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

### 2.2. Preparation of W/O/W multiple emulsion

Insulin emulsions were prepared by a two-step emulsification procedure using a homogenizer (Ace Homogenizer, Nihonseiki Kaisha Ltd., Tokyo, Japan) according to a method reported in a previous paper [24]. Designated amounts of insulin were dissolved in 200 μL of 0.1 N HCl, and then phosphate-buffered saline (PBS, pH 7.4) containing gelatin (5 % of the inner aqueous phase) 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 required. The weight was finally adjusted to 2 g, and this solution used for the inner aqueous phase. The concentrations of each component in the oily phase were as follows: DL-α-tocopherol, 0.06 %; egg yolk phospholipids (phosphatidylcholine: phosphatidylethanolamine, 7:3), 5 %; Span 80, 20 %.

Free or glycerides of fatty acids were also added to the oily phase, and the concentration was fixed at 0, 0.5, 1, 2 or 5 % of the total weight of the emulsion. An appropriate amount of triolein was added to adjust the weight of the oily phase, and 8 g of the oily phase was finally yielded. The inner aqueous phase was added to the oily phase, and then the first emulsification was carried out to form W/O emulsion using the homogenizer. Thirty grams of purified water containing 3 % Tween 80 was used for the outer aqueous phase. After addition of the primary W/O emulsion to the outer aqueous phase, the second emulsification was performed to form the W/O/W multiple emulsion. The weight ratio of each phase was, inner aqueous phase: oily phase: outer aqueous phase, 1:4:15.

As for the insulin emulsion containing PF 127, firstly, an insulin emulsion containing DHA (weight ratio of inner aqueous phase: oily phase: outer aqueous phase, 1:4:5) was prepared in the same way as described above. A stock PF 127 solution was prepared by dissolving designated amounts of PF 127 in purified water on ice, and kept at 5-10 °C to avoid gelation. Cold insulin emulsions were gently added to the PF 127 solution at a weight ratio of 1 to 1, and then the mixture was stirred until it became homogeneous. The final concentrations of PF 127 and DHA were fixed at 15 and 2 % of the total weight, respectively.

### 2.3. *In situ* absorption experiments

All the animal studies were performed in compliance with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals at Hoshi University. Male Wistar rats weighing 180-220 g were purchased from Tokyo Laboratory Animal Science Co., Ltd. (Tokyo, Japan). The animals were housed in environmentally controlled rooms at 23 ± 1 °C and 55 ± 5 % relative humidity, and allowed free access to water and food during acclimatization. The rats were fasted for 48 h prior to the experiments. Following anesthetization with an i.p. injection of 50 mg/kg sodium pentobarbital (Dainippon

Pharmaceutical Co., Ltd., Osaka, Japan), the rats were restrained in a supine position on a thermostatically controlled board at 37 °C. A 5 cm loop of the ileum, the colon or the rectum was identified and ligated at both ends. The ileal loop was made at the neighboring part of the cecum, and the colonic loop was made at the ascending colon. Subsequently, they were carefully returned to their original location inside the peritoneal cavity. The rats were further left on the board at 37 °C for 1 h to recover from the elevated blood glucose level due to the surgical operation. Insulin solution or insulin emulsion was administered directly into each loop through a stomach sonde needle for rats (KN-348, Natsume Seisakusyo Ltd., Tokyo, Japan). The insulin doses were 10, 20 and 50 IU/kg body weight, and the doses of insulin were changed by adjusting the amount of emulsion. A 0.2 mL sample of blood was taken from the jugular vein 5 min before the administration. Subsequent blood samples were taken at specified periods after dosing. Serum was separated by centrifugation at 13,400×g for 1 min and kept at -20 °C until analysis. The serum glucose level was determined using a glucose B-Test kit. Post-dose levels of the serum glucose were expressed as a percentage of the pre-dose level. The percentage change in the serum glucose levels was taken as the percentage of the pre-dose level subtracted from 100. The cumulative percentage change in the serum glucose level was calculated by summing, using the trapezoidal method, the areas below the baseline levels in the percentage change versus time curves for 0-4 h (the area above the glucose level-time curve: $AAC_{glucose}$). The pharmacological availability was calculated according to the method described by Morishita *et al* [26]. Insulin PBS solutions were administered subcutaneously at doses of 0.5, 1.0, 2.0 and 3.0 IU/kg body weight. Blood samples were collected from the jugular vein at designated periods. $AAC_{glucose}$ values were obtained, and then the dose-response curve between the logarithm of s.c. insulin doses and $AAC_{glucose}$ was estimated. The pharmacological availability, the relative bioavailability compared to s.c. route, was

calculated using the dose-response curve.

Insulin absorption was further studied in the same *in situ* system, but using the rectal segments pretreated with insulin free emulsion. This study was designed to evaluate the reversibility of the emulsion's insulin absorption enhancement effects. The W/O/W multiple emulsions containing 2 % capric acid and DHA, and 3 % glycocholic acid PBS solution (positive control) were used as the test solutions. The rectal loop was exposed to 0.5 mL of test solution for 0.5 or 3 h. Following exposure, the loop was gently rinsed with 20 mL of warmed PBS, and subsequently 0.5 mL of insulin PBS solution was administered to the loop at a dose of 10 IU/kg body weight, and the *in situ* insulin absorption studies were performed. Each mean value of $AAC_{glucose}$ was calculated. The reversibility was calculated as the percent change in the $AAC_{glucose}$ value as follows. The percent change in the $AAC_{glucose}$ value = $AAC_{glucose}$ of insulin solution after the exposure of insulin free emulsion / $AAC_{glucose}$ of insulin emulsion containing fatty acid × 100. In the case of glycocholic acid, the $AAC_{glucose}$ of the insulin solution was divided by the $AAC_{glucose}$ of the co-administration of insulin and glycocholic acid.

### 2.4. *In vivo* single and multiple absorption experiments

Male Wistar rats weighing 180-220 g were purchased from Sankyo Labo Service Co., (Tokyo, Japan). The rats were fasted for 48 h prior to the experiments. In the case of experiments using insulin emulsion as the dosage form, rats were housed in cages and were given water ad libitum, except for at the time of administration and blood sampling during the experimental periods. The rats were restrained in a supine position during administration and at each blood sampling under non-anesthesia. On the other hand, in the case of experiments using an insulin emulsion containing PF 127 hydrogel, the rats were anesthetized with the i.p. injection of 50 mg/kg sodium pentobarbital, and restrained in a supine position

throughout the experiment on a thermostatically controlled board at 37 °C. Insulin emulsions were administered rectally through the stomach sonde needle for rats. The insulin doses were 5, 10 and 20 IU/kg body weight. The blood samples were withdrawn at designated periods, and the determination of the serum glucose levels was performed as described above. In addition, the serum insulin levels were determined using enzyme immunoassay (IMX System, Abbott Japan Co., Ltd., Tokyo, Japan). The basal endogenous insulin level was subtracted from all insulin levels measured following insulin administration. The area under the insulin level-time curves for 0-4 h ($AUC_{insulin}$) was determined using the trapezoidal method.

As for the multiple administration experiments, each emulsion was rectally administered once a day at 10:00 a.m. for 10 days. The dose of insulin was fixed at 5 IU/kg body weight. On the first and tenth day, the *in vivo* insulin absorption experiments were performed. Immediately after the absorption experiment on the tenth day, the rectums were excised to evaluate their mucosal damage. The isolated intestines were fixed in 10 % neutral carbonate-buffered formalin, paraffin-sectioned, and stained with hematoxylin and eosin. All tissues were analyzed using light microscopy and scored based on the histopathological findings.

## 3. Results and Discussion

### 3.1. Enhancement effect of unsaturated fatty acids contained in insulin emulsion on rectal insulin absorption

Figures 3 and 4 show the changes in the serum insulin and glucose levels following the rectal administration of insulin emulsions containing various fatty acids in the *in vivo* absorption study. The concentration of fatty acids contained in the emulsion was fixed at 2 % of the total weight, and the insulin dose was 5 or 10 IU/kg. An insulin emulsion containing SA, 18-carbon saturated fatty acid, was used as a control. As shown in Figs. 3 and 4, the rectal insulin absorption was improved markedly, and a marked hypoglycemic effect was induced by the administration of insulin emulsions containing unsaturated fatty acids, such as OA, EPA and DHA. The peak insulin levels and the lowest glucose levels appeared at 0.25-0.5 and 0.5 h after administration, respectively. The increase in the insulin level and the reduction in the serum glucose level were progressively pronounced with the increase in the insulin dose.

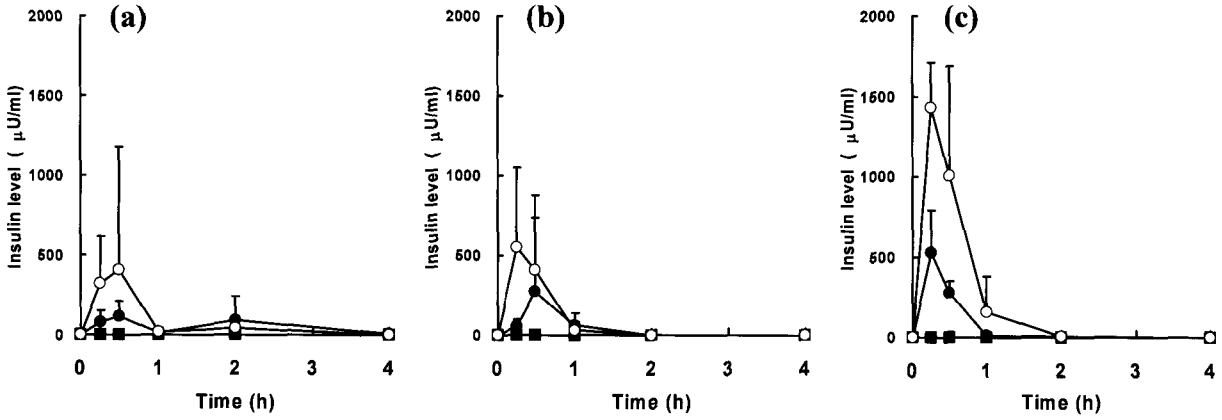


Figure 3. Change in serum insulin level following the rectal administration of insulin emulsion containing 2 % (a) OA, (b) EPA and (c) DHA. Each value represents the mean ± S.D. of five to seven rats.
Key: (■) 5 IU/kg of insulin emulsion containing stearic acid (control); (●) 5 and (○) 10 IU/kg of insulin emulsion, respectively.

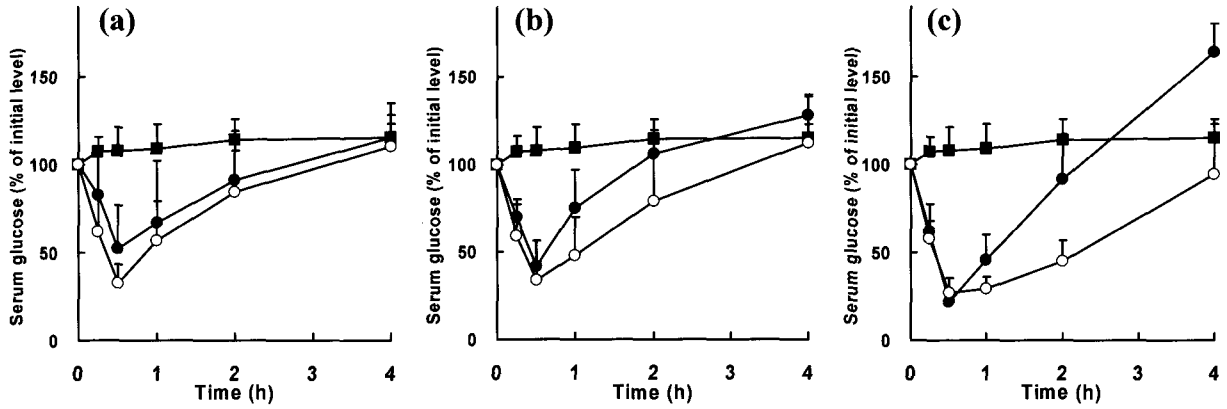


Figure 4. Change in serum glucose level following the rectal administration of insulin emulsion containing 2 % (a) OA, (b) EPA and (c) DHA. Each value represents the mean ± S.D. of five to seven rats.
Key: (■) 5 IU/kg of insulin emulsion containing stearic acid (control); (●) 5 and (○) 10 IU/kg of insulin emulsion, respectively.

Following these results, the values of $AAC_{glucose}$ were calculated. It was clearly observed that the hypoglycemic effects were in an insulin dose related manner (Fig. 5). Notably, significant relationships were shown between the insulin dose and $AAC_{glucose}$ after the administration of the insulin emulsion containing EPA and DHA.

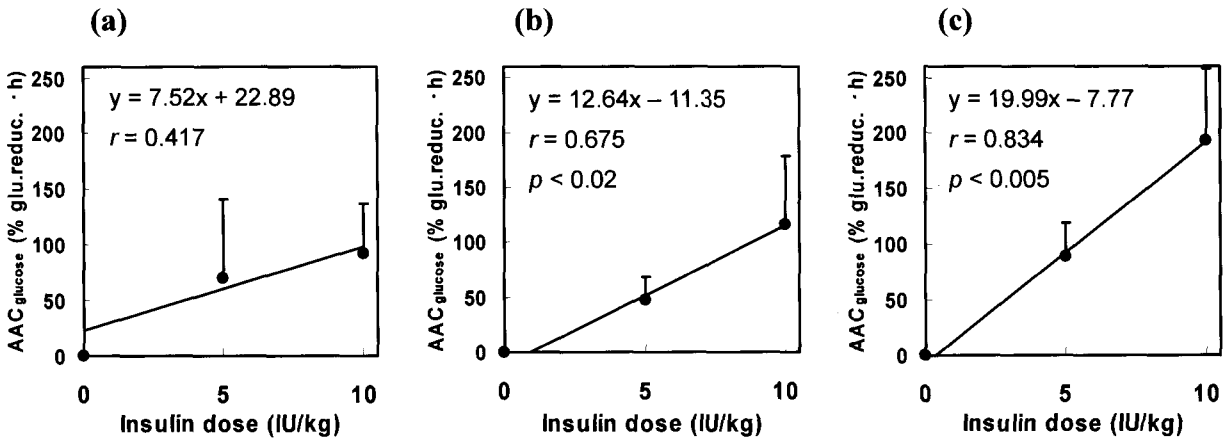


Figure 5. Relationship between insulin dose and $AAC_{glucose}$ following the rectal administration of insulin emulsion containing 2 % (a) OA, (b) EPA and (c) DHA. Each value represents the mean ± S.D. of five to seven rats.

The values for $AAC_{glucose}$ and the pharmacological availability are summarized in Table 1. The values of pharmacological availability induced by the emulsion containing DHA and EPA were greater than that of the emulsion containing OA. These results coincided with those obtained from the previous *in situ* studies [24,26].

Table 1. Pharmacological availability of insulin emulsion incorporating unsaturated fatty acids

| | $AAC_{glucose}$ (% glu. reduc.· h) | Pharmacological availability (%) |
|---|---|---|
| OA | 92.3 ± 44.4 | 7.7 ± 3.3 |
| EPA | 116.6 ± 61.6 | 11.0 ± 7.0 |
| DHA | 193.7 ± 63.8 | 25.4 ± 17.0 |

Insulin emulsions were administered rectally at 10 IU/kg of insulin.
Each value represents the mean ± S.D. of five to seven rats.

Further, the dose-dependent effect of DHA was evaluated by assessing the values of $AUC_{insulin}$ and $AAC_{glucose}$ after the rectal administration of insulin emulsions containing various concentrations of DHA. The insulin dose was fixed at 5 IU/kg. These values increased with the increase in the DHA concentration in the range of up to 1 %, and they reached saturation point at over 1 % (Fig. 6). Considering the previous findings that 18-carbon unsaturated fatty acids did not promote the insulin absorption from the intestine at 1 % [26], DHA will be expected to enhance insulin absorption at smaller amounts than 18-carbon unsaturated fatty acids.

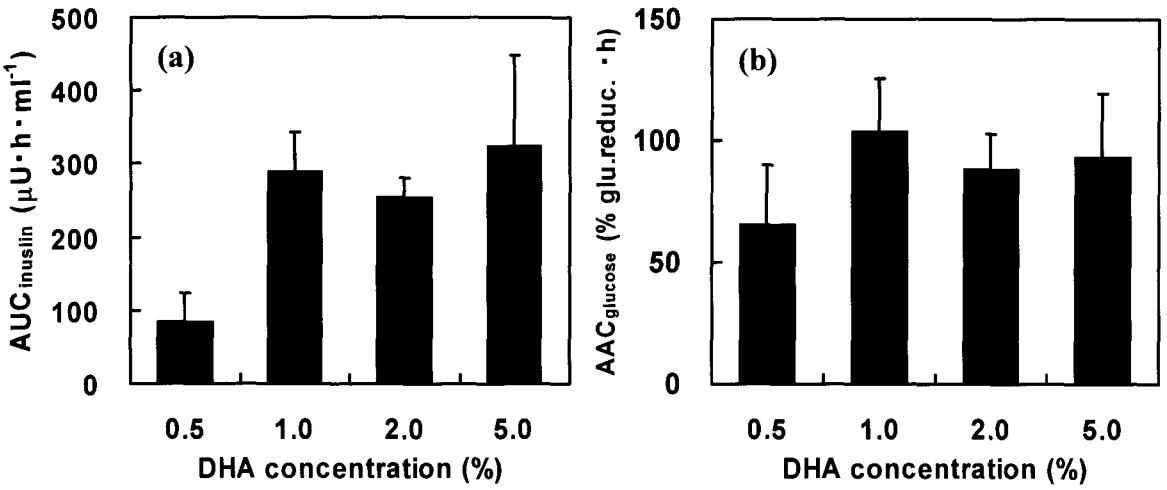


Figure 6. Comparison of (a) $AUC_{insulin}$ and (b) $AAC_{glucose}$ following the rectal administration of insulin emulsion containing various concentrations of DHA (5 IU/kg). Each value represents the mean ± S.D. of three to four rats.

As well as free fatty acids, it was reported previously that monoglyceride has an enhancement effect on intestinal absorption of several drugs [38,42]. Sekine *et al.* reported that the rectal absorption of cefmetazole sodium was promoted by the co-administration of medium chain fatty acid monoglycerides [42]. Based on these findings, the enhancement effects of glycerides of DHA were evaluated. Figure 7 shows the hypoglycemic effects following the *in situ* rectal administration of the emulsion containing glycerides of DHA, and Table 2 lists the values of $AAC_{glucose}$ and pharmacological availability. The administration of the emulsion containing DHA-TG (20 IU/kg) did not induce any hypoglycemic effect. Small decreases in the serum glucose levels following the administration of the insulin emulsion containing DHA-MG (20 IU/kg) were observed, and the lowest glucose level was approximately 90 % of the initial level. Meanwhile, a marked hypoglycemic effect was shown after the administration of the insulin emulsion containing DHA, despite a lower insulin dose (10 IU/kg).

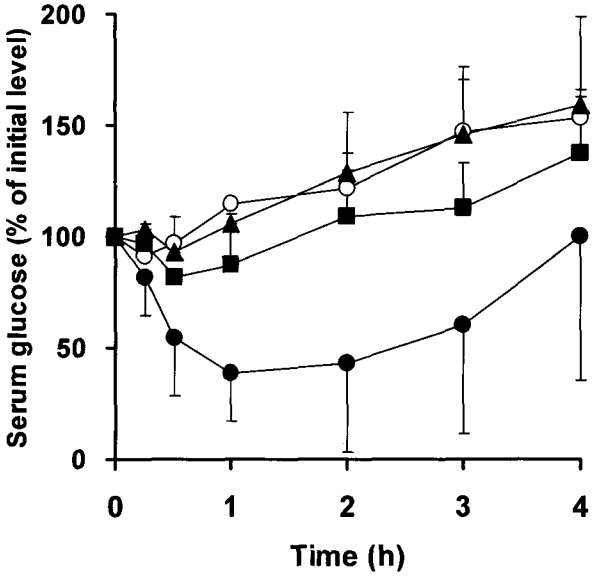


Figure 7. Change in serum glucose level following the *in situ* rectal administration of insulin emulsion containing 2 % glycerides of DHA. Each value represents the mean ± S.D. of five to nine rats.
Key: (○) 50 IU/kg of insulin solution (control); insulin emulsion containing (▲) DHA-TG (20 IU/kg of insulin); (■) DHA-MG (20 IU/kg of insulin); (●) DHA (10 IU/kg of insulin).

Table 2. Pharmacological availability of insulin emulsion containing glycerides of DHA

| Fatty acid | Insulin dose (IU/kg) | $AAC_{glucose}$ (% glu. reduc.· h) | Pharmacological availability (%) |
|---|---|---|---|
| DHA | 10 | 247.4 ± 254.6 | 29.0 ± 29.9 |
| DHA-MG | 20 | 37.4 ± 16.4 | 1.6 ± 0.7[a] |
| DHA-TG | 20 | 10.0 ± 1.7 | 1.2 ± 0.2[a] |

Each value represents the mean ± S.D. of five to nine rats.
[a] $p < 0.05$, vs DHA.

According to the results, the insulin absorption enhancement efficacies among various forms of DHA were in the order of DHA-TG ≤ DHA-MG << DHA. In general, dietary fats (> 90 %) exist as triglycerides in nature, and they are absorbed after digestion by pancreatic lipase. The pancreatic lipase system attacks the triglyceride with a high degree of positional specificity. Lipolysis occurs predominantly at the sn-1 and sn-3 positions, yielding two free

fatty acids and a 2-monoglyceride [43]. PUFAs and their 2-monoglyceride form are known to be easily absorbed from the intestinal membrane [43]. Taken together, it is likely that the absorbability of fatty acids is related to their enhancement effects on insulin absorption.

### 3.2. Comparison of the enhancement effect of DHA on insulin absorption with those of medium chain fatty acids

Capric acid and lauric acid are medium chain fatty acids having 10 and 12 carbon alkyl chains, respectively. Several studies have demonstrated that these fatty acids regulate the permeability of Caco-2 monolayers and the intestinal epithelium, and enhance the absorption of hydrophilic markers, such as sodium fluorescein [40,41]. This study compared the enhancement effects of long chain unsaturated fatty acids on the intestinal insulin absorption with those of medium chain fatty acids. Figure 8 shows the changes in the serum glucose level following the *in situ* administration of the emulsions (10 IU/kg) containing various fatty acids into the rectal loop. Insulin PBS solution (50 IU/kg) was used as a control. As shown in Fig. 8, all the emulsions containing fatty acids induced marked hypoglycemic effects, with the lowest glucose levels appearing 1-2 h after the administration. The hypoglycemic effects induced by the emulsions containing EPA and DHA were pronounced (Figs. 8 d and e). Table 3 shows the very high pharmacological availability of the insulin emulsion containing DHA, and the values were significantly higher than those obtained from the emulsion containing medium chain fatty acids.

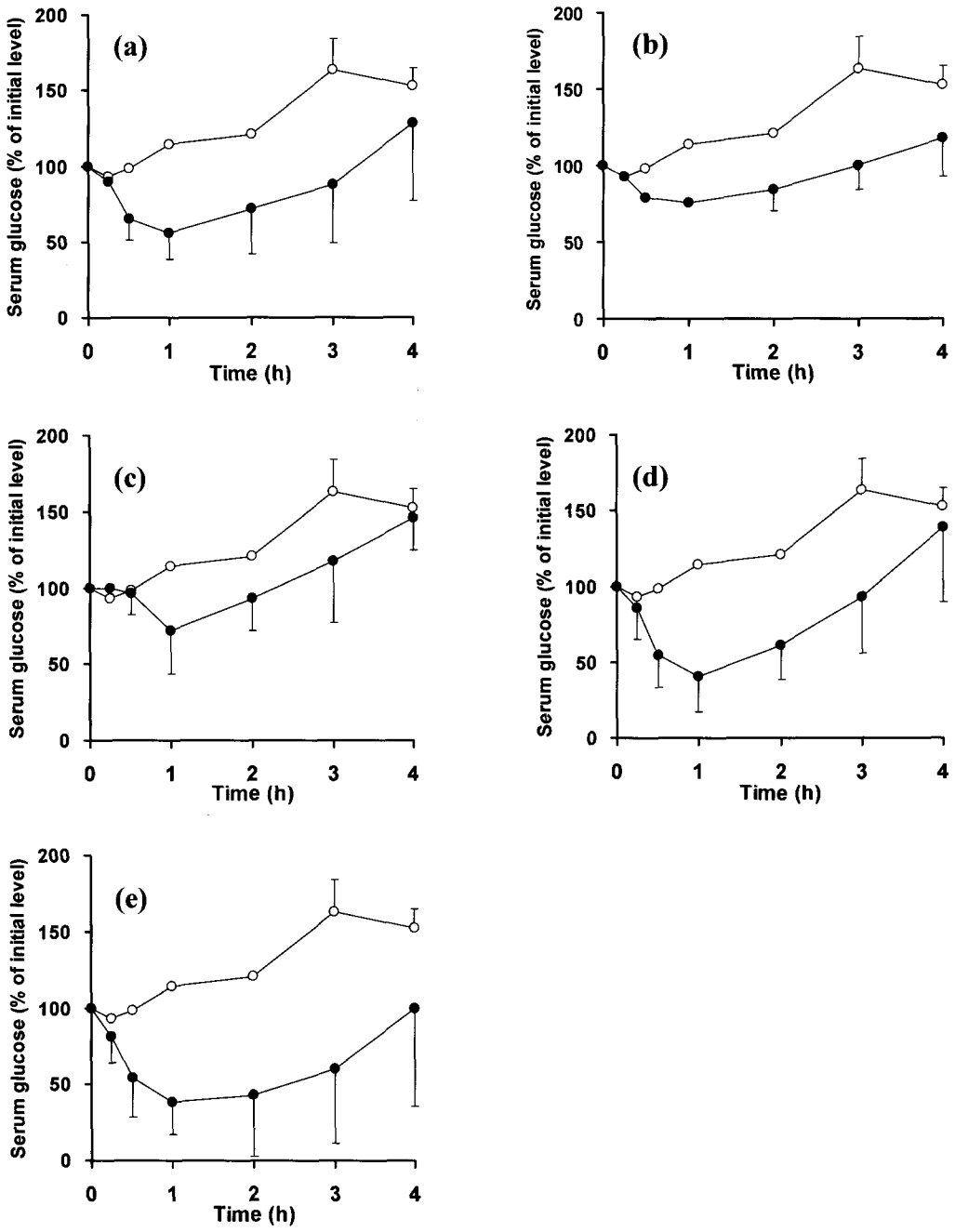


Figure 8. Change in serum glucose level following the *in situ* rectal administration of insulin emulsion containing 2 % (a) capric acid, (b) lauric acid, (c) OA, (d) EPA and (e) DHA. Each value represents the mean ± S.D. of five to nine rats.

Key: (○) 50 IU/kg of insulin solution (control); (●) 10 IU/kg of insulin emulsion, respectively.

Table 3. Pharmacological availability of insulin emulsion containing various fatty acids

| Fatty acid | $AAC_{glucose}$ (% glu. reduc.· h) | Pharmacological availability (%) |
|---|---|---|
| Capric acid | 68.2 ± 79.0 | 4.4 ± 5.1[a] |
| Lauric acid | 50.8 ± 25.3 | 3.7 ± 1.8[a] |
| OA | 56.3 ± 15.6 | 3.9 ± 1.1[a] |
| EPA | 153.6 ± 133.9 | 10.8 ± 9.5 |
| DHA | 247.4 ± 254.6 | 29.0 ± 29.9 |

Insulin emulsions were administered into rectal loop at 10 IU/kg of insulin.
Each value represents the mean ± S.D. of four to nine rats. [a]$p< 0.05$, vs DHA.

### 3.3. Site-dependent hypoglycemic effect of the emulsion containing DHA

There are many reports that absorption enhancers act more strongly in the large intestine than in the small intestine [39,44,45]. Previous studies in our laboratory also documented that insulin absorption from emulsions containing 18-carbon unsaturated fatty acids occurred site dependently, and more markedly in the lower parts of the gastrointestinal tract, such as the colon and rectum [24,25]. This study evaluated the site-dependent enhancement effect of DHA on intestinal insulin absorption. Figure 9 shows the changes in the serum glucose level-time profiles following the *in situ* administration of the insulin emulsions (50 IU/kg) containing DHA into the ileum, the colon and the rectum. The enhancement effect of DHA on insulin absorption was shown in all loops, and the effects in the colon and rectum were stronger than in the ileum. The effect of DHA was similar to those of 18-carbon unsaturated fatty acids. These site-dependent effect is probably related to differences in the biological functions of the intestinal tract and physical properties of the mucosal membrane, such as the composition of lipids and membrane fluidity [18,38,46,47]. In addition, it is known that the activity of the proteases in the large intestine is lower than that in the small intestine [37,48], and this contributes to the site-dependent effect to a certain extent. As the results of this

study, it was confirmed that the large intestine was a suitable site for insulin delivery using this carrier system.

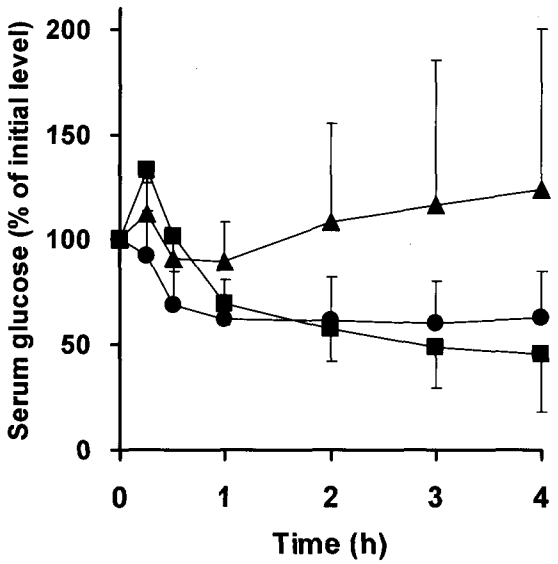


Figure 9. Change in serum glucose level following the *in situ* administration of insulin emulsion into various intestinal loops. Insulin emulsion containing 2 % DHA was administered at 50 IU/kg of insulin. Each value represents the mean ± S.D. of four to seven rats.
Key: (▲) intra-ileal; (■) -colonic; (●) -rectal administration of the emulsion.

### 3.4. Reversibility of the absorption modifying effect of the emulsions

The changes in the $AAC_{glucose}$ values following pretreatment with insulin free emulsions containing capric acid and DHA, and glycocholic acid solution are shown in Table 4. Glycocholic acid, the major bile acid in humans, was used as a positive control. There are a number of reports that bile acids and bile salts increase intestinal drug absorption by altering the functions of the mucosal membrane [49-52]. As shown in Table 4, in the case of glycocholic acid, there was no significant reduction in the $AAC_{glucose}$ values between the 0.5 h pretreatment group and the co-administration group, with the enhancement effect on insulin

absorption remaining. Since it is well known that the application of bile acids induces mucosal damages [53-56], the continuance of the enhancement effect was probably due to the mucosal damage caused by treatment with glycocholic acid. In addition, when the pretreatment with glycocholic acid solution was prolonged to 3.0 h, the value of $AAC_{glucose}$ markedly decreased. The reduction of the enhancement effect may be attributed to running out of the administered glycocholic acid. Swenson *et al.* reported the intestinal wall damage caused by bile salt treatment recovered to the control level within 3 h of the cessation [56]. On the other hand, a marked reduction in the values of $AAC_{glucose}$ was observed by the pretreatment with the emulsion containing DHA and capric acid for 0.5 h, and their enhancement effects were diminished rapidly. Therefore, it was suggested that the action of DHA on the intestinal membrane occurred temporarily and was highly reversible, and the co-administration with insulin and DHA is necessary for improvement of intestinal insulin absorption.

Table 4. Effect of the emulsion pretreatment on rectal insulin absorption

| Sample | Pretreatment period (h) | Percent change in $AAC_{glucose}$ value |
|---|---|---|
| W/O/W multiple emulsion containing capric acid | - | 100.0 ± 43.4 |
| | 0.5 | 22.1 ± 24.3 |
| | 3.0 | 31.8 ± 27.3 |
| W/O/W multiple emulsion containing DHA | - | 100.0 ± 10.0 |
| | 0.5 | 20.5 ± 2.5 |
| | 3.0 | 16.8 ± 1.4 |
| Glycocholic acid solution | - | 100.0 ± 20.7 |
| | 0.5 | 73.2 ± 44.3 |
| | 3.0 | 17.2 ± 15.1 |

Each value represents the mean ± S.D.

### 3.5. Hypoglycemic effect and toxicity induced by the multiple administrations of insulin emulsion

Multiple rectal administrations of insulin emulsions containing various fatty acids for 10 days were performed, and the hypoglycemic effects were examined. Figure 10 shows the time profiles of the serum glucose level following the administration of insulin emulsions containing OA and DHA on the first or tenth day. Insulin emulsion without unsaturated fatty acids was used as a control in this study. As for the emulsion containing DHA, a marked hypoglycemic effect was observed after the multiple applications, and the $AAC_{glucose}$ values on the first and the tenth day were almost the same. Although the $AAC_{glucose}$ values did not change during multiple administrations, the minimal glucose level tended to decrease. Similar trends were observed for the emulsion containing OA.

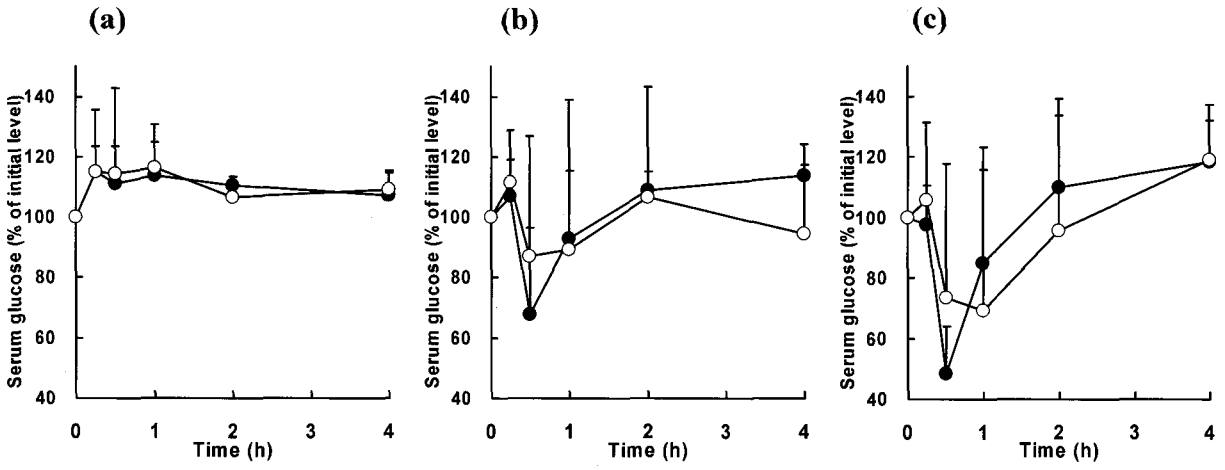


Figure 10. Time profiles of serum glucose level following 10 days multiple administrations of insulin emulsion (a) without fatty acid (control) and containing 2 % (b) OA, or (c) DHA. Insulin emulsions were administered rectally at 5 IU/kg of insulin. Each value represents the mean ± S.D. of five rats.
Key: (●) First day, and (○) tenth day of multiple applications.

Moreover, the rectal mucosal damage following the multiple applications of insulin emulsions was investigated. Light micrographs of the rectal mucosa following the multiple applications of the emulsions are shown in Fig. 11, and tissue damage scores based on the histopathological findings are summarized in Table 5. Multiple applications of insulin emulsions containing DHA and OA induced no or very slight mucosal damage in the rectum. Since these fatty acids are contained in natural sources and are usually obtained through diet, their applications would not be accompanied by mucosal damage. Additionally, Horie *et al.* reported that PUFAs, such as DHA, protected the small intestine from the mucosal damage caused by the oral administration of the antitumor drug, methotrexate [57]. Therefore, these fatty acids can be safely used as absorption enhancers.

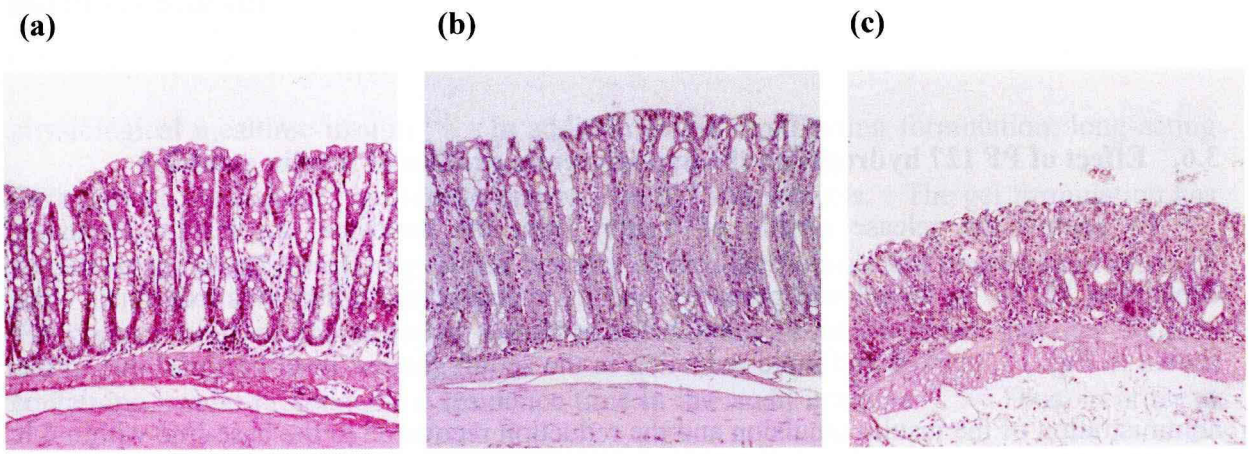


Figure 11. Light micrographs (H & E stain, ×100) of rat rectal mucosa following 10 days multiple applications of insulin emulsion (a) without fatty acid and containing (b) OA, and (c) DHA.

Table 5. Microscopical findings of the rectum following 10 days multiple applications of insulin emulsion containing fatty acids

| Microscopic findings | Fatty acid | | |
|---|---|---|---|
| | Control[a] | OA | DHA |
| *Mucosa* | | | |
| Degeneration | 0 | 0 | 1 |
| *Lamina propria mucosa* | | | |
| Cellular infiltration | 0 | 0 | 1 |
| Fibrosis | 0 | 0 | 0 |
| *Submucosa* | | | |
| Edema | 0 | 0 | 0 |
| Cellular infiltration | 0 | 0 | 0 |

Score: 0, no change; 1, very slight; 2, slight; 3, moderate; 4, marked.
Insulin emulsions were administered rectally at 5 IU/kg of insulin.
[a]W/O/W multiple emulsion without fatty acids

### 3.6. Effect of PF 127 hydrogel on the hypoglycemic profiles of insulin emulsion

To control the release profile of insulin from the emulsion, an insulin emulsion containing PF 127 hydrogel was prepared, and its hypoglycemic effect was examined. As shown in Fig. 12, marked and rapid reduction in the serum glucose level occurred due to the administration of the insulin emulsion and the reduction recovered to the base-line within 4 h after the administration. In contrast, a gradual decrease in the serum glucose level was observed following the administration of insulin emulsion containing PF 127 hydrogel, and was maintained up to 6 h after the administration. The nadir of the serum glucose-time curve was broadened.

The time profile of the glucose reduction provided by the insulin emulsion was similar to that of the only insulin monomeric analogue, insulin Lispro [58]. It was reported that the pharmacokinetic and pharmacodynamic profiles of insulin Lispro closely mimic those of

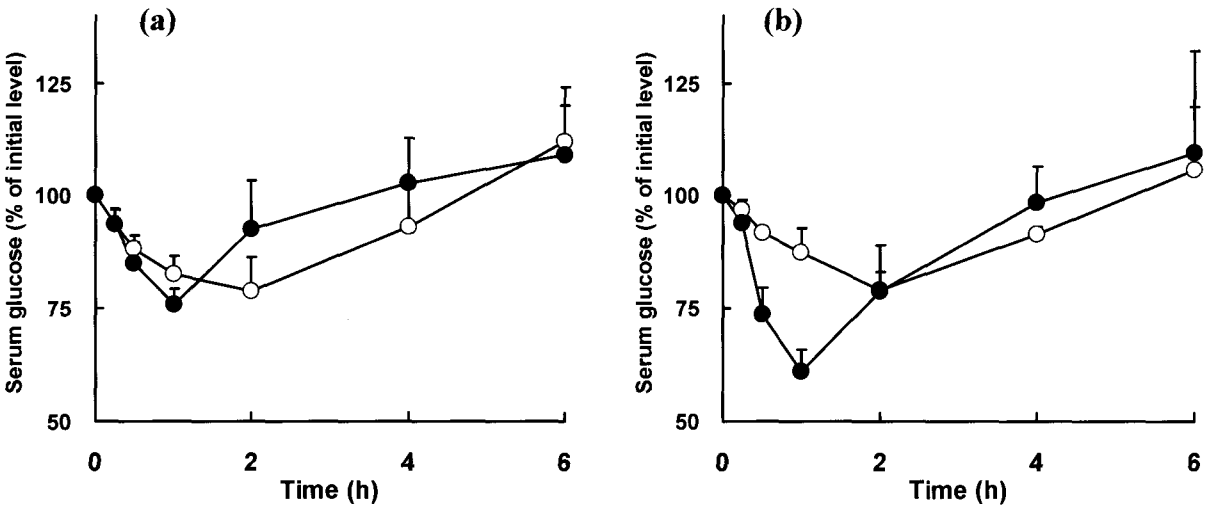


Figure 12. Effect of Pluronic F127 hydrogel contained in the emulsion on time profiles of the serum glucose level. The formulation was administered rectally at (a) 5 or (b) 20 IU/kg of insulin. Each value represents the mean ± S.D. of four to six rats.
Key: (●) Insulin emulsion containing 2 % DHA, (○) insulin emulsion containing 2 % DHA and PF127 hydrogel.

physiological mealtime insulin [2]. In addition to the rapid-acting formulation, long-acting formulations are required in order to generate basal insulin levels. The gel formulation has the advantage of sustained drug release because of its adhesiveness and prolongation effect of the contact time to the mucosal membrane. Kimura *et al.* reported that gel spheres containing insulin prolonged the residence time in the small intestine [59]. Thus, in order to control the insulin release, a gel formulation that combined an insulin emulsion with a PF 127 hydrogel was prepared. PF127 is a block copolymer composed of poly(ethylene oxide) and poly(propylene oxide), and can form micelles in water. Moderately concentrated solution of PF127 in water exists as liquid at refrigerator temperatures and hydrogel at physiological temperatures. PF127 hydrogel has generated considerable interest for drug delivery applications because it enables the rapid conversion of a solution into a hydrogel after administration into the body cavity. Barichello *et al.* evaluated PF127 hydrogel containing

insulin and concluded that these formulations were useful for the preparation of a controlled delivery system [60]. Figure 12 clearly shows that a prolonged hypoglycemic effect was induced by an insulin emulsion containing PF 127 hydrogel. These results suggested that insulin emulsion possesses the capacity for varying the drug release by using the PF 127 hydrogel.

## 4. Conclusions

To investigate the possibility of PUFAs as absorption enhancers for intestinal insulin delivery, the effectiveness and the toxicity were evaluated with an *in vivo* and *in situ* study. Marked insulin absorption and a hypoglycemic effect were shown within a very short time after the rectal administration of insulin emulsion containing unsaturated fatty acids. The hypoglycemic effects were in an insulin dose related manner. The enhancement effects of PUFAs, in particular DHA, were much more pronounced than that of oleic acid, and reached saturation point at 1 % of the total amount of emulsion. The effect of DHA was outstanding compared to that of its glyceride forms or medium chain fatty acids. In addition, the effect of DHA was site-dependent, and the action of DHA in the large intestine, such as the colon and rectum, was stronger than in the small intestine, such as the ileum. Considering the clinical use of insulin preparations, a multiple administration study was conducted. During the multiple rectal applications of the insulin emulsion to rats for 10 days, the hypoglycemic effects were successfully obtained, and no or very slight mucosal damage was observed in the excised rectum. Moreover, to manipulate the glucose reduction profile, a gel formulation that combined the insulin emulsion with PF127 hydrogel was prepared, and the hypoglycemic effect was evaluated. As expected, the sustained hypoglycemic effect was shown by the administration of this gel formulation.

# CHAPTER 2

## Structural change in lipid bilayer induced by the treatment with fatty acids

## 1. Introduction

In Chapter 1, it was confirmed that PUFAs, particularly DHA, had strong enhancement effects on intestinal insulin absorption. These effects occurred within very short time after administration, and the action was reversible without tissue damage. Although the mechanism responsible remains unclear, the previous study in our laboratory suggested that the effect of PUFAs was attributed to their actions on the transcellular pathway rather than the paracellular pathway because the tissue membrane resistance values did not change with treatment [25]. When an emulsion is administered into the intestinal lumen, the fatty acids contained in the emulsion will be taken up readily into the lipid bilayer of the intestinal mucosa, causing a change in the lipid packing. In general, the structural change in the lipid bilayer is a critical factor for the transcellular pathway. There are many reports that perturbation of the lipid-packing order increases drug permeability [27,29,61]. In addition, recent studies have shown that altering membrane fluidity influences carrier-mediated transport, such as P-glycoprotein-mediated efflux [62-64]. Because DHA and EPA have multiple double bonds, they should change the lipid packing drastically. Such changes in the lipid bilayer structure should cause modifications of the biological membrane function, and it is important to identify the action of fatty acids on the lipid bilayer structure.

This Chapter investigated changes in the physical properties of the lipid bilayer by treating model bilayer with various fatty acids, such as SA, OA, EPA, and DHA. Because the plasma membrane structure is too complex, DPPC liposomes were used as a model of the lipid bilayer. The structural changes in the lipid bilayer induced by fatty acids were assessed by measuring changes in the phase transition temperature, fluorescence anisotropy, and detergent insolubility. To evaluate whether fatty acids incorporated in the formulation act successfully on the lipid bilayer, the same experiments were also performed using a W/O/W multiple emulsion containing fatty acids.

## 2. Materials and Methods

### 2.1. Materials

Gelatin, triolein, Span 80, DL-α-tocopherol, OA, Tween 80, egg yolk phospholipids (phosphatidylcholine and phosphatidylethanolamine), DHA and EPA were the same as those described in Chapter 1. DPPC, SA, polyoxyethylene (10) octylphenyl ether (Triton X-100), phospholipid C-Test kit, and nonesterified fatty acid (NEFA) C-Test kit were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of analytical grade and commercially available.

### 2.2. Preparation of DPPC liposomes

DPPC dissolved in chloroform was pipetted into a flask, and the chloroform removed by evaporation at room temperature under a nitrogen stream. This procedure resulted in the formation of a thin lipid film on the inside wall of the flask. The film was stored overnight in a vacuum desiccator to ensure the complete evaporation of chloroform. Ten milliliters of PBS was added to the flask and the DPPC was hydrated for 30 min. The suspension was sonicated for 10 min at about 60 °C using a bath-type sonicator. The liposome suspension was prepared at a total lipid concentration of 10 mM and stored at room temperature until used in the experiments.

### 2.3. Differential scanning calorimeter measurement

Fatty acid suspensions and W/O/W multiple emulsions containing fatty acids were used as the treatment solutions. Fatty acid suspensions were prepared by suspending designated amounts of fatty acids, such as SA, OA, EPA, and DHA, in a mixture of methanol and PBS (1:1, v/v). W/O/W multiple emulsions were prepared according to the method described in

Chapter 1. The concentration of fatty acid was fixed at 2 % of total weight of emulsion. The weight ratio of each phase was, inner aqueous phase: oily phase: outer aqueous phase, 1:4:15.

Fatty acids were applied to the DPPC bilayer as described by Maitani *et al* [65]. Two hundred and fifty microliters of treatment solution was added to 1000 μL of DPPC liposome suspensions and this mixture was incubated for 2 h at 37 °C. Samples were centrifuged at 13,400×g for 2 min to separate the supernatant and pellet. The obtained pellets of known weight (approximately 5 mg) were placed in aluminum pans for the differential scanning calorimeter (DSC) measurement. DSC measurements were performed with a Thermo Plus DSC 8230 (Rigaku Co. Ltd., Tokyo, Japan). The scan rate was set at 1 °C/min. The phase transition temperature was determined as the peak temperature.

### 2.4. Fluorescence anisotropy measurement

The DPPC bilayer was labeled with DPH by adding 10 μL of 10 mM freshly prepared DPH stock solution in tetrahydrofuran to 1000 μL of liposome suspension and then incubating at 37 °C for 2 h in the dark to complete the labeling. The treatment with fatty acids in DPH-labeled liposomes was performed as described above. The pellets were collected by centrifugation at 13,400×g for 2 min. The obtained pellets were resuspended in PBS and the absorbance at 400 nm was set <0.50 to permit the measurement of fluorescence. The fluorescence anisotropy of DPH in the DPPC bilayer was measured with a fluorescence spectrophotometer (Hitachi F-450, Hitachi Co. Ltd., Tokyo, Japan) at an excitation wavelength of 351 nm and an emission wavelength of 430 nm. The steady-state fluorescent anisotropy was calculated using the following equation:

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$

where $r$ is anisotropy, and $I_{VV}$ and $I_{VH}$ are the intensity measured in directions parallel and

perpendicular to the polarized exciting light, respectively.

### 2.5. Detergent resistance studies

After application of fatty acids to the DPPC liposome suspension, 750 μL of 10 % Triton X-100 solution was added to 1250 μL of a DPPC liposome suspension treated with fatty acid, and the sample was incubated at 25 °C for 2 h. The sample was centrifuged at 13,400×g for 2 min to separate the supernatant and pellet. The supernatant was removed, and the pellets were resuspended in an equal volume of fresh PBS. The sample was further diluted 100 times with PBS and the optical density (OD) at 400 nm was measured using a spectrophotometer (U-best 30, Japan Spectroscopic Co., Ltd., Tokyo, Japan). The OD at 400 nm of a freshly prepared DPPC liposome suspension was also measured and this value was used as the initial level. Detergent insolubility was calculated as the OD after the addition of Triton X-100 divided by the initial level.

### 2.6. Determination of the composition of fatty acids in DPPC bilayer treated with fatty acids

Application of unsaturated fatty acids to DPPC liposomes was performed using fatty acid suspensions. The treatment amount of each fatty acid was fixed at 30 mol% relative to the amount of DPPC. The pellets were collected by centrifugation at 13,400×g for 2 min and the pellets were resuspended in an equal volume of fresh PBS. DPPC and fatty acid concentration were determined using a phospholipid C-Test kit and a NEFA C-Test kit, and the composition of fatty acids in DPPC bilayer was calculated.

## 3. Results and Discussion

### 3.1. Changes in the physical properties of the DPPC bilayer induced by treatment with fatty acids

Figure 13 shows the DSC curves of the DPPC bilayer treated with various fatty acids. For the DPPC bilayer treated with the mixture of PBS and methanol (control), the sharp endothermic peak due to the phase transition from the gel phase to the liquid-crystalline phase was observed at 40.8 °C. This temperature is lower than the phase transition temperature of pure DPPC reported by other researchers [61,66], and the reduction in phase transition temperature occurred probably because of the effect of methanol in the treatment solution. Tran *et al.* reported a similar effect induced by ethanol [67]. In addition, it had been already confirmed in a preliminary study that DPPC liposomes without any treatment (blank) had a phase transition at about 42 °C (data not shown). Treatment with unsaturated fatty acids, such as OA, EPA, and DHA, shifted the phase transition temperature to a lower value and broadened the sharp endothermic peak (Fig. 13). The phase transition temperatures of the DPPC bilayer treated with 30 mol% of fatty acids were 41.3 °C for SA, 37.5 °C for OA, 36.3 °C for EPA, and 36.2 °C for DHA.

Figure 14 shows the effects of fatty acids (30 mol%) on the fluorescence anisotropy of DPH in the DPPC bilayer. Treatment with unsaturated fatty acids shifted the phase transition of the DPPC bilayer to a lower temperature and broadened the phase transitions compared with those of the control. These results were consistent with those obtained from the DSC study (Fig. 13). Treatment with unsaturated fatty acids markedly decreased the values of fluorescence anisotropy of DPH in the gel phase.

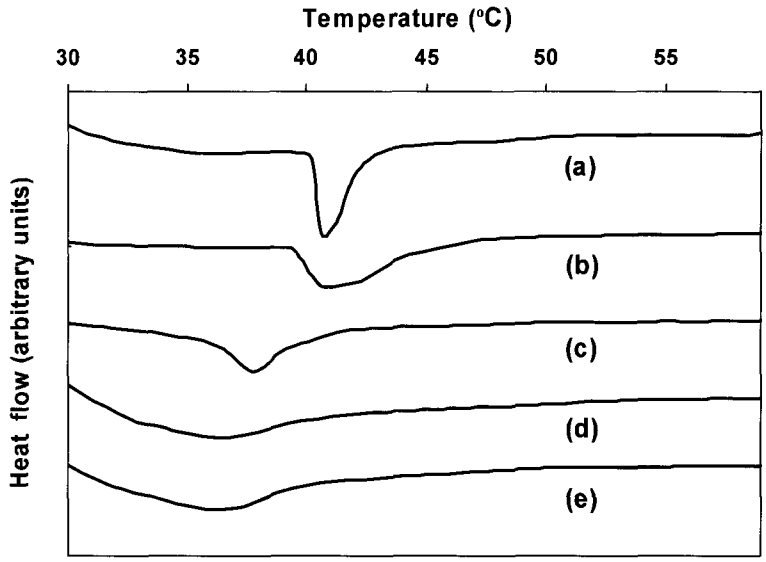


Figure 13. Differential scanning calorimetry thermograms of the DPPC bilayer after treatment with fatty acids. Thirty mol percent of fatty acid relative to the amount of DPPC was applied to the DPPC bilayer in the form of a suspension. The temperature was scanned at 1 °C/min. (a) control, (b) SA, (c) OA, (d) EPA, and (e) DHA.

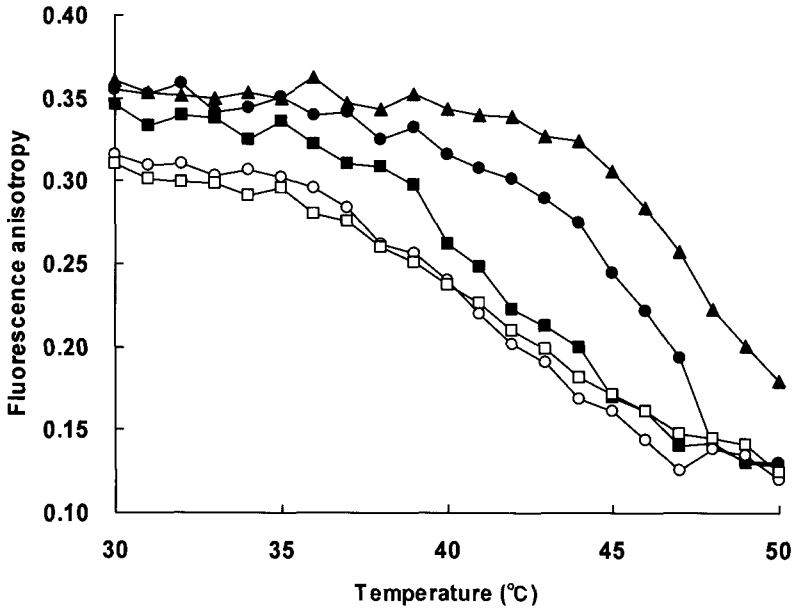


Figure 14. Fluorescence anisotropy of DPH in the DPPC bilayer after treatment with fatty acids. Thirty mol percent of fatty acids relative to the amount of DPPC was applied to the DPPC bilayer in the form of a suspension. The temperature was scanned at 1 °C/min. Key: (●) control, (▲) SA, (■) OA, (○) EPA, and (□) DHA.

Furthermore, this study was investigated the effects of the amount of fatty acids used in the treatment on the phase transition temperature and fluorescence anisotropy of DPH. Increasing the molar fraction of unsaturated fatty acids induced a notable progressive decrease in the phase transition temperatures and the values of fluorescence anisotropy of DPH (Figs. 15 and 16). The modifications induced by DHA and EPA were most pronounced. These changes are caused by the interaction between fatty acids and the DPPC bilayer. DPPC, which has two 16-carbon saturated chains, forms a substantially more tightly packed lipid bilayer. Once applied to the DPPC bilayer, unsaturated fatty acids are taken up into the lipid bilayer in a dose-dependent manner, reducing the van der Waals interactions between the phospholipid hydrocarbon chains because of their kinked structure [29]. The decreases in the phase transition temperature and the value of fluorescence anisotropy reflect this mechanism. Conversely, SA treatment caused a slight increase in the phase transition temperature and the values of fluorescence anisotropy of DPH (Figs. 15 and 16).

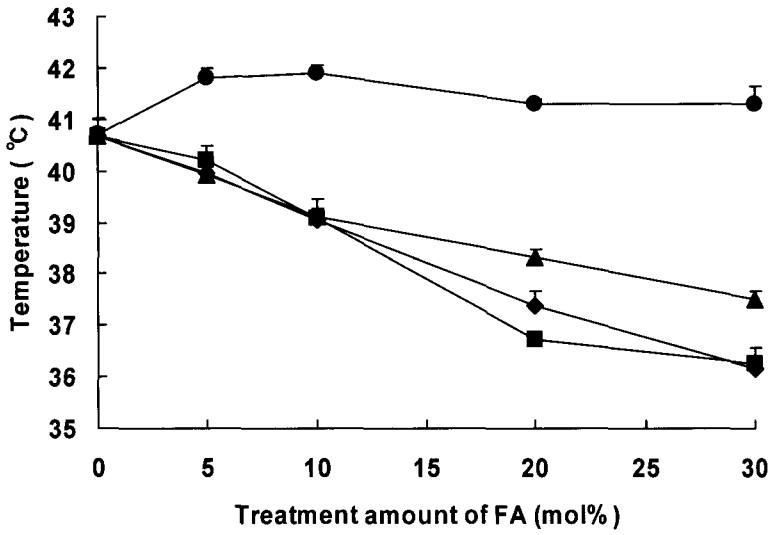


Figure 15. Effect of the treatment amount of fatty acids on the phase transition temperature of the DPPC bilayer. An adequate amount of fatty acids was applied to the DPPC bilayer in the form of a suspension. Each value represents the mean ± S.D. of three experiments. Key: (●) SA, (▲) OA, (■) EPA, and (♦) DHA.

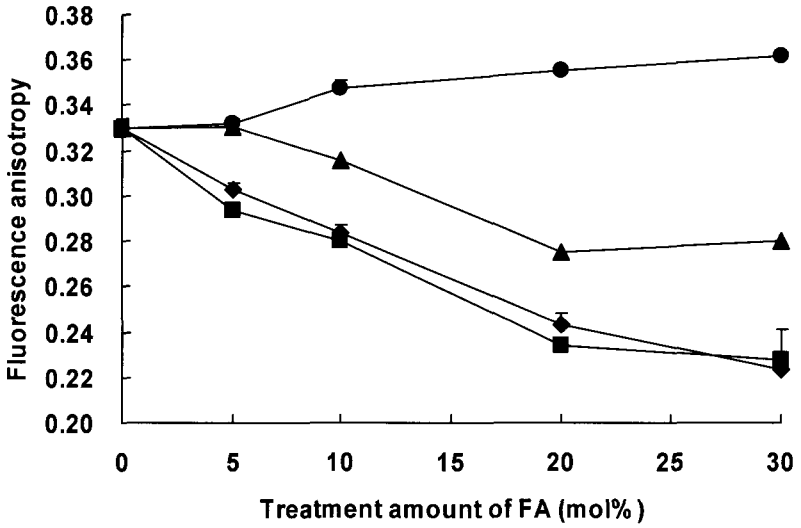


Figure 16. Effect of the treatment amount of fatty acids on fluorescence anisotropy of DPH in the DPPC bilayer at 37 °C. An adequate amount of fatty acids was applied to the DPPC bilayer in the form of a suspension. Each value represents the mean ± S.D. of three experiments. Key: (●) SA, (▲) OA, (■) EPA, and (♦) DHA.

Because SA is a saturated fatty acid composed of a longer hydrocarbon chain than DPPC, the packing of the DPPC bilayer might be tighter after treatment with SA. The detergent insolubility of the DPPC bilayer treated with fatty acids was also assessed. This method is based on the observation that ordered lipid domains tend to resist solubilization by nonionic detergents, such as Triton X-100, whereas disordered fluid domains dissolve in these detergents. The insoluble membrane fraction can be regarded as tightly packed domains that were present in the sample before the addition of detergent. London *et al.* used detergent insolubility as an index of the formation of ordered lipid domains (lipid raft) in a model lipid bilayer [68,69]. It was found that fatty acid treatment influenced the distribution of the ordered lipid domain in the membrane. Table 6 shows the detergent insolubility of the DPPC bilayer treated with 30 mol% of fatty acids. Considerable amounts of insoluble membrane fractions were obtained from the control and SA treatment conditions (Table 6), indicating that these membranes were composed of a large amount of ordered and rigid

regions. In contrast, the DPPC bilayer treated with unsaturated fatty acids was nearly completely dissolved in Triton X-100. The lowest level of detergent insolubility was observed with DHA and EPA treatment, suggesting that the tightly packed membrane of the DPPC bilayer was altered in the disordered fluid membrane by treatment with unsaturated fatty acids, particularly DHA and EPA.

Table 6. Detergent insolubility of the DPPC bilayer after treatment with fatty acids

| | Detergent insolubility $(OD_{400nm}+TX\text{-}100)/(OD_{400nm}-TX\text{-}100)$ |
|---|---|
| Control[a] | 0.501 ± 0.027 |
| SA | 0.612 ± 0.016 |
| OA | 0.092 ± 0.006 |
| EPA | 0.030 ± 0.002 |
| DHA | 0.030 ± 0.004 |

[a]Mixture of PBS and methanol (1:1 v/v %)

Thirty mol percent of fatty acids relative to the amount of DPPC was applied to the DPPC bilayer in the form of a suspension. Each value represents the mean ± S.D. of three determinations.

TX-100, Triton X-100.

These results confirmed that the unsaturated fatty acids changed the lipid bilayer structure markedly. The modifications of the bilayer physical properties were more pronounced with DHA and EPA than with OA treatment. The order of potency of the modifications of the physical properties induced by unsaturated fatty acids agreed with the order of their absorption enhancement effect observed in Chapter 1. It is speculated that the potency of the modifications depends on the molecular structure and amount of fatty acids incorporated into the lipid bilayer. To examine the possible mechanism responsible for the differences in potency, the composition of fatty acids in the DPPC bilayer after incubation

with unsaturated fatty acids was determined. As shown in Table 7, each fatty acid comprised over 25 mol% of the bilayer lipid component, and the relative composition was nearly identical for all fatty acids tested. This finding indicates that the different actions of the various unsaturated fatty acids can be attributed mainly to changes in the bilayer molecular structure, such as a kinked conformation contributed by the double bond, and not to the amount of fatty acids incorporated into the lipid bilayer.

Table 7. Composition of fatty acids in the DPPC bilayer after treatment with unsaturated fatty acids

| | Fatty acid (mol%) | DPPC (mol%) |
|---|---|---|
| OA | 27.0 ± 0.3 | 73.0 ± 0.3 |
| EPA | 26.4 ± 0.4 | 73.6 ± 0.4 |
| DHA | 25.9 ± 0.2 | 74.1 ± 0.2 |

Thirty mol percent of fatty acids relative to the amount of DPPC was applied to the DPPC bilayer in the form of a suspension. Each value represents the mean ± S.D. of three determinations.

### 3.2. Effect of fatty acids contained in W/O/W multiple emulsions on membrane fluidity

Figure 17 shows the DSC curves of the DPPC bilayer treated with emulsions containing various fatty acids. A sharp endothermic peak was observed at about 42 °C, and a broad endothermic peak also obtained at below 40 °C in the DPPC bilayer treated with the emulsion without fatty acid (control). The sharp endothermic peak reflects the phase transition of pure DPPC domains and the broad endothermic peak reflects the disordered fluid domains that were altered by the treatment with emulsion in the membrane. The emulsion in itself induced a marked change in the lipid bilayer structure because the emulsion contains large amounts of unsaturated lipids, such as triolein and egg yolk phospholipids. The endothermic peak at about 42 °C decreased after treatment with the emulsion containing OA and disappeared completely after treatment with the emulsion containing DHA or EPA.

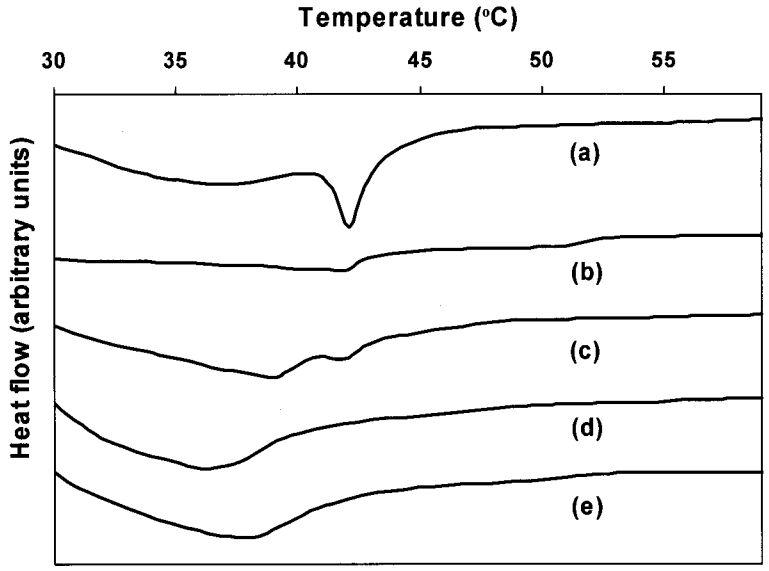


Figure 17. Differential scanning calorimetry thermograms of the DPPC bilayer after treatment with a W/O/W multiple emulsion. Emulsion containing 2 % fatty acid was applied to the DPPC bilayer. The temperature was scanned at 1 °C/min. (a) control, (b) SA, (c) OA, (d) EPA, and (e) DHA.

Table 8 lists the fluorescence anisotropy values of DPH in the bilayer treated with the emulsions at 37 °C. These values decreased markedly with treatment with emulsion containing unsaturated fatty acids. Table 9 summarizes the detergent insolubility values of the DPPC bilayer treated with emulsion. A large amount of DPPC was dissolved in Triton X-100 in all samples, which showed only very low levels of insolubility. These results demonstrate that unsaturated fatty acids act efficiently on the DPPC bilayer, even when included in emulsion form, which obviously changed the physical properties of the DPPC bilayer.

Table 8. Fluorescence anisotropy of DPH in the DPPC bilayer after treatment with W/O/W multiple emulsions containing fatty acids at 37 °C

| | Fluorescence anisotropy |
|---|---|
| Control[a] | 0.305 ± 0.007 |
| SA | 0.346 ± 0.002 |
| OA | 0.278 ± 0.005 |
| EPA | 0.208 ± 0.006 |
| DHA | 0.210 ± 0.005 |

[a]W/O/W multiple emulsion without fatty acids

Each value represents the mean ± S.D. of three determinations.

Table 9. Detergent insolubility of the DPPC bilayer after treatment with W/O/W multiple emulsions containing fatty acids

| | Detergent insolubility $(OD_{400nm}+TX\text{-}100)/(OD_{400nm}-TX\text{-}100)$ |
|---|---|
| Control[a] | 0.045 ± 0.002 |
| SA | 0.101 ± 0.030 |
| OA | 0.012 ± 0.005 |
| EPA | 0.007 ± 0.003 |
| DHA | 0.008 ± 0.008 |

[a]W/O/W multiple emulsion without fatty acids

Each value represents the mean ± S.D. of three determinations.

TX-100, Triton X-100.

## 4. Conclusions

The structural changes in a DPPC bilayer induced by treatment with various fatty acids were clarified by determining the phase transition temperature, fluorescence anisotropy of DPH, and detergent insolubility. Treatment with unsaturated fatty acid broadened and shifted the phase transitions of the DPPC bilayer to a lower temperature. The phase transition temperature and the value of fluorescence anisotropy of DPH at 37 °C decreased progressively with increasing treatment amounts of unsaturated fatty acid. A large amount of the DPPC bilayer treated with unsaturated fatty acid was dissolved in Triton X-100, yielding a low level of detergent insolubility. These modifications of the bilayer physical properties were most pronounced with DHA and EPA treatment. These results show that unsaturated fatty acids, particularly DHA and EPA, induce a marked change in the lipid bilayer structure. The composition of fatty acids in the DPPC bilayer was similar after treatment with various unsaturated fatty acids, suggesting that the different actions of unsaturated fatty acids are attributed to change in the molecular structure (e.g., kinked conformation by double bonds). This study further explored the change in physical properties induced by fatty acids dispersed in the W/O/W multiple emulsion and found that unsaturated fatty acids acted efficiently on the DPPC bilayer, even when incorporated in the emulsion form.

# CHAPTER 3

## Formulation optimization of W/O/W multiple emulsion as a carrier of insulin intestinal delivery according to a novel optimization technique

## 1. Introduction

Chapters 1 and 2 investigated the feasibility of unsaturated fatty acids as absorption enhancers for intestinal insulin absorption. It was clarified that unsaturated fatty acids, especially DHA, possessed the great activity for promoting insulin absorption, and it was related to the structural change in lipid bilayer. This chapter focuses on the formulation design of W/O/W multiple emulsion using a novel optimization technique. In general, pharmaceutical formulations are composed of many components and undergo manufacturing processes, and numerous factors are involved in generating their various characteristics. Since the relationships between the factors and characteristics are complex, undesirable formulations are sometimes obtained. Therefore, it is important to identify the relationships between the causal factors and individual characteristics, and to optimize these variables for the formulation's purpose. The W/O/W multiple emulsion is also a complex system, including many components and manufacturing processes. Although the absorption enhancer must play a principal role in the pharmacological effect, other causal factors may exist, because there are several reports that numerous factors influenced the pharmacological effect of the final formulation [70,71]. In addition to the pharmacological effect, many characteristics should be considered in the design of pharmaceutical formulations. The stability of the resulting formulation is an important characteristic, because its appearance and homogeneity should be preserved during long-term storage. Physical properties are also important characteristics because they may affect crucial characteristics, such as the pharmacological effect and stability, although their optimum values are not always required [72-74].

The objective of this study was to identify the relationships between the formulation factors and characteristics, and optimize the formulation of the emulsion for mucosal insulin delivery. Firstly, model formulations were prepared according to the L16 orthogonal

experimental design. OA was used as an absorption enhancer in this study. Response variables, such as the inner droplet size, viscosity, stability and hypoglycemic effect, were measured, and then the contributions of the causal factors and their interactions to response variables were clarified by means of ANOVA analysis. The orthogonal experimental design is known to be an efficient method to clarify the relationship of multivariate data [75-77]. Furthermore, the formulation optimization of W/O/W multiple emulsion was performed by means of a computer optimization technique incorporating a multivariate spline interpolation (MVS) [78,79].

## 2. Materials and Methods

### 2.1. Materials

Gelatin, triolein, Span 80, Tween 80, DL-α-tocopherol, egg yolk phospholipids (phosphatidylcholine and phosphatidylethanolamine) and OA were the same as those described in Chapter 1 and 2. Crystalline porcine insulin (27.3 IU/mg) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of analytical grade.

### 2.2. Preparation of W/O/W multiple emulsion

The emulsions were prepared according to the method described in previous Chapters. The L16 orthogonal experimental design for five factors and the two-factor spherical second-order composite experimental design were applied to prepare the model formulations. The preparation conditions of model formulations are listed in Tables 10 and 11. For the orthogonal experimental design, the gelatin concentration in inner aqueous phase, insulin amount in 2 g of inner aqueous phase, volume ratio of outer aqueous phase to the total, OA concentration in W/O/W multiple emulsion and agitation time of the second emulsification process were selected as the causal factors. For the composite spherical experimental design, the ratio of the outer aqueous phase to the total volume and agitation time of the second emulsification process were selected as the causal factors.

Table 10. Formulations for insulin emulsion based on orthogonal experimental design

| Formulation number | A Gelatin (%) | B Insulin (Unit) | C Outer aqueous phase (%) | D Oleic acid (%) | F Agitation time (min) |
|---|---|---|---|---|---|
| Rp. 1 | 0 | 200 | 66.7 | 0 | 2 |
| Rp. 2 | 0 | 200 | 66.7 | 5 | 5 |
| Rp. 3 | 0 | 200 | 85.7 | 0 | 5 |
| Rp. 4 | 0 | 200 | 85.7 | 5 | 2 |
| Rp. 5 | 0 | 600 | 66.7 | 0 | 5 |
| Rp. 6 | 0 | 600 | 66.7 | 5 | 2 |
| Rp. 7 | 0 | 600 | 85.7 | 0 | 2 |
| Rp. 8 | 0 | 600 | 85.7 | 5 | 5 |
| Rp. 9 | 10 | 200 | 66.7 | 0 | 5 |
| Rp. 10 | 10 | 200 | 66.7 | 5 | 2 |
| Rp. 11 | 10 | 200 | 85.7 | 0 | 2 |
| Rp. 12 | 10 | 200 | 85.7 | 5 | 5 |
| Rp. 13 | 10 | 600 | 66.7 | 0 | 2 |
| Rp. 14 | 10 | 600 | 66.7 | 5 | 5 |
| Rp. 15 | 10 | 600 | 85.7 | 0 | 5 |
| Rp. 16 | 10 | 600 | 85.7 | 5 | 2 |

Table 11. Formulations for insulin emulsion based on composite spherical experimental design for two factors

| Formulation number | $X_1$[a] | $X_2$[a] | Outer aqueous phase (%)[b] | Agitation time (min)[b] |
|---|---|---|---|---|
| Rp. 1 | -1 | -1 | 62.9 | 2.44 |
| Rp. 2 | -1 | 1 | 62.9 | 4.56 |
| Rp. 3 | 1 | -1 | 77.1 | 2.44 |
| Rp. 4 | 1 | 1 | 77.1 | 4.56 |
| Rp. 5 | $-\sqrt{2}$ | 0 | 60.0 | 3.50 |
| Rp. 6 | $\sqrt{2}$ | 0 | 80.0 | 3.50 |
| Rp. 7 | 0 | $-\sqrt{2}$ | 70.0 | 2.00 |
| Rp. 8 | 0 | $\sqrt{2}$ | 70.0 | 5.00 |
| Rp. 9 | 0 | 0 | 70.0 | 3.50 |
| Rp. 10 | 0 | 0 | 70.0 | 3.50 |

[a]Coded form, [b]Physical units.

## 2.3. Droplet size measurement

The size of the inner droplets of the emulsion was measured in freshly prepared samples. The mean diameter of the emulsion was determined using a dynamic laser light scattering instrument (ELS-800, Otsuka Electronics Co., Ltd., Osaka, Japan). The emulsion was diluted (approximately 500 times) with purified water before the measurement.

## 2.4. Viscosity measurement

The viscosity measurement of the fresh emulsion was carried out using a cone and plate type rheometer (Rheomat 15, Contraves Industrial Products Ltd., Middlwsex, UK) at 25 °C and a shear rate of 7.8 $s^{-1}$.

## 2.5. Stability of the emulsions

The stability of the emulsions was evaluated by a turbidity measurement method to determine the phase separation described by Pearce and Kinsella [80]. In the turbidity measurement method, the emulsion was packed into a syringe (1 mL volume) immediately after preparation and was stored standing vertically at 4 °C. Samples were taken from the lower side of the syringe at 0, 1, 3, 5 and 10 days after preparation, diluted 100 times with purified water, and then the turbidity was measured using a spectrophotometer (Ubest-30, Japan Spectroscopic Co., Ltd., Tokyo, Japan) at 600 nm. A decrease in the homogeneity of the emulsion (i.e., a decrease in the emulsion stability) was expressed as a percentage of the initial value of the turbidity. The extent of the stability response of the emulsion was calculated as the area above the curve of the percentage change in the turbidity levels ($AAC_{turbidity}$) for 0-10 days using the trapezoidal method.

## 2.6. *In vivo* insulin absorption experiments

Male Wistar rats weighing 180-200 g were purchased from the Sankyo Lab Service Co. Ltd. (Tokyo, Japan). The rats were fasted for 48 h prior to the experiments. Following anesthetization with the i.p. injection of sodium pentobarbital (50 mg/kg), the rats were restrained in a supine position on a thermostatically controlled board at 37 °C. Then, insulin emulsions were administered directly into the rectum. Insulin doses were changed by adjusting the emulsion amount. In the orthogonal experimental design study and the OA's dose-dependency study, the insulin doses were fixed at 10 and 20 IU/kg of body weight, respectively. Blood samples (0.1 mL) were withdrawn from the jugular vein at 5 min prior to administration and 0.5, 1, 2, 3 and 4 h following administration. Blood glucose levels were determined using a Novo Assist® Plus (Novo Nordisk Pharma., Ltd., Tokyo, Japan). The post-dose serum glucose levels were expressed as a percentage of the pre-dose levels. The hypoglycemic response was evaluated using $AAC_{glucose}$, calculated from the time curve of the blood glucose levels, similar to the stability study. The mean values and standard deviations were calculated from five rats.

## 2.7. Data analysis

Results of the L16 orthogonal experimental design for five causal factors were statistically analyzed based on the ANOVA technique using a computer program, STATISTICA (Statsoft, Tulsa, OK, USA). As for the optimization study, the optimum formulation was estimated using a simultaneous optimization technique, in which MVS was incorporated. A two-factor composite second-order spherical experimental design was employed to select the model formulation. The data measured for the model formulations were analyzed with a computer program, dataNESIA (Yamatake Corp., Tokyo, Japan). The MVS has recently been recognized as a superior method for the high precision modeling of

multi-dimensional data points [81]. MVS is basically a boundary element method [82]. Green functions are used for interpolating the minimum curvature of multi-dimensional data points [82]. As usual, observational data include experimental error. To avoid the problem of over estimation, the multi-dimensional data surface, including the experimental error, is estimated as the sum of interpolation with a Green function in a linear polynomial equation (thin-plate approximation) [83]. The smoothing parameter, which is the ratio of the Green function interpolation and thin-plate approximation, is automatically estimated using a generalized cross-validation technique. The optimum formulation was estimated based on the standardized Euclidian distance described previously [84].

## 3. Results and Discussion

### 3.1. ANOVA analysis

Owing to ANOVA analysis, the relationships between the formulation factors and characteristics were clarified. The experimental values of each response variable are listed in Table 12, and ANOVA tables for the individual characteristics are shown in Tables 13-16. As shown in Table 13, the volume ratio of the outer aqueous phase significantly affected the diameter of the inner droplet (i.e., W/O emulsion dispersed in the outer aqueous phase), and its contribution index was 20.3 % (Table 13). The decrease in the volume ratio of the outer aqueous phase resulted in the increase in the diameter of inner droplet. This was probably due to the aggregation of inner droplets. Span 80 and Tween 80, the non-ionic surfactants contained in emulsion as emulsifiers, locate at the interface of the oily phase and outer aqueous phase, respectively. These surfactants can interact with each other via van der Waals forces. This interaction contributes to the strength of the interfacial film and stabilizes the emulsion system against particle coalescence. The dispersion of inner droplets in the outer aqueous phase occurs due to the interaction between Span 80 and Tween 80 [85]. When the density of the inner droplet in the continuous phase is going to increase, inner droplets are allowed to interact with each other, resulting in the formation of aggregates all over the emulsion. The aggregation is reversible and the aggregates can be broken down by gentle shaking, since the van der Waals force is a very weak interaction. In fact, preliminary experiments showed that the viscosity of emulsions composed of a lower volume of outer aqueous phase decreased with the prolongation of the agitation time, or the increase in the shear rate, indicating that they were thixotropic fluids, while the viscosity of emulsions possessing a higher volume of the outer aqueous phase did not show any change (data not shown). These changes in viscosity strongly support the hypothesis.

In contrast to the droplet size, various factors and their interactions affected the

viscosity of the emulsion significantly (Table 14). The viscosity of the emulsion increased with the increase in the gelatin concentration in the inner aqueous phase, the decrease in the volume ratio of the outer aqueous phase and the OA amount, and the shortening of the agitation time. Among all of these factors, the volume ratio of the outer aqueous phase's contribution was clearly the most predominant, and all of the interactions significantly affected the viscosity of the emulsion. At a lower volume of outer aqueous phase, the inner droplets aggregated with each other via a low binding force, the van der Waals force, and they formed a network structure in the sample. In addition, the oily phase is obviously more viscous than the aqueous phase. Therefore, the physical interactions of inner droplets and the stickiness of the oily phase resulted in the high viscosity. The gelatin solution is much more viscous, so it is reasonable that the viscosity of the emulsion was increased by the addition of gelatin to the inner aqueous phase. The decrease in the amount of OA in the oily phase indicates the increase in the amount of triolein, because it is the component to adjust the weight of the oily phase. Since the molecular weight of triolein is approximately three times greater than that of OA, triolein is much more viscous than OA. Thus, the change in the composition of the emulsion caused the increase in its viscosity. In addition, the viscosity of the emulsion increased with the shortening of the agitation time. Considering the high shear stress caused by the homogenizer, it is thought that the agitation process greatly affected the forming emulsion and then changed the rheological property of the final products.

Table 12. Experimental values of response variables of insulin emulsion prepared according to orthogonal design

| Formulation number | Droplet size (nm) | Viscosity (mPa·s) | Turbidity $AAC_{turbidity}$ (% reduc.· day) | Hypoglycemic effect $AAC_{glucose}$ (% glu. reduc.· h) |
|---|---|---|---|---|
| Rp. 1 | 455.6 ± 123.1 | 36.6 | 47.4 ± 41.7 | 24.2 ± 25.9 |
| Rp. 2 | 492.6 ± 93.4 | 6.9 | 680.4 ± 13.0 | 42.4 ± 19.2 |
| Rp. 3 | 262.8 ± 18.5 | 5.2 | 600.3 ± 25.4 | 26.3 ± 36.7 |
| Rp. 4 | 287.5 ± 51.4 | 6.8 | 762.1 ± 11.2 | 48.4 ± 40.3 |
| Rp. 5 | 590.5 ± 209.1 | 14.1 | 634.3 ± 14.7 | 18.0 ± 14.6 |
| Rp. 6 | 397.4 ± 112.2 | 34.3 | 484.5 ± 48.6 | 50.2 ± 36.3 |
| Rp. 7 | 409.0 ± 73.9 | 9.9 | 776.6 ± 14.6 | 2.5 ± 4.3 |
| Rp. 8 | 347.9 ± 33.4 | 5.7 | 766.6 ± 10.1 | 39.7 ± 43.6 |
| Rp. 9 | 377.7 ± 59.1 | 29.4 | 370.0 ± 103.6 | 34.3 ± 22.6 |
| Rp. 10 | 316.0 ± 34.8 | 38.6 | 531.2 ± 101.2 | 57.7 ± 30.8 |
| Rp. 11 | 338.3 ± 27.0 | 8.9 | 768.1 ± 3.8 | 41.8 ± 25.1 |
| Rp. 12 | 273.9 ± 62.1 | 5.4 | 594.2 ± 6.5 | 57.1 ± 26.4 |
| Rp. 13 | 539.2 ± 91.5 | 76.0 | 22.5 ± 19.7 | 0.9 ± 1.9 |
| Rp. 14 | 334.0 ± 64.9 | 22.2 | 661.0 ± 15.3 | 82.5 ± 40.4 |
| Rp. 15 | 263.1 ± 9.4 | 6.3 | 606.9 ± 17.4 | 31.4 ± 20.6 |
| Rp. 16 | 410.9 ± 143.3 | 6.0 | 783.8 ± 5.4 | 83.4 ± 51.6 |

Each value represents the mean ± S.D.

Table 13. ANOVA table for inner droplet size of insulin emulsion prepared according to orthogonal design

| Factor | | DF[a] | MS[b] | $F_0$[c] | Contribution index |
|---|---|---|---|---|---|
| A | (Gelatin) | 1 | 19035.4 | 1.8 | 1.9 |
| B | (Insulin) | 1 | 29723.5 | 2.9 | 4.2 |
| C | (Outer aqueous phase) | 1 | 103445.4 | 10.0** | 20.3 |
| D | (Oleic acid) | 1 | 17687.5 | 1.7 | 1.6 |
| F | (Agitation time) | 1 | 5593.8 | 0.5 | 0.0 |
| A*B | | 1 | 3.2 | 0.0 | 0.0 |
| A*C | | 1 | 15155.0 | 1.5 | 1.1 |
| A*D | | 1 | 10.9 | 0.0 | 0.0 |
| A*F | | 1 | 31244.4 | 3.0 | 4.6 |
| B*C | | 1 | 303.4 | 0.0 | 0.0 |
| B*D | | 1 | 7634.5 | 0.7 | 0.0 |
| B*F | | 1 | 6637.0 | 0.6 | 0.0 |
| C*D | | 1 | 27603.7 | 2.7 | 3.8 |
| C*F | | 1 | 18505.5 | 1.8 | 1.8 |
| D*F | | 1 | 10132.8 | 1.0 | 0.0 |
| Error | | 16 | 10351.8 | - | 60.8 |
| Total | | 32 | | | 100.0 |

[a]Degrees of freedom, [b]Mean square, [c]Observed $F$ value.

**$p < 0.01$

Table 14. ANOVA table for viscosity of insulin emulsion prepared according to orthogonal design

| Factor | | DF[a] | MS[b] | $F_0$[c] | Contribution index |
|---|---|---|---|---|---|
| A | (Gelatin) | 1 | 669.8 | 15.6** | 5.2 |
| B | (Insulin) | 1 | 168.4 | 3.9 | 1.0 |
| C | (Outer aqueous phase) | 1 | 5191.8 | 120.6** | 42.8 |
| D | (Oleic acid) | 1 | 460.6 | 10.7** | 3.5 |
| F | (Agitation time) | 1 | 1857.5 | 43.2** | 15.1 |
| A*B | | 1 | 48.0 | 1.1 | 0.0 |
| A*C | | 1 | 708.8 | 16.5** | 5.5 |
| A*D | | 1 | 163.8 | 3.8 | 1.0 |
| A*F | | 1 | 14.0 | 0.3 | 0.0 |
| B*C | | 1 | 139.5 | 3.2 | 0.8 |
| B*D | | 1 | 30.0 | 0.7 | 0.0 |
| B*F | | 1 | 143.7 | 3.3 | 0.8 |
| C*D | | 1 | 285.6 | 6.6* | 2.0 |
| C*F | | 1 | 1346.8 | 31.3** | 10.8 |
| D*F | | 1 | 119.4 | 2.8 | 0.6 |
| Error | | 16 | 43.0 | - | 10.7 |
| Total | | 32 | | | 100.0 |

[a]Degrees of freedom, [b]Mean square, [c]Observed $F$ value.

*$p < 0.05$, **$p < 0.01$

With respect to final products, one of the most important considerations is stability. Stability of this emulsion system is characterized by an absence of coalescence of the internal phase, an absence of creaming, and maintaining its elegance in terms of appearance, such as odor, color and other physical properties. The stability of the emulsion was evaluated from the $AAC_{turbidity}$ values calculated by the turbidity change-time curve, which expressed the extent of the turbidity decrease, and a stable emulsion was defined as one having a low $AAC_{turbidity}$ value. As shown in Table 15, the stability of the emulsion was significantly influenced by all the causal factors. The stability of the emulsion increased with the increase in the gelatin concentration, the decrease in the insulin, the OA amount and the volume ratio of the outer aqueous phase, and the shortening of the agitation time. The effect of the gelatin contained in the inner aqueous phase on the stability of the emulsion systems was found in our previous study [23]. As well as the droplet size and viscosity, the most significant factor affecting the stability of the emulsion is the volume ratio of the outer aqueous phase (Table 13). The network formations of the inner droplets via van der Waals forces decrease the dynamic activity of themselves, resulting in the stabilization of the emulsion. Moreover, the ANOVA analysis suggested some relationship between the viscosity and the stability of the emulsion, since the preparation conditions for a viscous emulsion were very much similar to that required for a stable emulsion.

Table 15. ANOVA table for stability of insulin emulsion prepared according to orthogonal design

| Factor | | DF[a] | MS[b] | $F_0$[c] | Contribution index |
|---|---|---|---|---|---|
| A | (Gelatin) | 1 | 32209.2 | 18.7** | 1.2 |
| B | (Insulin) | 1 | 27427.7 | 15.9** | 1.0 |
| C | (Outer aqueous phase) | 1 | 930134.5 | 540.4** | 35.5 |
| D | (Oleic acid) | 1 | 387684.8 | 225.2** | 14.8 |
| F | (Agitation time) | 1 | 101954.8 | 59.2** | 3.8 |
| A*B | | 1 | 108585.2 | 63.1** | 4.1 |
| A*C | | 1 | 2244.1 | 1.3 | 0.0 |
| A*D | | 1 | 5271.0 | 3.1 | 0.1 |
| A*F | | 1 | 44032.0 | 25.6** | 1.6 |
| B*C | | 1 | 238.5 | 0.1 | 0.0 |
| B*D | | 1 | 3005.2 | 1.8 | 0.1 |
| B*F | | 1 | 40565.5 | 23.6** | 1.5 |
| C*D | | 1 | 238600.2 | 138.6** | 9.1 |
| C*F | | 1 | 595900.8 | 346.2** | 22.8 |
| D*F | | 1 | 39045.0 | 22.7** | 1.4 |
| Error | | 32 | 1721.3 | - | 3.0 |
| Total | | 48 | | | 100.0 |

[a]Degrees of freedom, [b]Mean square, [c]Observed $F$ value.

**$p < 0.01$

In the insulin absorption study, the addition of gelatin and OA significantly increased the insulin's pharmacological effect (Table 16). A previous study demonstrated that a gelatin free emulsion did not induce any hypoglycemic effect following ileal administration [23]. The high viscosity surrounding insulin molecules can protect the insulin degradation from enzymatic hydrolysis and prolong the residence time of insulin at the mucosal membrane. The contribution of OA to the pharmacological effect of the emulsion was outstanding, its contribution index being 23.4 % (Table 16). The pharmacological effect of the insulin emulsion was strongly affected by the absorption enhancement effect of the OA rather than by the physical properties of the emulsion, such as the viscosity or droplet size.

Table 16. ANOVA table for hypoglycemic effect of insulin emulsion prepared according to orthogonal design

| Factor | | DF[a] | MS[b] | $F_0$[c] | Contribution index |
|---|---|---|---|---|---|
| A | (Gelatin) | 1 | 5875.8 | 6.3* | 4.8 |
| B | (Insulin) | 1 | 175.3 | 0.2 | 0.0 |
| C | (Outer aqueous phase) | 1 | 130.1 | 0.1 | 0.0 |
| D | (Oleic acid) | 1 | 24824.8 | 26.6** | 23.4 |
| F | (Agitation time) | 1 | 159.5 | 0.2 | 0.0 |
| A*B | | 1 | 454.0 | 0.5 | 0.0 |
| A*C | | 1 | 982.5 | 1.1 | 0.1 |
| A*D | | 1 | 1225.5 | 1.3 | 0.3 |
| A*F | | 1 | 130.9 | 0.1 | 0.0 |
| B*C | | 1 | 28.0 | 0.0 | 0.0 |
| B*D | | 1 | 4818.8 | 5.2* | 3.8 |
| B*F | | 1 | 681.1 | 0.7 | 0.0 |
| C*D | | 1 | 260.0 | 0.3 | 0.0 |
| C*F | | 1 | 1354.6 | 1.5 | 0.4 |
| D*F | | 1 | 1079.5 | 1.2 | 0.1 |
| Error | | 64 | 935.1 | - | 67.1 |
| Total | | 80 | | | 100.0 |

[a]Degrees of freedom, [b]Mean square, [c]Observed $F$ value.

*$p < 0.05$, **$p < 0.01$

### 3.2. Formulation optimization

Based on the results of the orthogonal experimental design study, the optimum formulation of the emulsion, which had both a high pharmacological activity and stability as a pharmaceutical formulation, was estimated. To begin with, the OA concentration in the emulsion was determined by the consideration of its pharmacological effect and toxicity. As shown in Fig. 18, the hypoglycemic effects increased with the increase in the OA concentration in the range of 0 to 2 % of the emulsion. Particularly, a marked pharmacological effect was induced at an OA concentration of 2 %. Furthermore, it had been confirmed that no mucosal damage is induced by the multiple applications of insulin emulsion containing 2 % OA in Chapter 1. Therefore, the optimal OA concentration was decided at 2 %. Subsequently, model formulations were prepared according to the two-factor spherical second-order composite experimental design (Table 11), and their stability was determined. The volume ratio of the outer aqueous phase and the agitation time were selected as the causal factors, since these contributions to stability were very large, approximately 62.1 % in total, including their interactions (Table 15). The other variable factors were fixed as follows: OA, 2 % of total weight; gelatin, 5 % of the inner aqueous phase; insulin, 200 IU per 2 g of the inner aqueous phase. A three-dimensional diagram of the stability as a function of the volume ratio of the outer aqueous phase, and the agitation time is shown in Fig. 19. Nonlinear relationships between the causal factors and the response variables were represented by the response surface, as predicted by MVS (Fig. 19). With the decrease in the fraction of the outer aqueous phase and the shortening of the agitation time, the $AAC_{turbidity}$ values decreased, resulting in the increased stability of the emulsion.

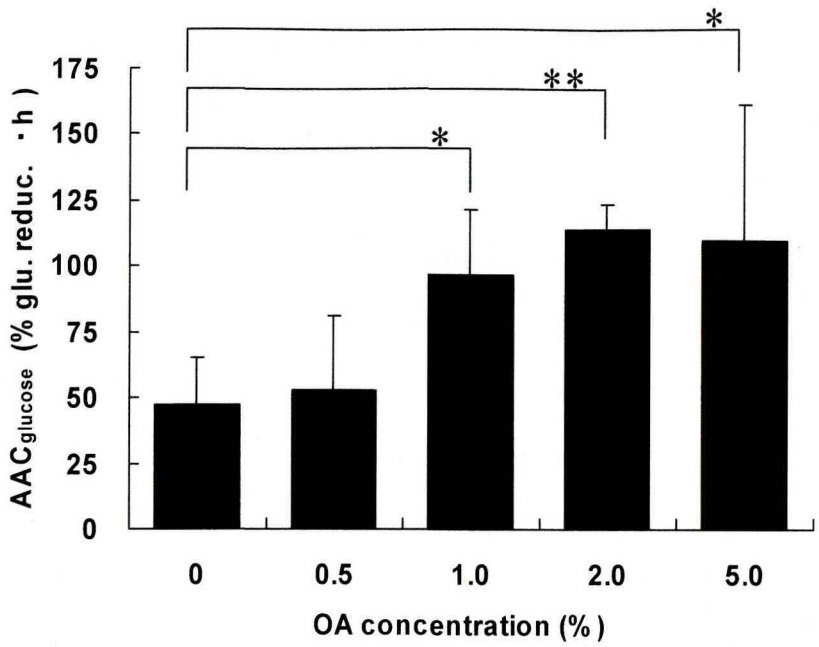


Figure 18. Comparison of hypoglycemic effects of insulin emulsion incorporating various concentrations of OA. Insulin emulsions were administered into the rectum at 20 IU/kg of insulin. Each value represents the mean ± S.D. $^{*}p<0.05$, $^{**}p<0.01$.

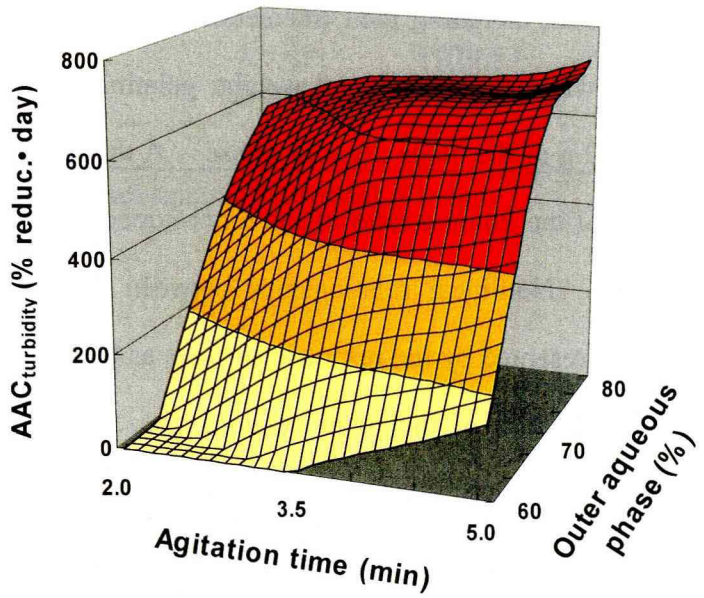


Figure 19. Response surface of stability of the insulin emulsion predicted by MVS.

Finally, the optimal formulation was estimated and evaluated. The values of the causal factors that give the minimal $AAC_{turbidity}$ value are listed in Table 17. Since the experimental value of $AAC_{turbidity}$ was almost zero, it was concluded that the predicted values were in agreement with the experimental values, and the formulation was thought to be highly stable. Thus, the optimum formulation, having a desirable pharmacological effect and high stability, was successfully obtained.

Table 17. Optimal formulation composition of insulin emulsion predicted by MVS and predicted and observed values of stability

| Factor | | Response | |
|---|---|---|---|
| Outer aqueous phase (%) | Agitation time (min) | $AAC_{turbidity}$ (% reduc.· day) | |
| | | Predicted value | Observed value[a] |
| 60.7 | 2.97 | 0.0 | 1.5 ± 1.6 |

[a]Mean ± S.D. of three determinations.

## 4. Conclusions

ANOVA analysis revealed the relationships between the formulation factors and the individual characteristics. The droplet size of the emulsion increased significantly with the decrease in the volume ratio of the outer aqueous phase to the total. Both the viscosity and the stability of the emulsion were affected by various causal factors and their interactions, and it was suggested that they had something to do with each other. As for the hypoglycemic effect, the most influential factor was the amount of OA, which was used as an absorption enhancer to the emulsion. Based on the response surface generated by MVS, the optimum formulation, having the highest stability and the most desirable pharmacological effect, was successfully prepared.

# SUMMARY

Insulin is a key hormone regulating the blood glucose level, and it is the mainstay of drug therapy for patients with insulin-dependent diabetes mellitus. All current insulin preparations are limited to the injection dosage form, so an alternative route, especially an oral route, of insulin delivery has been desired for a long time. However, it is difficult to deliver insulin via the gastrointestinal tract because of extensive proteolytic degradation by intestinal enzymes and insufficient membrane permeability due to its high molecular weight and low lipophilicity.

A W/O/W multiple emulsion is thought to be an efficient drug carrier because it can protect peptides and incorporate various types of absorption enhancers in each phase, in accordance with its solubility. In addition, PUFAs are well known functional lipids that possess various biological actions, and they are expected to be effective absorption enhancers for intestinal insulin delivery. The aim of this study was to establish a novel insulin enteral delivery system using a W/O/W multiple emulsion containing PUFAs.

In Chapter 1, the enhancement effect on intestinal insulin absorption and the mucosal damage induced by PUFAs were evaluated with an *in vivo* and *in situ* study. Marked insulin absorption and a hypoglycemic effect were shown within a very short time after the rectal administration of insulin emulsion. The hypoglycemic effects were in an insulin dose related manner. The enhancement effects of PUFAs, in particular DHA, were much more pronounced than that of oleic acid, and reached saturation point at 1 % of the total amount of emulsion. The effect of DHA was outstanding compared to those of its glyceride forms or medium chain fatty acids. In addition, the effect of DHA was site-dependent, and the action of DHA in the large intestine, such as the colon and rectum, was stronger than that in the small intestine, such as the ileum. Considering the clinical use of insulin preparations, a

multiple administration study was conducted. During the multiple rectal applications of the insulin emulsion for 10 days, the hypoglycemic effects were successfully obtained, and no or very slight mucosal damage was observed in the excised rectum. Moreover, to manipulate the glucose reduction profile, a gel formulation that combined the insulin emulsion with PF127 hydrogel was prepared, and the hypoglycemic effect was evaluated. As expected, the sustained hypoglycemic effect was shown by the administration of this gel formulation.

The structural change in the lipid bilayer is a critical factor for the transcellular pathway. In Chapter 2, the structural changes in a DPPC bilayer induced by treatment with various fatty acids were clarified by determining the phase transition temperature, fluorescence anisotropy of DPH, and detergent insolubility. Treatment with unsaturated fatty acid broadened and shifted the phase transitions of the DPPC bilayer to a lower temperature. The phase transition temperature and the value of fluorescence anisotropy of DPH at 37 °C decreased progressively with increasing treatment amounts of unsaturated fatty acid. A large amount of the DPPC bilayer treated with unsaturated fatty acid was dissolved in Triton X-100, yielding a low level of detergent insolubility. These modifications of the bilayer physical properties were most pronounced with DHA and EPA treatment. These data show that unsaturated fatty acids, particularly DHA and EPA, induce a marked change in the lipid bilayer structure. The composition of fatty acids in the DPPC bilayer was similar after treatment with various unsaturated fatty acids, suggesting that the different actions of unsaturated fatty acids are attributed to change in the molecular structure (e.g., kinked conformation by double bonds). This study further explored the change in physical properties induced by fatty acids dispersed in the W/O/W multiple emulsion and found that unsaturated fatty acids acted efficiently on the DPPC bilayer, even when incorporated in the emulsion form.

Numerous variable factors including components and the manufacturing process may

affect various responses of pharmaceutical formulations. For the design of pharmaceutical formulations, it is important to clarify the relationship between the causal factors and the individual characteristics, and to optimize these variables simultaneously. The aim of Chapter 3 was to optimize the formulation of the W/O/W multiple emulsion for intestinal mucosal insulin delivery. ANOVA analysis revealed the relationships between the formulation factors and the individual characteristics. The droplet size of the emulsion increased significantly with the decrease in the volume ratio of the outer aqueous phase to the total. Both the viscosity and the stability of the emulsion were affected by various causal factors and their interactions, and it was suggested that they had something to do with each other. As for the hypoglycemic effect, the most influential factor was the amount of OA, which was used as an absorption enhancer to the emulsion. Based on the response surface generated by MVS, the optimum formulation, having the highest stability and the most desirable pharmacological effect, was successfully prepared.

In conclusion, it was found in this study that PUFAs are valuable functional fatty acids which enable to improve the intestinal insulin absorption without evoking any mucosal damages. This study also clarified that W/O/W multiple emulsion is useful drug delivery carrier. Results observed in this study provide great benefits for those researching in oral delivery of peptide drugs.

# ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude and deepest appreciation to Professor Kozo Takayama and Associate Professor Mariko Morishita (Department of Pharmaceutics, Hoshi University) for their guidance and advice in my research work and preparing this dissertation.

Secondly, I wish to express my gratitude and appreciation to the former president of Hoshi University, Dr. Tsuneji Nagai for his valuable assistance in my research work.

I would like to also thank Mr. Yoshiyuki Chiba, Mr. Shinji Tokiwa (Central Research Laboratory, Nippon Suisan Kaisya, Ltd.), Mr. Koichi Isowa (The Center of Japan Biological Chemistry Co., Ltd.), Ms. Hisae Watanabe (Department of Pharmaceutics, Hoshi University) and Mr. Chosei Kaseda (R&D Headquarters, Yamatake Co.) for their helpful guidance and assistance in my research work.

Moreover, I would like to thank Professor Yoshie Maitani (Fine Drug Targeting Research Laboratory, Hoshi University), Dr. Yasuko Obata (Department of Pharmaceutics, Hoshi University), Dr. Yoshinobu Aoki (Research Center, Kyorin Pharmaceutical Co., Ltd.) and Dr. Koji Nakamura (R&D Center, Terumo Co., Ltd.) for their valuable suggestions and fruitful discussions.

Furthermore, I am grateful to Dr. Takanori Saitoh and Dr. Hideaki Okabe (Lintec Co.) for providing me with the opportunity to engage in the research and development of pharmaceutical products.

I would also like to acknowledge Dr. Masaru Hoshi and Mr. Eiji Nogami (Technology Administration, Lintec Co.) for kindness and precious suggestions.

In addition, I am grateful to all members of the Research Laboratory of Lintec Corporation, and all my colleagues of the Department of Pharmaceutics of Hoshi University for their kindness and assistance.

Finally, I wish to express my sincere gratitude to my parents and my wife. This thesis would not have been fulfilled without their encouragement and support of my study and life.

# REFERENCES


1) Madsbad, S., Insulin analogues: have they changed insulin treatment and improved glycaemic control?, *Diabetes Metab. Res. Rev.*, **18**, S21-28 (2002).
2) Hoffman, A., Ziv, E., Pharmacokinetic considerations of new insulin formulations and routes of administration., *Clin. Pharmacokinet.*, **33**, 285-301 (1997).
3) Owens, D. R., Zinman, B., Bolli, G., Alternative routes of insulin delivery., *Diabet. Med.*, **20**, 886-898 (2003).
4) Takei, I., Kasatani, T., Future therapy of diabetes mellitus., *Biomed. Pharmacother.*, **58**, 578-581 (2004).
5) King, M. J., Badea, I., Solomon, J., Kumar, P., Gaspar, K. J., Foldvari, M., Transdermal delivery of insulin from a novel biphasic lipid system in diabetic rats., *Diabetes Technol. Ther.*, **4**, 479-488 (2002).
6) Mitragotri, S., Blankschtein, D., Langer, R., Ultrasound-mediated transdermal protein delivery., *Science*, **269**, 850-853 (1995).
7) Lalej-Bennis, D., Boillot, J., Bardin, C., Zirinis, P., Coste, A., Escudier, E., Chast, F., Peynegre, R., Slama, G., Selam, J. L., Six month administration of gelified intranasal insulin in 16 type 1 diabetic patients under multiple injections: efficacy vs subcutaneous injections and local tolerance., *Diabetes Metab.*, **27**, 372-377 (2001).
8) Heinemann, L., Linkeschova, R., Rave, K., Hompesch, B., Sedlak, M., Heise, T., Time-action profile of the long-acting insulin analog insulin glargine (HOE901) in comparison with those of NPH insulin and placebo., *Diabetes Care*, **23**, 644-649 (2000).
9) Farr, S. J., McElduff, A., Mather, L. E., Okikawa, J., Ward, M. E., Gonda, I., Licko, V., Rubsamen, R. M., Pulmonary insulin administration using the AERx system: physiological and physicochemical factors influencing insulin effectiveness in healthy fasting subjects., *Diabetes Technol. Ther.*, **2**, 185-197 (2000).
10) Skyler, J. S., Cefalu, W. T., Kourides, I. A., Landschulz, W. H., Balagtas, C. C., Cheng, S. L., Gelfand, R. A., Efficacy of inhaled human insulin in type 1 diabetes mellitus: a randomised proof-of-concept study., *Lancet*, **357**, 331-335 (2001).
11) Hermansen, K., Ronnemaa, T., Petersen, A. H., Bellaire, S., Adamson, U., Intensive therapy with inhaled insulin via the AERx insulin diabetes management system: a 12-week proof-of-concept trial in patients with type 2 diabetes., *Diabetes Care*, **27**, 162-167 (2004).
12) Carino, G. P., Mathiowitz, E., Oral insulin delivery., *Adv. Drug Deliv. Rev.*, **35**, 249-257 (1999).
13) Modi, P., Mihic, M., Lewin, A., The evolving role of oral insulin in the treatment of

diabetes using a novel RapidMist System., *Diabetes Metab. Res. Rev.*, **18**, S38-42 (2002).

14) Madison, L. L., Combes, B., Unger, R. H., Kaplan, N., The relationship between the mechanism of action of the sulfonylureas and the secretion of insulin into the portal circulation., *Ann. N. Y. Acad. Sci.*, **74**, 548-556 (1959).

15) Gwinup, G., Elias, A. N., Domurat, E. S., Insulin and C-peptide levels following oral administration of insulin in intestinal-enzyme protected capsules., *Gen. Pharmacol.*, **22**, 243-246 (1991).

16) Mathiowitz, E., Jacob, J. S., Jong, Y. S., Carino, G. P., Chickering, D. E., Chaturvedi, P., Santos, C. A., Vijayaraghavan, K., Montgomery, S., Bassett, M., Morrell, C., Biologically erodable microspheres as potential oral drug delivery systems., *Nature*, **386**, 410-414 (1997).

17) Krauland, A. H., Guggi, D., Bernkop-Schnurch, A., Oral insulin delivery: the potential of thiolated chitosan-insulin tablets on non-diabetic rats., *J. Controlled Release*, **95**, 547-555 (2004).

18) Yamamoto A., Muranishi S., Rectal drug delivery systems improvement of rectal peptide absorption by absorption enhancers, protease inhibitors and chemical modification., *Adv. Drug Deliv. Rev.*, **28**, 275-299 (1997).

19) Shichiri, M., Kawamori, R., Yoshida, M., Etani, N., Hoshi, M., Short-term treatment of alloxan-diabetic rats with intrajejunal administration of water-in-oil-in-water insulin emulsions., *Diabetes*, **24**, 971-976 (1975).

20) Cunha, A. S., Grossiord, J. L., Puisieux, F., Seiller, M., Insulin in w/o/w multiple emulsions: preparation characterization and determination of stability towards proteases in vitro., *J. Microencapsul.*, **14**, 311-319 (1997).

21) Engel, R. H., Riggi, S. J., Fahrenbach, M. J., Insulin: intestinal absorption as water-in-oil-in-water emulsions., *Nature*, **219**, 856-857 (1968).

22) Oba, N., Sugimura, H., Umehara, Y., Yoshida, M., Kimura, T., Yamaguchi, T., Evaluation of an oleic acid water-in-oil-in-water-type multiple emulsion as potential drug carrier via the enteral route., *Lipids*, **27**, 701-705 (1992).

23) Matsuzawa, A., Morishita, M., Takayama, K., Nagai, T., Absorption of insulin using water-in-oil-in-water emulsion from an enteral loop in rats., *Biol. Pharm. Bull.*, **18**, 1718-1723 (1995).

24) Suzuki, A., Morishita, M., Kajita, M., Takayama, K., Isowa, K., Chiba, Y., Tokiwa, S., Nagai, T., Enhanced colonic and rectal absorption of insulin using a multiple emulsion containing eicosapentaenoic acid and docosahexaenoic acid., *J. Pharm. Sci.*, **87**, 1196-1202 (1998).

25) Kajita, M., Morishita, M., Takayama, K., Chiba, Y., Tokiwa, S., Nagai, T., Enhanced enteral bioavailability of vancomycin using water-in-oil-in-water multiple emulsion

incorporating highly purified unsaturated fatty acid., *J. Pharm. Sci.*, **89**, 1243-1252 (2000).

26) Morishita, M., Matsuzawa, A., Takayama, K., Isowa, K., Nagai, T., Improving insulin enteral absorption using water-in-oil-in-water emulsion., *Int. J. Pharm.*, **172**, 189-198 (1998).

27) Turunen, T. M., Urtti, A., Paronen, P., Audus, K. L., Rytting, J. H., Effect of some penetration enhancers on epithelial membrane lipid domains: evidence from fluorescence spectroscopy studies., *Pharm. Res.*, **11**, 288-294 (1994).

28) Zheng, J. Y., Fulu, M. Y., Decrease of genital organ weights and plasma testosterone levels in rats following oral administration of leuprolide microemulsion., *Int. J. Pharm.*, **307**, 209-215 (2006).

29) Stillwell, W., Wassall, S. R., Docosahexaenoic acid: membrane properties of a unique fatty acid., *Chem. Phys. Lipids*, **126**, 1-27 (2003).

30) Jump, D. B., The biochemistry of n-3 polyunsaturated fatty acids., *J. Biol. Chem.*, **277**, 8755-8758 (2002).

31) Carroll, K. K., Dietary fats and cancer., *Am. J. Clin. Nutr.*, **53**, 1064S-1067S (1991).

32) Zerouga, M., Stillwell, W., Stone, J., Powner, A., Jenski, L. J., Phospholipid class as a determinant in docosahexaenoic acid's effect on tumor cell viability., *Anticancer Res.*, **16**, 2863-2868 (1996).

33) McLennan, P., Howe, P., Abeywardena, M., Muggli, R., Raederstorff, D., Mano, M., Rayner, T., Head, R., The cardiovascular protective role of docosahexaenoic acid., *Eur. J. Pharmacol.*, **300**, 83-89 (1996).

34) Dyerberg, J., Bang, H. O., A hypothesis on the development of acute myocardial infarction in Greenlanders., *Scand. J. Clin. Lab. Invest.*, **42**, 7-13 (1982).

35) Leaf, A., Weber, P. C., Cardiovascular effects of n-3 fatty acids., *N. Engl. J. Med.*, **318**, 549-557 (1988).

36) Menkes, J. H., Alter, M., Steigleder, G. K., Weakley, D. R., Sung, J. H., A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration., *Pediatrics*, **29**, 764-779 (1962).

37) Lee, V. H., Yamamoto, A., Kompella, U. B., Mucosal penetration enhancers for facilitation of peptide and protein drug absorption., *Crit. Rev. Ther. Drug Carrier. Syst.*, **8**, 91-192 (1991).

38) Muranishi, S., Absorption enhancers.,*Crit. Rev. Ther. Drug. Carrier. Syst.*, **7**, 1-33 (1990).

39) Uchiyama, T., Sugiyama, T., Quan, Y. S., Kotani, A., Okada, N., Fujita, T., Muranishi, S., Yamamoto, A., Enhanced permeability of insulin across the rat intestinal membrane by various absorption enhancers: their intestinal mucosal toxicity and absorption-enhancing mechanism of n-lauryl-beta-D-maltopyranoside., *J. Pharm.*

*Pharmacol.*, **51**, 1241-1250 (1999).

40) Sekine, M., Sasahara, K., Kojima, T., Hasegawa, K., Okada, R., Improvement of bioavailability of poorly intestinally absorbed drugs from medium-chain glyceride base. Enhancement of the rectal absorption of cefmetazole sodium in rabbits., *Chem. Pharm. Bull.*, **32**, 4189-4192 (1984).

41) Mattson, F. H., Beck, L. W., The specificity of pancreatic lipase for the primary hydroxyl groups of glycerides., *J. Biol. Chem.*, **219**, 735-740 (1956).

42) Lindmark, T., Kimura, Y., Artursson, P., Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in Caco-2 cells., *J. Pharmacol. Exp. Ther.*, **284**, 362-369 (1998).

43) Sawada, T., Ogawa, T., Tomita, M., Hayashi, M., Awazu, S., Role of paracellular pathway in nonelectrolyte permeation across rat colon epithelium enhanced by sodium caprate and sodium caprylate., *Pharm. Res.*, **8**, 1365-1371 (1991).

44) Fetih, G., Lindberg, S., Itoh, K., Okada, N., Fujita, T., Habib, F., Artersson, P., Attia, M., Yamamoto, A., Improvement of absorption enhancing effects of n-dodecyl-beta-D-maltopyranoside by its colon-specific delivery using chitosan capsules., *Int. J. Pharm.*, **293**, 127-135 (2005).

45) Zheng, Y., Qiu, Y., Lu, M. F., Hoffman, D., Reiland, T. L., Permeability and absorption of leuprolide from various intestinal regions in rabbits and rats., *Int. J. Pharm.*, **185**, 83-92 (1999).

46) Brasitus, T. A., Schachter, D., Lipid composition and fluidity of rat enterocyte basolateral membranes. Regional differences., *Biochim. Biophys. Acta*, **774**, 138-146 (1984).

47) Brasitus, T. A., Dudeja, P. K., Regional differences in the lipid composition and fluidity of rat colonic brush-border membranes., *Biochim. Biophys. Acta*, **819**, 10-17 (1985).

48) Yamamoto, A., Taniguchi, T., Rikyuu, K., Tsuji, T., Fujita, T., Murakami, M., Muranishi, S., Effects of various protease inhibitors on the intestinal absorption and degradation of insulin in rats., *Pharm. Res.*, **11**, 1496-1500 (1994).

49) O'Reilly, J.R., Corrigan, O.I., O'Driscoll, C.M., The effect of mixed micellar systems, bile salt/fatty acids, on the solubility and intestinal absorption of clofazimine (B663) in the anaesthetised rat., *Int. J. Pharm.*, **109**, 147-154 (1994).

50) Yamashita, S., Masada, M., Nadai, T., Kimura, T., Effect of adjuvants on charge-selective permeability and electrical resistance of rat jejunal membrane., *J. Pharm. Sci.*, **79**, 579-583 (1990).

51) O'Reilly, J. R., Corrigan, O. I., O'Driscoll, C. M., The effect of simple micellar systems on the solubility and intestinal absorption of clofazimine (B663) in the anaesthetised rat., *Int. J. Pharm.*, **109**, 137-146 (1994).

52) Werner, U., Kissel, T., Reers, M., Effects of permeation enhancers on the transport of a

peptidomimetic thrombin inhibitor (CRC 220) in a human intestinal cell line (Caco-2)., *Pharm. Res.*, **13**, 1219-1227 (1996).

53) Halpern, M. D., Holubec, H., Saunders, T. A., Dvorak, K., Clark, J. A., Doelle, S. M., Ballatori, N., Dvorak, B., Bile acids induce ileal damage during experimental necrotizing enterocolitis., *Gastroenterology*, **130**, 359-372 (2006).

54) Zhao, D. L., Hirst, B. H., Bile salt-induced increases in duodenal brush-border membrane proton permeability, fluidity, and fragility., *Dig. Dis. Sci.*, **35**, 589-595 (1990).

55) Casellas, F., Mourelle, M., Papo, M., Guarner, F., Antolin, M., Armengol, J. R., Malagelada, J. R., Bile acid induced colonic irritation stimulates intracolonic nitric oxide release in humans., *Gut*, **38**, 719-723 (1996).

56) Swenson, E. S., Milisen, W. B., Curatolo, W., Intestinal permeability enhancement: efficacy, acute local toxicity, and reversibility., *Pharm. Res.*, **11**, 1132-1142 (1994).

57) Horie, T., Nakamaru, M., Masubuchi, Y., Docosahexaenoic acid exhibits a potent protection of small intestine from methotrexate-induced damage in mice., *Life Sci.*, **62**, 1333-1338 (1998).

58) Howey, D. C., Bowsher, R. R., Brunelle, R. L., Woodworth, J. R., [Lys(B28), Pro(B29)]-human insulin. A rapidly absorbed analogue of human insulin., *Diabetes*, **43**, 396-402 (1994).

59) Kimura, T., Sato, K., Sugimoto, K., Tao, R., Murakami, T., Kurosaki, Y., Nakayama, T., Oral administration of insulin as poly(vinyl alcohol)-gel spheres in diabetic rats., *Biol. Pharm. Bull.*, **19**, 897-900 (1996).

60) Barichello, J. M., Morishita, M., Takayama, K., Chiba, Y., Tokiwa, S., Nagai, T., Enhanced rectal absorption of insulin-loaded Pluronic F-127 gels containing unsaturated fatty acids., *Int. J. Pharm.*, **183**, 125-132 (1999).

61) Roach, C., Feller, S. E., Ward, J. A., Shaikh, S. R., Zerouga, M., Stillwell, W., Comparison of cis and trans fatty acid containing phosphatidylcholines on membrane properties., *Biochemistry*, **43**, 6344-6351 (2004).

62) Wu, S. J., Robinson, J. R., Transcellular and lipophilic complex-enhanced intestinal absorption of human growth hormone., *Pharm Res*, **16**, 1266-1272 (1999).

63) Rege, B. D., Kao, J. P., Polli, J. E., Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers., *Eur. J. Pharm. Sci.*, **16**, 237-246 (2002).

64) Sinicrope, F. A., Dudeja, P. K., Bissonnette, B. M., Safa, A. R., Brasitus, T. A., Modulation of P-glycoprotein-mediated drug transport by alterations in lipid fluidity of rat liver canalicular membrane vesicles., *J. Biol. Chem.*, **267**, 24995-25002 (1992).

65) Maitani, Y., Nakamura, K., Suenaga, H., Kamata, K., Takayama, K., Nagai, T., The enhancing effect of soybean-derived sterylglucoside and beta-sitosterol beta-D-glucoside on nasal absorption in rabbits., *Int. J. Pharm.*, **200**, 17-26 (2000).

66) Zhao, L., Feng, S. S., Go, M. L., Investigation of molecular interactions between paclitaxel and DPPC by Langmuir film balance and differential scanning calorimetry., *J. Pharm. Sci.*, **93**, 86-98 (2004).
67) Tran, R., Ho, S., Dea, P., Effects of ethanol on lipid bilayers with and without cholesterol: the distearoylphosphatidylcholine system., *Biophys. Chem.*, **110**, 39-47 (2004).
68) Wang, J., Megha,, London, E., Relationship between sterol/steroid structure and participation in ordered lipid domains (lipid rafts): implications for lipid raft structure and function., *Biochemistry*, **43**, 1010-1018 (2004).
69) Xu, X., London, E., The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation., *Biochemistry*, **39**, 843-849 (2000).
70) Constantinides, P. P., Scalart, J. P., Lancaster, C., Marcello, J., Marks, G., Ellens, H., Smith, P. L., Formulation and intestinal absorption enhancement evaluation of water-in-oil microemulsions incorporating medium-chain glycerides., *Pharm. Res.*, **11**, 1385-1390 (1994).
71) Constantinides, P. P., Welzel, G., Ellens, H., Smith, P. L., Sturgis, S., Yiv, S. H., Owen, A. B., Water-in-oil microemulsions containing medium-chain fatty acids/salts: formulation and intestinal absorption enhancement evaluation., *Pharm. Res.*, **13**, 210-215 (1996).
72) Olivieri, L., Seiller, M., Bromberg, L., Besnard, M., Duong, T. N., Grossiord, J. L., Optimization of a thermally reversible W/O/W multiple emulsion for shear-induced drug release., *J. Controlled Release*, **88**, 401-412 (2003).
73) Muguet, V. V., Seiller, M., Barratt, G., Clausse, D., Marty, J. P., Grossiord, J. L., W/O/W multiple emulsions submitted to a linear shear flow: Correlation between fragmentation and release., *J. Coll. Interface Sci.*, **218**, 335-337 (1999).
74) Muguet, V., Seiller, M., Barratt, G., Hebel, N., Marty, J.P., Grossiord, J.L., Influence of the fragmentation of multiple globules on the rheological properties of W/O/W multiple emulsions., *J. Rheology,* **44**, 379-395 (2000).
75) Levison, K. K., Takayama, K., Isowa, K., Okabe, K., Nagai, T., Formulation optimization of indomethacin gels containing a combination of three kinds of cyclic monoterpenes as percutaneous penetration enhancers., *J. Pharm. Sci.*, **83**, 1367-1372 (1994).
76) Wang, Y. M., Sato, H., Adachi, I., Horikoshi, I., Optimization of the formulation design of chitosan microspheres containing cisplatin., *J. Pharm. Sci.*, **85**, 1204-1210 (1996).
77) Abuzarur-Aloul, R., Gjellan, K., Sjolund, M., Graffner, C., Critical dissolution tests of oral systems based on statistically designed experiments. III. In vitro/in vivo correlation for multiple-unit capsules of paracetamol based on PLS modeling., *Drug Dev. Ind. Pharm.*, **24**, 371-383 (1998).

78) Ogawa, N., Morishita, M., Takayama, K., Nagai, T., Quantitative evaluation of in vitro release medium to predict transfer of flunisolide into nasal mucosa of rabbits from ointments, *S. T. P. Pharma Sci.*, **13**, 259-263 (2003).
79) Surini, S., Akiyama, H., Morishita, M., Nagai, T., Takayama, K., Release phenomena of insulin from an implantable device composed of a polyion complex of chitosan and sodium hyaluronate., *J. Controlled Release*, **90**, 291-301 (2003).
80) Pearce, K.N., Kinsella, J.E., Emulsifying properties of proteins: Evaluation of a turbidimetric technique., *J. Agric. Food Chem.*, **26**, 716-723 (1978).
81) Tetteh, J., Howells, S., Metcalfe, E., Suzuki, T., Optimisation of radial basis function neural networks using biharmonic spline interpolation., *Chemometr. Intell. Lab. Syst.*, **41**, 17-29 (1998).
82) Sandwell, D.T., Biharmonic spline interpolation of GEOS-3 and Seasat altimeter data., *Geophys. Res. Lett.*, **14**, 139-142 (1987).
83) Wahba, G., Spline models for observational data, *Society for Industrial and Applied Mathematics, Philadelphia, PA*, (1990).
84) Takayama, K., Fujikawa, M., Obata, Y., Morishita, M., Neural network based optimization of drug formulations., *Adv. Drug Deliv. Rev.*, **55**, 1217-1231 (2003).
85) Boyd, J., Parkinson, C., Sherman, P., Factors affecting emulsion stability, and the HLB [hydrophilic-liphophilic balance] concept., *J. Coll. Interface Sci.*, **41**, 359-370 (1972).