European Journal of Pharmaceutics and Biopharmaceutics 74 (2010) 223–232

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Nano-sized water-in-oil-in-water emulsion enhances intestinal absorption of calcein, a high solubility and low permeability compound

Kenjiro Koga [a,*], Nobuo Takarada [a], Kanji Takada [b]

[a] Department of Clinical Pharmaceutics, Hokuriku University, Kanazawa, Japan
[b] Department of Pharmacokinetics, Kyoto Pharmaceutical University, Kyoto, Japan

ARTICLE INFO

Article history:
Received 7 April 2009
Accepted in revised form 8 September 2009
Available online 13 September 2009

Keywords:
w/o/w emulsion
Nanoparticle
BCS class III
Emulsifier
Intestinal absorption

ABSTRACT

Our goal was to develop safe and stable multilayer emulsions capable of enhancing intestinal absorption of biopharmaceutics classification system (BCS) class III drugs. First, w/o emulsions were prepared using calcein as a model BCS class III compound and condensed ricinoleic acid tetraglycerin ester as a hydrophobic emulsifier. Then water-in-oil-in-water (w/o/w) emulsions were prepared with shirasu porous glass (SPG) membranes. Particle size analyses and calcein leakage from oil droplets in w/o/w emulsions led us to select stearic acid hexaglycerin esters (HS-11) and Gelucire 44/14 as hydrophilic emulsifiers. Analyses of the absorption-enhancing effects of w/o/w emulsions on intestinal calcein absorption in rats showed that calcein bioavailability after intraduodenal (i.d.) administration of HS-11 or Gelucire 44/14 + polyvinyl alcohol (PVA) w/o/w emulsions prepared with 0.1-μm pore-sized SPGs was significantly higher than that of the calcein control. However, serum calcein concentration *vs.* time profiles after i.d. administration of w/o/w emulsions prepared with 1.1-μm and 30-μm pore-sized SPGs and an emulsion prepared with a calcein-containing outer water phase were comparable to control profiles. These results suggested that HS-11 or Gelucire 44/14 + PVA are safe outer water phase additives and that 0.1-μm pore-sized SPGs are important for preparing w/o/w emulsions that enhanced intestinal calcein absorption.

1. Introduction

The oral absorption of a drug strongly depends on the water solubility of the drug and on the drug's membrane permeability by passive diffusion, endocytosis, intake *via* transporters, etc. in the intestinal tract. Based on a simple theory involving two parameters—solubility and permeability—the biopharmaceutics classification system (BCS) was proposed as an *in vitro* evaluation method to assess intestinal absorption of various types of compounds [1,2]. Drugs with high solubility and low permeability are classified as BCS class III drugs. Ordinarily, BCS class III drugs are not adapted to oral formulations. Therefore, oral formulations of BCS class III drugs require the addition of an absorption enhancer [3] and/or unique pharmaceutical means of drug delivery [4,5] in order to enhance the bioavailability (*BA*) of the drugs. An example of the latter case is the concept of multiple emulsion formulations [6,7], which generally involves the encapsulation of a highly hydrophilic drug in a water-in-oil-in-water (w/o/w) or a solid-in-oil-in-water (s/o/w) emulsion. The drug contained in the inner water phase is coated with oil, and then dispersed in the outer water phase. If the drug is absorbed directly as an oil droplet from the intestinal tract, the *BA* of the drug will increase remarkably [8]. Technical problems encountered with this pharmaceutical preparation include size optimization, encapsulation, and thermodynamic stability [9]. Although size optimization is easily attained through a high-pressure emulsifier, the encapsulation efficiency of a drug in a multiple emulsion system decreases due to high shearing stress. Recently, a convenient membrane emulsification technique that takes into account both size optimization and drug encapsulation has been developed [10–12]. In this novel procedure, a w/o or s/o emulsion is ejected to the outer phase by low shearing stress using shirasu porous glass (SPG) membranes with a uniform pore size.

The aim of the present study was to prepare a characteristic w/o/w emulsion with safe emulsifiers to enhance the intestinal absorption of calcein, a model BCS class III compound. Although the membrane permeability of calcein may be affected by chelating with metal ion such as calcium, calcein was already used as a model compound for intestinal absorption enhancement evaluation [13] and permeability studies in epithelial membrane [14]. Especially, it is postulated that the influence of metal ion can be ignored in our experiments, that is, 100 mM phosphate-buffered solution, pH 7.4, was used as a solvent in water phase containing calcein.

* Corresponding author. Department of Clinical Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3, Kanagawa-machi, Kanazawa 920-1181, Japan. Tel.: +8176 229 1165; fax: +8176 229 2781.
E-mail address: k-koga@hokuriku-u.ac.jp (K. Koga).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.09.004

Because calcein is difficult to form a chelate with metal ion in the presence of phosphate salts.

2. Materials and methods

2.1. Materials

Condensed ricinoleic acid tetraglycerin ester (CR-310), a hydrophobic emulsifier, was a gift from Sakamoto Yakuhin Kogyo Co., Ltd. (Osaka, Japan). Stearic acid hexaglycerin esters (HS-9 and HS-11), lauric acid decaglycerin ester (L-7D), and stearic acid decaglycerin esters (S-24D and S-28D), all hydrophilic emulsifiers, were gifts from Mitsubishi Kagaku Foods Corp. (Tokyo, Japan). Gelucire 44/14 and Labrasol (Gattefosse Corp., Saint-Priest, France), a hydrophilic emulsifier, were gifts from CBC Co., Ltd. (Tokyo, Japan). Calcein was purchased from Dojindo Laboratories (Kumamoto, Japan). Soybean oil, polyvinyl alcohol (PVA; degree of polymerization was approximately 500), *N*-acetyl-L-cysteine, and polyoxyethylene (10) octylphenyl ether (Triton X-100) were purchased from Wako Pure Chemical Industries (Osaka, Japan). QCU-1, 2-hydroxypropyl methyl methacrylate, water-soluble methacrylate (Quetol-523) and methyl methacrylate were purchased from Nisshin EM (Tokyo, Japan). Other chemicals were of HPLC or reagent grade.

2.2. Animals

Six-week-old Male Sprague Dawley rats were purchased from Sankyo Labo Service Corporation, Inc. (Toyama, Japan). The rats were treated in accordance with the Guidelines for Animal Experimentation from Hokuriku University and were fed standard laboratory chow and had free access to water. Food was withheld from the rats for 16 h before they were used in the absorption studies. For *in vivo* experiments, rats were divided into eight groups, with each group consisting of four rats (250–285 g body weight).

2.3. Preparation of w/o/w emulsion

Calcein solution (10 mg/ml), an inner water phase, was prepared by dissolving calcein with 100 mM phosphate-buffered solution (PBS), pH 7.4, in w/o/w emulsion. Soybean oil (2.50 g) and CR-310 (1.00 g) were mixed at 60 °C for 15 min to equalize, and then the mixture was cooled to room temperature. The soybean oil/CR-310 mixture represented a uniform phase. An aliquot (1.5 g) of calcein solution (10 mg/ml) was put in the oil phase mixture and was homogenized at 16,000 rpm for 3 min using a Polytron (PT-MR 3100, Kinematica AG, Littau/Luzern, Switzerland) to make a w/o emulsion. A w/o emulsion without calcein was also prepared with 100 mM PBS, pH 7.4.

Hydrophilic emulsifier solutions (1.0%, w/v)—i.e., HS-9, HS-11, S-24D, S-28D, L-7D, Gelucire 44/14, and Labrasol—were prepared with 5.0% glucose solution using a bath-type sonicator. The hydrophilic emulsifier solutions (1.0%, w/v) containing PVA (1.0%, w/v) were also prepared with 5.0% glucose solution. These emulsifier solutions were used as an outer water phase in the w/o/w emulsion.

SPG emulsifier apparatus and the setting conditions are shown in Fig. 1. The w/o emulsion (approximately 5 ml) was infused into a stainless steel syringe equipped with a shirasu porous glass (SPG) Membrane Emulsifier (DH-01, SPG Technology Co., Ltd., Miyazaki, Japan). A 20-ml glass vial containing the outer water phase (9.0 ml) was set in the SPG device. Three kinds of SPGs (pore size: 0.1, 1.1, and 30 μm), 2.5 cm length and 8 mm inner diameter, were used to prepare the w/o/w emulsion. The SPG membranes were preliminarily soaked in a beaker with outer water phase and then

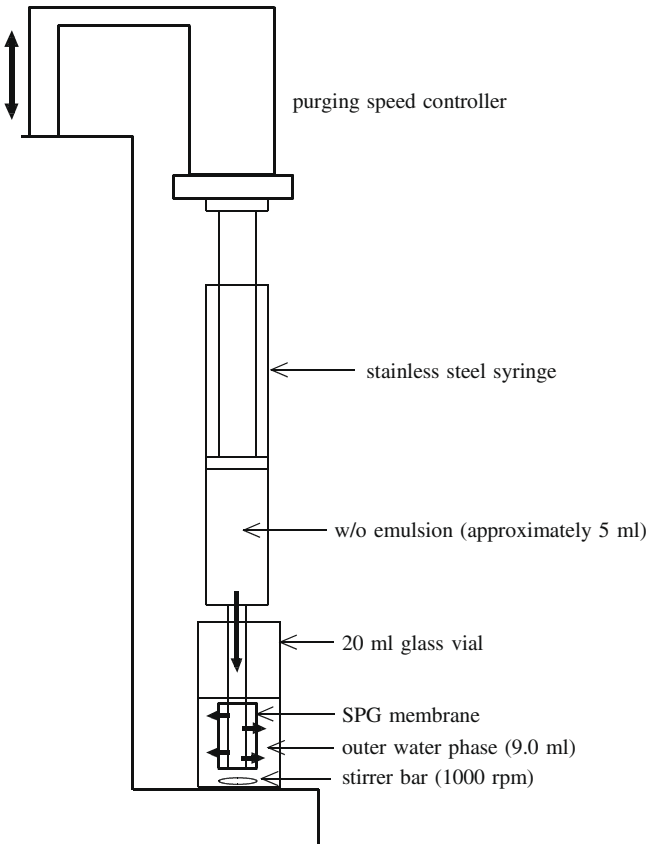

Fig. 1. SPG membrane emulsifier.

set to the emulsifier apparatus. The purging speed of the syringe (the extrusion flow rate of w/o emulsion to the outer water phase) was set to 0.1, 1.0, and 1.0 ml/h for 0.1, 1.1, and 30-μm pore-sized SPG membranes, respectively. The stirring speed of the magnetic bar (15 mm in length) in the 20-ml glass vial was constant at 1000 rpm for all conditions. The working time to prepare the w/o/w emulsion was at least 1.2 h and 12 h for speeds of 1.0 ml/h and 0.1 ml/h, respectively. The prepared w/o emulsions are extruded to outer water phase *via* SPG membrane according to the purging speed of the syringe. The extruded w/o emulsions are dispersed into outer water phase by the shearing stress using a stirrer bar and interfacial tension between outer water phase and w/o emulsion phase.

Preparation processes of w/o/w emulsions encapsulating calcein in inner water phase and w/o/w emulsions containing calcein in outer water phase are shown in Fig. 2. Calcein concentration in both types of w/o/w emulsions was adjusted to 1.0 mg/ml by adding the outer water phase or 5.0% glucose solution containing 1.0% emulsifier. For all formulations, the preparation of w/o/w emulsions in each *in vitro* experiment was carried out by three triplicates.

2.4. Calcein concentration in w/o/w emulsion

Prepared w/o/w emulsion (1.5 ml) and chloroform (2.0 ml) were mixed in a centrifuge tube. The mixture was shaken for 15 min at the maximum speed of a Vortex Shaker (VR-36, Taitec Corp., Koshigaya, Japan), and thereafter, it was centrifuged at 1600*g* for 15 min. A mixture of the water phase (20 μl) and 100 mM PBS, pH 7.4, (180 μl), was aliquoted into the wells of a 96-well plate. The fluorescence intensity of the samples was measured at an excitation (Ex) wavelength of 485 nm and emission

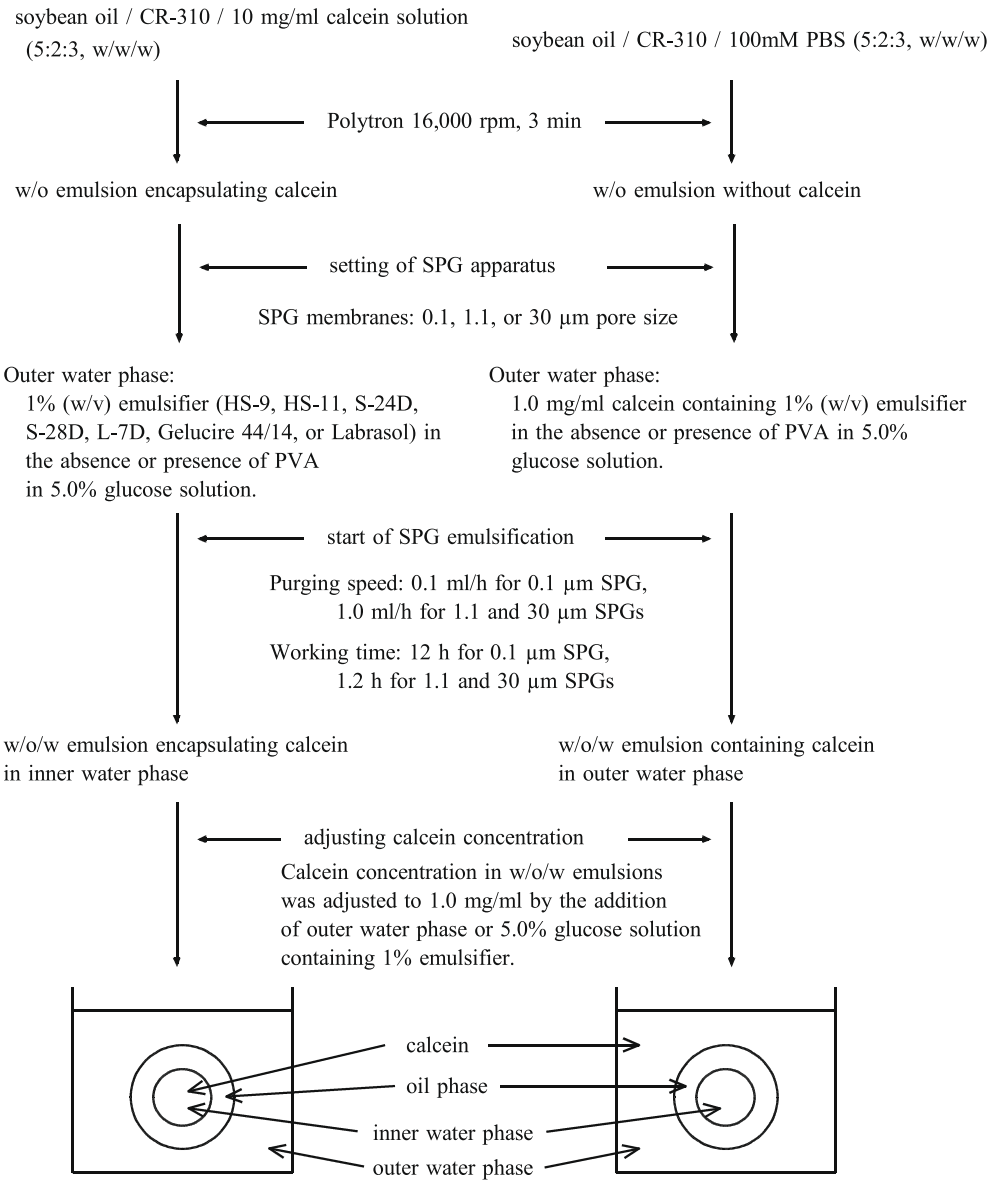

Fig. 2. Preparation processes of w/o/w emulsions.

(Em) wavelength of 527 nm using a Fluorescent Microplate Reader (Fluoreskan Ascent CF, Thermo Labsystems AB, Stockholm, Sweden).

2.5. Leakage of calcein from the inner water phase

The concentrations of calcein that had leaked from the w/o/w emulsion 1 h, 3 and 7 days after the preparation (F_{out}) were measured as the calcein concentration at the bottom phase after separation of the outer water phase and w/o emulsion. Separation was carried out by centrifuging according to a method used to measure encapsulation efficiency [15]. Briefly, the prepared w/o/w emulsion (1.5 ml) was centrifuged at 16,100*g* for 10 min. A mixture of the bottom phase (20 μl) and 100 mM PBS, pH 7.4, (180 μl), was aliquoted into the wells of a 96-well plate. The fluorescence intensities of both samples were measured using a Fluorescent Microplate Reader. Standard calibration samples were prepared based on both experimental processes. Total calcein concentration in w/o/w emulsion (F_{total}) was measured as described in Section 2.4. The F_{total} values were all 1.0 mg/ml as shown in Fig. 2, and each coefficient valiance was less than 3.0%.

The leakage of calcein from the inner water phase of the w/o/w emulsion was calculated using the following equation:

$$\text{Leakage of calcein } (\%) = F_{out}/F_{total} \times 100$$

where F_{out} and F_{total} represent the fluorescence intensities of calcein in the outer water phase and in the total water phase of w/o/w emulsion, respectively.

2.6. Particle size

The particle sizes and weight-weighed average diameter (*dw*)/number-weighed average diameter (*dn*) ratio of oil droplets in w/o/w emulsions prepared using a 0.1-μm pore-sized SPG membrane were analyzed using a Submicron Particle Sizer (NICOMPTM 380, Agilent Technologies, Inc., Santa Barbara, CA, USA) 1 h, 3 and 7 days after the preparation. All formulations 3 and 7 days after the preparation were stirred for 10 s using a vortex mixer, then were mea-

sured the particle sizes. Size polydispersity index (*PI*) is ordinarily calculated by the ratio of first cumulant value (c_1) and second cumulant value (c_2), i.e., $PI = c_2/c_1^2$ [16]. In our experiments, the *dw* and *dn* values calculated using a multimodal method (a cumulant analysis) [17,18] were used as the *PI*. The *dw*/*dn* ratio indicates the degree of polydispersity [19,20]. Namely, the *dw*/*dn* = 1.00 is monodispersity. The size distribution range is broad with an increase in *dw*/*dn* ratio. The particles of oil droplets prepared using 1.1-μm and 30-μm pore-sized SPG membranes were observed using an optical microscope (Axiovert 40 CFL/HBo 50, Carl Zeiss Japan, Tokyo, Japan), and the diameter of particles was calculated with image analysis software, Sigma Scan Pro for Windows (Systat Software Inc., Point Richmond, CA, USA).

2.7. Turbidity of w/o/w emulsion

The w/o/w emulsions containing HS-11, Gelucire 44/14, L-7D, S-24D, or S-28D were prepared according to the method described earlier. As the control sample, w/o/w emulsion without emulsifier in the outer water phase was also prepared. Each prepared w/o/w emulsion was placed into a 10-ml centrifuge glass tube. Immediately, a mixture of the bottom phase (0.1 ml) and 5.0% glucose solution (0.5–2.0 ml) was aliquoted into the wells of a 96-well plate, and the absorbance at 650 nm was measured 0, 1 h, 1, 3, and 7 days after preparation using a Multiplate Reader (Sunrise Rainbow RC, Tecan Japan Co., Ltd., Kawasaki, Japan), according to the method of Pirzadeh et al. [21].

2.8. In vivo experiments

Rats were anesthetized with an intraperitoneal injection of urethane (3.0 g/kg). After the hair of the abdomen and the inner upper arm of the rats were shaved, the rats were kept in a supine position on an operation plate and secured in place with vinyl tape.

For intravenous (i.v.) bolus injection studies, calcein solution (1.0 mg/ml) dissolved in 100 mM PBS, pH 7.4, was administered into the right subclavian vein (0.66 mg/kg). Blood samples (0.25 ml) were collected from the left subclavian vein 2, 5, 15, 30, 45, 60, 120, 180, 240, 360, and 480 min after administration. Once blood samples clotted at room temperature, they were centrifuged at 1400*g* for 15 min in order to obtain serum samples.

For intraduodenal (i.d.) administration studies, the abdominal incision of rats was made with a surgical knife, and the duodenum was carefully exteriorized. Calcein solution (1.0 mg/ml) dissolved in 5.0% glucose solution (for the control group) and w/o/w emulsion containing calcein (1.0 mg/ml as the concentration in w/o/w emulsion) were administered into the duodenum (1.0 mg/kg) with a 1.0-ml syringe and 22G 1/2″ needle. The duodenum was immediately returned to the abdominal cavity, and the abdominal incision was closed with a surgical clip. Blood samples (0.25 ml) were collected from the left subclavian vein 0.5, 1, 2, 3, 4, 6, and 8 h after administration. Serum samples were obtained as described earlier. The w/o/w emulsions were administered to rats within 2 h after the solutions were prepared with the SPG device.

2.9. Calcein concentration in serum

A mixture of serum samples (100 μl), 100 mM PBS, pH 7.4, (80 μl), and 10% Triton X-100 solution (20 μl) was aliquoted into the wells of a 96-well plate. After shaking, the fluorescence intensity was measured as described earlier.

2.10. Calculation of bioavailability

The *BA* of calcein after i.d. administration was calculated using the following equation:

$$BA\ (\%) = (AUC_{id} \times D_{iv})/(AUC_{iv} \times D_{id}) \times 100$$

where AUC_{iv} and AUC_{id} represent the area under the serum calcein concentration *vs.* time curve (*AUC*) after i.v. and i.d. administration, respectively. The *AUC* was calculated using the trapezoidal rule up to 8 h and with serum calcein concentration. D_{iv} and D_{id} represent the calcein dose per kg when the calcein solutions were administered i.v. and i.d., respectively.

2.11. Histopathology

Pharmaceutical formulations with surfactants induce infrequently mucosal damage, i.e. local toxicity, in intestinal tract [22]. For the mucosal damage, histopathological assessment is clear observation, but not enough for cytotoxicity studies. We carried out the studies of mucosal damage according to the method of Miyake et al. [23]. A portion of the intestine (approximately 3 cm in length) from the middle of the rat's small intestine was carefully excised 8 h after administering 5.0% glucose solution (control) and three different kinds of w/o/w emulsions, which were prepared by adding HS-11, Gelucire 44/14, or Gelucire 44/14 + PVA to 5.0% glucose solution. As a positive control for mucosal damage, a mixture of *N*-acetyl-L-cysteine (NAC) and Triton X-100 was administered to rats according to the method of Takatsuka et al. [24]. Concretely, the mixture solution (0.25 ml) containing 5 mg NAC and 5 mg Triton X-100 was infused into rat jejunum. The portion of jejunum segments exposed to the mixture solution was carefully excised at 1 h after the administration and then was washed with 5.0% glucose solution (3 ml). All the dissected intestines were cut into 5-mm pieces with a stainless steel razor blade (FH-20, Feather, Osaka, Japan). All pieces were placed in a fixative solution (4.0% paraformaldehyde containing 1.0% glutaraldehyde dissolved in PBS, pH 7.4) for 4 h at room temperature and agitated constantly. After discarding the fixative, the intestine pieces were sequentially incubated in the following solutions under constant agitation: PBS for 30 min, 50% ethanol for 30 min, 70% ethanol for 1 h, 90% ethanol for 1 h, 95% ethanol for 1 h, 100% ethanol for 1 h, ethanol/HQM (1:1, v/v) for 1 h, and HQM for 4.5 h. HQM is a mixture of hydroxypropyl methyl methacrylate/Quetol-523/methyl methacrylate/QCU-1 (65:10:25:0.1, volume ratio). All of these procedures were carried out in a total volume of 10 ml at room temperature. Each piece of intestine was placed into a flat gelatin capsule (No. 00, Lilly Co., Nisshin EM) filled with HQM, then cured for 20 h at 60 °C. The resulting block of embedded tissue was cut into 2-μm thick sections with a glass knife and rotary microtome (PR-50, Yamato Kohki Kogyo, Saitama, Japan). Sections were mounted onto glass slides and then stained with hematoxylin and eosin (H&E). After drying, we assessed the stained intestinal sections for pathological changes and obtained images using a microscope equipped with a digital camera (Axiovert 40 CFL/HBo 50, Carl Zeiss Japan, Tokyo, Japan).

2.12. Statistical analysis

Statistical analyses were conducted using Tukey test for multiple comparisons and using Student's *t*-tests for comparisons between two groups. The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Effect of emulsifier on droplet size in w/o/w emulsions

The droplet size in emulsions is affected by the type of emulsifier [25] and the blend ratio of two types of emulsifiers [26]. In fact, the initial (1 h after each preparation) droplet sizes in w/o/w emulsions prepared using 0.1-μm pore-sized SPG membranes were dif-

ferent as shown in Table 1. The initial droplet sizes in emulsions prepared by adding HS-9, S-28D, or S-24D were remarkably larger than that in the absence of emulsifier. Those in emulsions prepared by adding Gelucire 44/14, Labrasol, or L-7D were similar to that in the absence of emulsifier. The initial droplet size in the emulsion containing HS-11 was the smallest, being similar to the SPG membrane pore size (0.1 μm). It was reported that the droplet size and size distribution in emulsions prepared using SPG membranes were strongly related with membrane wettability (contact angle between the SPG membrane surface and dispersed phase) [27]. They concluded that the droplet size is independent on the membrane pore size in the condition of contact angle >45°. Therefore, it is suggested that the reason that HS-9, S-28D, and S-24D were inconvenient for the preparation of nano-sized emulsions using SPG membranes is related to the membrane wettability.

In general, the droplet size in emulsions increases gradually through the process of aggregation and coalescence of dispersed phase with the progress of the time. Actually, the droplet sizes 7 days after the preparations of w/o/w emulsions increased compared to those of initial droplet sizes. Especially, the size distribution of w/o/w emulsions with HS-9, S-28D, HS-11, S-24D, or PVA showed two peaks, suggesting that aggregation or coalescence of oil droplet was observed. When w/o/w emulsions with HS-9, S-28D, HS-11, S-24D, or PVA 7 days after the preparation were dispersed using a bath-type sonicator for 3 min, the average particle size of emulsion with HS-11 only was remarkably shifted to small size (average diameter 326 ± 184 nm). On the other hand, no pronounced differences were observed in emulsions with HS-9, S-28D, S-24D, or PVA. From these results, it is predicted that a part of aggregated emulsion with HS-11 was reversibly dispersed by thermodynamic force using a sonicator. And coalescence of oil droplets may be strongly occurred for emulsions with HS-9, S-28D, S-24D, or PVA. Although the leakage of calcein from inner water phase in w/o/w emulsions may increase with operation such as sonication, the aggregation of oil droplets will be prevented reversibly. These results suggested that HS-11 was the best emulsifier among the seven emulsifiers tested under our experimental conditions. Droplet size in emulsion is determined by the oil component and surfactant but not physicochemical parameters, such as temperature, viscosity, and interfacial tension [28]. For our w/o emulsion, soybean oil and CR-310 were used as the oil component and the hydrophobic emulsifier, respectively. Therefore, we presumed that the oil droplet size in the w/o/w emulsion could be determined by adding a hydrophilic emulsifier to the outer water phase. Furthermore, the oil droplet size in w/o/w emulsion also depends on the hydrophilic relationship between the SPG surface and components in the outer water phase. If the outer water phase components decrease the hydrophilicity of the SPG surface, then the contact angle of the oil droplet will also increase. Therefore, when w/o emulsions are passed through a SPG membrane to the outer water phase, the droplet size in w/o/w emulsions will increase from the theory of membrane wettability [27].

PVA reportedly acts as a stabilizer for the preparation of nanoparticles [29–31]. To examine how PVA affects the particle size of oil droplets in w/o/w emulsions, we prepared w/o/w emulsions containing either HS-11 + PVA or Gelucire 44/14 + PVA. We observed no difference in the initial average particle size of oil droplets in w/o/w emulsions containing HS-11 and those containing HS-11 + PVA (Table 1), suggesting that the average particle sizes of both emulsions were the same as the SPG pore size (0.1 μm) and that it was difficult to decrease the droplet size to less than 0.1 μm. However, 7 days after the preparation, the average particle size in w/o/w emulsion with HS-11 + PVA was half the size of that in w/o/w emulsions with HS-11. Therefore, it appeared that PVA did not affect the initial droplet size in w/o/w emulsions containing HS-11, but inhibited the increase in apparent particle size, aggregation, 7 days after the preparation. On the other hand, the initial average particle size in the w/o/w emulsion containing Gelucire 44/14 + PVA was 143 ± 29 nm, which was half the size of particles in the w/o/w emulsion containing Gelucire 44/14, moreover, 7 days after the preparation, the average particle size in w/o/w emulsion with Gelucire 44/14 + PVA was half the size of that in w/o/w emulsions with Gelucire 44/14, suggesting that PVA promoted the release of small droplets in the w/o emulsion from inside of the SPG membrane to the outer water phase and inhibited an increase in particle size in time dependent. One possible reason for the decreased droplet size is that the outer water phase containing Gelucire 44/14 + PVA decreased the contact angle of the oil droplet onto the SPG surface. From these results, we concluded that HS-11 and Gelucire 44/14 were good hydrophilic emulsifiers for preparing w/o/w emulsions.

Table 1
Average particle size and size distribution of w/o/w emulsions prepared using a 0.1-μm pore-sized SPG membrane.

Emulsifier	HLB	m.p. (°C)	Average particle size (nm)			*dw*/*dn*
			1 h	3 days	7 days	1 h
None	–	–	322 ± 65	2047 ± 1021	3975 ± 1279	1.02
HS-9	9	50–55	1055 ± 179	1272 ± 280[a]	1360 ± 355[a]	1.03
S-28D	10	50–55	1750 ± 229	1992 ± 549[a]	2003 ± 839[a]	1.02
HS-11	11	50–55	105 ± 20	491 ± 166	638 ± 322[a]	1.10
S-24D	11	50–55	788 ± 166	1055 ± 446[a]	1237 ± 551[a]	1.03
Gelucire 44/14	14	42–46	316 ± 63	501 ± 121	534 ± 115	1.01
Labrasol	14	<0	376 ± 76	380 ± 94	388 ± 86	1.08
L-7D	17	<0	350 ± 58	429 ± 105	572 ± 217	1.02
PVA	–	>300	319 ± 66	883 ± 107	1212 ± 876[a]	1.01
HS-11 + PVA	–	–	117 ± 25	242 ± 79	324 ± 186	1.08
Gelucire 44/14 + PVA	–	–	143 ± 29	206 ± 103	284 ± 116	1.09

HLB, hydrophile–lipophile balance; *dw* and *dn* represent weight-weighed average diameter and number-weighed average diameter, respectively. Melting point (m.p.) is softened or solidified temperature of compound. PVA (Polymerization degree about 500) was used as an emulsion stabilizer. The particle sizes were determined 1 h, 3 and 7 days after the preparation. The *dw*/*dn* values were calculated from the particle sizes of 1 h after the preparation. Average particle sizes represent means ± SD of three determinations for three different preparations.

[a] Two peaks were observed in size distribution.

3.2. Effect of emulsifier on calcein release from the inner water phase

Table 2 shows the leakage of calcein from the inner water phase of various w/o/w emulsions encapsulating calcein. When an emulsifier was not added to the outer water phase, calcein leakages from the w/o/w emulsion at 3 and 7 days were 38.2 ± 4.4% and 45.6 ± 4.3%, respectively. Calcein leakages in w/o/w emulsions with HS-9, S-28D, S-24D, Gelucire 44/14, PVA, or Gelucire 44/14 + PVA 3 and 7 days after the preparations were significantly low compared with that in w/o/w emulsions without surfactant in outer water phase. The results shown in Tables 1 and 2 indicate that calcein leakage increased as droplet size in the w/o/w emulsion decreased. There is an interesting theoretical relationship between particle size and calcein leakage except for w/o/w emulsion with Gelucire 44/14: when the volume of the oil phase remains the same, the surface area tangent to the outer water phase increases as droplet size of the emulsion decreases. Therefore, calcein leakage from w/o/w emulsions containing HS-9, S-28D, or S-24D was small compared to calcein leakage from those containing other emulsifiers.

Calcein leakage from oil droplets in the w/o/w emulsion containing Gelucire 44/14 decreased remarkably at 1 h, 3 and 7 days. However, the calcein leakage from emulsions containing Gelucire 44/14 + PVA was greater than that from emulsions containing Gelucire 44/14. Although the inhibitory effect of PVA on calcein release from oil droplets in the w/o/w emulsions was not observed,

Table 2
Leakage of calcein from the inner water phase of w/o/w emulsions encapsulating calcein 1 h, 3 and 7 days after the preparation.

Emulsifier	Leakage of calcein (%)		
	1 h	3 days	7 days
None	12.7 ± 3.2	38.2 ± 4.4	45.6 ± 4.3
HS-9	5.8 ± 1.4*	12.2 ± 2.0*	16.6 ± 2.3*
S-28D	4.7 ± 0.9*	9.6 ± 1.9*	11.7 ± 2.1*
HS-11	18.2 ± 3.7	33.5 ± 5.1	40.1 ± 3.9
S-24D	5.0 ± 0.6*	9.8 ± 1.6*	13.0 ± 2.4*
Gelucire 44/14	3.3 ± 0.8*	7.9 ± 1.2*	9.4 ± 1.8*
Labrasol	10.4 ± 2.8	22.0 ± 6.8	26.1 ± 3.3
L-7D	11.2 ± 3.3	35.5 ± 6.7	41.9 ± 5.4
PVA	11.8 ± 3.5	18.2 ± 4.6*	21.4 ± 2.5*
HS-11 + PVA	10.1 ± 4.2	36.9 ± 4.4	43.3 ± 4.8
Gelucire 44/14 + PVA	8.3 ± 2.2	20.4 ± 4.8*	26.3 ± 1.7*

A 0.1-μm pore-sized SPG membrane was used to prepare the w/o/w emulsions. PVA was used as an emulsion stabilizer. Data represent the means ± SD of three determinations for three different preparations.
* $p < 0.05$ *vs.* emulsifier none group by Tukey test.

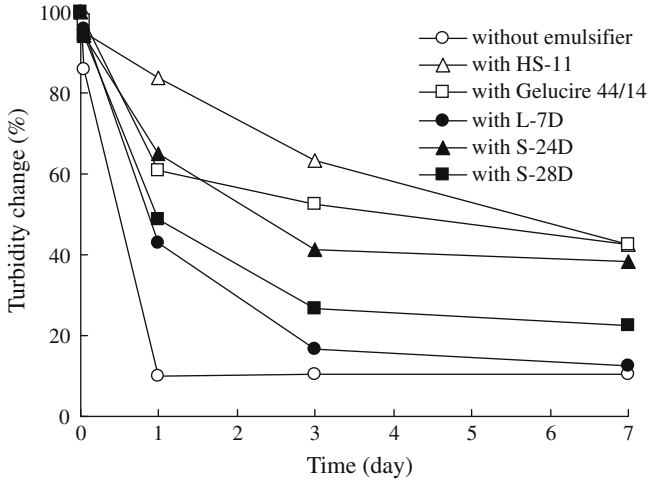

Fig. 3. Turbidity changes in w/o/w emulsions in the absence and presence of emulsifiers. Each surfactant (1.0%, w/v) was added to outer water phase for the preparation of w/o/w emulsions encapsulating calcein. Calcein concentrations in w/o/w emulsions were all adjusted to 1.0 mg/ml. The without emulsifier formulation indicated in symbol does not contain a surfactant in outer water phase. Each data point represents the mean of three determinations for three different preparations. Standard deviations were too small to display.

the theory between particle size and calcein leakage also can be used to explain the increase in calcein leakage from the w/o/w containing Gelucire 44/14 + PVA. Furthermore, calcein leakage from oil droplets in the w/o/w emulsion containing HS-11 was similar to that from the w/o/w emulsion lacking an emulsifier, even though the droplet size of the HS-11 w/o/w emulsion was very small. This suggests that HS-11 inhibits calcein release from oil droplets in the w/o/w emulsion. Especially, the calcein leakages in w/o/w emulsions with Gelucire 44/14 and with HS-11 were relatively related to not only the average droplet size but also the distribution characteristics of surfactants maintaining tightly w/o particles in w/o/w emulsions.

From the calcein leakage results, we concluded that Gelucire 44/14 was the best emulsifier for preparing w/o/w emulsions.

3.3. Turbidity changes in w/o/w emulsions

In general, we observed phase behavioral changes, such as creaming, aggregation, and coalescence of droplets in emulsions under steady-state conditions. Especially, nano-sized emulsions are thermodynamically unstable compared to micron-sized emulsions [9]. Nano-sized soybean oil droplets encapsulating the water phase (w/o emulsions) were thought to move toward the upper phase during steady-state conditions. Thereafter, the appearance of the bottom phase of the w/o/w emulsion may gradually change from being white turbid to translucent or transparent. In fact, the turbidity of the bottom phase in the w/o/w emulsion lacking emulsifiers rapidly decreased (Fig. 3). On the other hand, the turbidity of the bottom phase in w/o/w emulsions containing emulsifiers slowly decreased, depending on the time elapsed. The turbidity changes of emulsions containing HS-11 in experimental duration were less than those of the control sample and emulsions with other emulsifiers. Moreover, the turbidity change in the emulsions with HS-11 showed a characteristic profile, that is, the turbidity change proportionately decreased dependent on the time. The differences of the turbidity change between 3 days and 7 days in emulsions with HS-11 and Gelucire 44/14 were 21.0% and 9.7%, respectively. In contrast, the differences of the turbidity change of emulsions with L-7D, S-24D, or S-28D were less than 4% in the same interval of 3–7 days. These results indicated that the movement toward the upper phase (creaming) of oil droplets in emulsions with HS-11 was slower than those in other emulsions. The turbidity change may be related to the aggregation mechanism of oil droplets. Because a part of emulsions with HS-11 was redispersed with sonication (Section 3.1). Namely, it seems that oil droplets in emulsions with HS-11 might be difficult to be mutually interacted compared with emulsions containing other emulsifiers. Thus, it is considered that the interparticle interaction of oil droplets is closely related to the stability of emulsions. Therefore, it is concluded that HS-11 enhanced the thermodynamic stability of the w/o/w emulsions in this experimental section.

3.4. Effect of emulsifier on intestinal absorption of calcein

On the basis of the droplet size results, calcein leakage, and turbidity changes in w/o/w emulsions containing various emulsifiers, we selected w/o/w emulsions containing HS-11, Gelucire 44/14, and Gelucire 44/14 + PVA for the *in vivo* absorption experiments in rats. Calcein solution (1.0 mg/ml) was used as a control formulation. Stability of the w/o/w emulsions such as prevention of calcein leakage must be guaranteed in *in vivo* experiments. Because the calcein leakage from w/o droplets will be a negative factor for the absorption-enhancing effect of emulsifiers on the intestinal absorption of calcein in rats. The w/o/w emulsions were administered to rats within 2 h after the preparation based on the method that the sampling period in pharmacokinetics studies was set to 8 h. Namely, it was considered that the influence of stability of the w/o/w emulsions for the intestinal absorption of calcein will be almost disregarded based on the results of changes in turbidity after 24 h.

Fig. 4 shows the serum calcein concentration *vs.* time profile after i.d. administration of 1.0 mg/kg calcein solution and three kinds of w/o/w emulsions encapsulating calcein. After the administration of calcein solution (control), the maximum serum calcein concentration, C_{max}, was 13.7 ± 5.4 ng/ml. Thereafter, calcein gradually disappeared from the systemic circulation. The serum calcein concentration *vs.* time profile obtained after the administration of w/o/w emulsion containing Gelucire 44/14 was similar to that obtained from the control experiment. On the other hand, serum calcein concentrations after administration of w/o/w emulsions containing either HS-11 or Gelucire 44/14 + PVA were higher than those obtained from the other formulations. Especially, C_{max} of calcein from the HS-11 and Gelucire 44/14 + PVA formulations were 118 ± 47 ng/ml and 88.5 ± 20.3 ng/ml 90 min and 30 min after administration, respectively. Furthermore, *BA* values after the administration of calcein solution and w/o/w emulsions containing

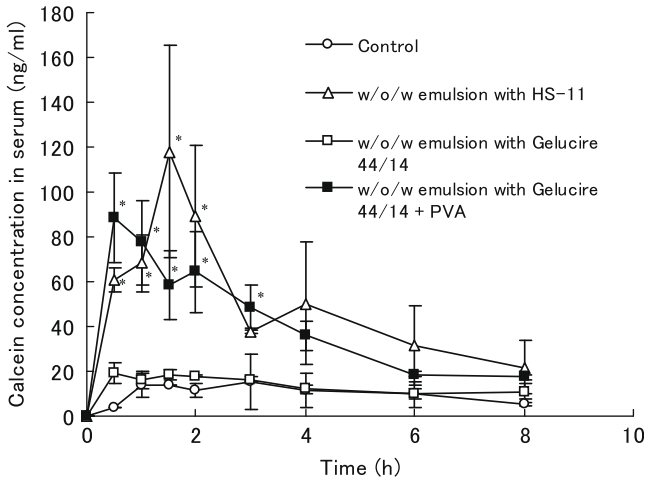

Fig. 4. Serum calcein concentration *vs.* time profiles after intraduodenal administration into rats. The dose of calcein was 1.0 mg/kg. Each surfactant (1.0%, w/v) and PVA (1.0%, w/v) was added to outer water phase. Control formulation was 1.0 mg/ml calcein solution, and others were w/o/w emulsions encapsulating calcein. Calcein concentrations in w/o/w emulsions were all adjusted to 1.0 mg/ml. Each data point with error bar represents the mean ± SD of four experiments for same preparation. $^{*}p < 0.05$ *vs.* control group; Tukey test.

HS-11, Gelucire 44/14, or Gelucire 44/14 + PVA were 1.84 ± 0.4%, 12.2 ± 5.9%, 2.8 ± 0.5%, and 8.0 ± 3.3%, respectively. The w/o/w emulsions containing either HS-11 or Gelucire 44/14 + PVA significantly enhanced calcein absorption from rat small intestine. These results suggest that the absorption-enhancing effect of emulsifiers on the intestinal absorption of calcein may be ascribed to the droplet size effect in the w/o/w emulsions but not to the type of emulsifiers used, because the serum calcein concentrations were enhanced by the w/o/w emulsion containing Gelucire 44/14 + PVA (initial average particle size: 143 nm) but not by the w/o/w emulsion containing Gelucire 44/14 (initial average particle size: 316 nm). It is difficult to clarify whether PVA inhibited the release of calcein from w/o droplets in the w/o/w emulsions or not. Because from the results in Tables 1 and 2, the difference of the particle size of w/o droplets will affect certainly the calcein leakage from the w/o droplets. As the theoretical hypothesis based on calculation, the ratio of both average particle sizes was 2.2 (316/143). Since the volume of oil phase in w/o/w emulsions is same, the ratio of surface area per droplet was 4.8 (as the surface of one droplet's sphere), the ratio of oil droplet number per w/o/w emulsion volume was 0.094 (as a volumetric ratio which divided w/o emulsion volume by one droplet volume), and the ratio of total surface area per w/o emulsion volume was calculated to be 0.45. On the other hand, the ratio of initial calcein leakage in w/o/w emulsions with Gelucire 44/14 in the absence and presence of PVA was 0.40 (=3.3/8.3). The initial calcein leakage ratio (0.40) calculated from both w/o/w emulsions with different average particle size was similar to the total surface area ratio (0.45). Therefore, the calculated data suggested that PVA did not significantly inhibit the leakage of calcein from the w/o droplets. We thus predicted, based on droplet size, that oil droplets in the w/o/w emulsion containing Gelucire 44/14 may be unstable as the phase behavioral changes such as aggregation in the intestinal tract. Moreover, PVA worked not only as an emulsifier stabilizer, but also maintained the size of oil droplets in the intestinal tract after i.d. administration.

3.5. Effect of droplet size on intestinal absorption of calcein

How the emulsifiers influenced the intestinal absorption of calcein in rats led us to focus on the droplet size in w/o/w emulsion. Thus, we performed comparative studies investigating the effect of droplet size in w/o/w emulsions containing Gelucire 44/14 + PVA, but using different pore-sized SPG membranes. The average particle sizes of oil droplets in w/o/w emulsions prepared using 0.1-, 1.1-, and 30-μm pore-sized SPG membranes were 0.143 ± 0.029, 5.15 ± 1.02, and 13.9 ± 3.3 μm, respectively. Fig. 5 shows the serum calcein concentration *vs.* time profile after i.d. administration of different sized w/o/w emulsions encapsulating calcein (1.0 mg/kg calcein). After administration of w/o/w emulsions prepared with 1.1-μm and 30-μm pore-sized SPG membranes, the serum calcein concentrations were significantly lower than that obtained with a 0.1-μm pore-sized SPG membrane. The serum calcein concentration *vs.* time profiles after the administration of emulsions prepared with 1.1-μm and 30-μm pore-sized SPG membranes were almost the same as that obtained with calcein solution (Fig. 4). These results clearly show that oil droplet size is an important factor for enhancing the absorption of calcein. There are two possible absorption-enhancing mechanisms to explain how the oil droplets encapsulating calcein in w/o/w emulsion were rapidly absorbed: (1) via a paracellular route, and/or (2) via an intracellular route. Nanoparticles prepared with chitosan, as a drug carrier, are effectively permeated via a paracellular pathway [32,33], whereas latex nanoparticles [34] and nanogel containing folate [35] are remarkably permeated via a transcellular pathway. To the best of our knowledge, reports concerning the intestinal absorption mechanism of drugs through w/o/w emulsions do not exist. Our results, however, are supported by the finding that insulin absorption by rat small intestine depends on droplet size in a w/o/w emulsion [36]. Therefore, we concluded that the absorption-enhancing effect of w/o/w emulsions on calcein was strongly dependent on the oil droplet size.

3.6. Effect of encapsulating site on intestinal absorption of calcein

The observation that oil droplet size in w/o/w emulsions determined the absorption-enhancing effect on calcein from the rat small intestine prompted us to investigate how the encapsulating site affects the intestinal absorption of calcein under constant droplet size conditions. Two w/o/w emulsion formulations—(1) encapsulating calcein in the inner water phase and (2) dissolving calcein in the outer water phase—were prepared using a 0.1-μm

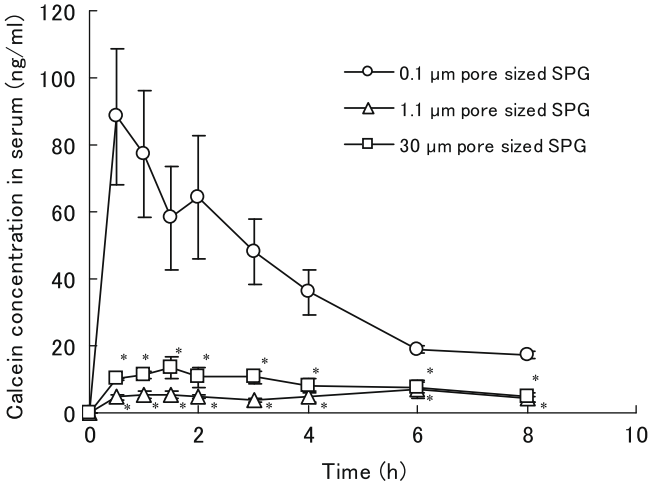

Fig. 5. Serum calcein concentration *vs.* time profiles in rats after intraduodenal administration of w/o/w emulsions containing Gelucire 44/14 + PVA prepared using three kinds of SPG membranes (0.1, 1.1, and 30 μm pore size). All formulations were w/o/w emulsions encapsulating calcein. Calcein concentrations were all adjusted to 1.0 mg/ml. The dose of calcein was 1.0 mg/kg. Each data point with error bar represents the mean ± SD of four experiments for same preparation. $^{*}p < 0.05$ *vs.* 0.1-μm pore-sized SPG group; Tukey test.

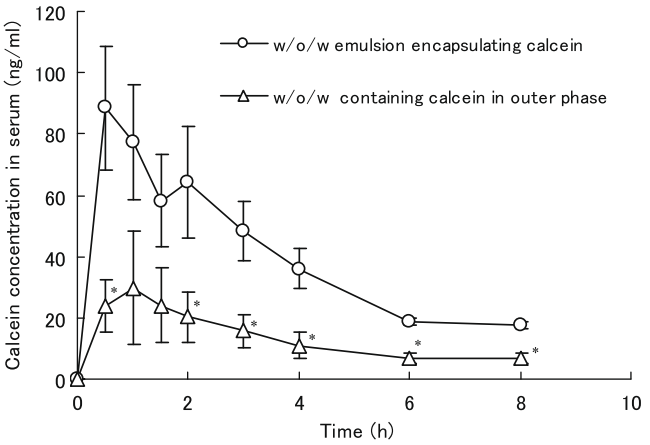

Fig. 6. Serum calcein concentration *vs.* time profiles in rats after intraduodenal administration of w/o/w emulsion encapsulating calcein and w/o/w emulsion in which calcein was dissolved in the outer water phase. The w/o/w emulsions were prepared by adding Gelucire 44/14 + PVA to outer water phase. Calcein concentrations were all adjusted to 1.0 mg/ml. The dose of calcein was 1.0 mg/kg. Each data point with error bar represents the mean ± SD of four experiments for same preparation. $^{*}p < 0.05$; Student's t-test.

pore-sized SPG membrane. The purpose of this experiment was to determine whether the encapsulation of calcein in w/o/w emulsion is critical for enhancing the intestinal absorption of calcein. Fig. 6 shows the serum calcein concentration *vs.* time profiles after administration of the w/o/w emulsions prepared by adding calcein in the inner or outer water phase. The calcein concentration in serum after the administration of w/o/w emulsion containing calcein in the outer water phase was significantly low compared to that of the w/o/w emulsion encapsulating calcein, almost similar to the control experiment (calcein solution; Fig. 4). *BA* value after the administration of the w/o/w emulsion containing calcein in outer water phase was 1.4-fold of that after the administration of control formulation, and was 0.33-fold of that after the administration of the w/o/w emulsion encapsulating calcein. Therefore, it became clear that calcein must be encapsulated into the oil droplets to enhance the intestinal absorption of calcein. On the other hand, there are reports that medium chain fatty acids such as caprylate (C8), caprate (C10) and laurate (C12) enhanced the intestinal membrane permeability of hydrophilic compounds *via* paracellular route [37,38]. However, soybean oil consists of oleic acid, linoleic acid, linolenic acid, palmitic acid, and their glycerides. Therefore, it is thought the difficulty that the permeation of oil droplets encapsulating calcein was enhanced *via* paracellular route in rat intestinal tract.

The finding that serum calcein concentration increased due to the encapsulation of calcein suggested that oil droplets encapsulating calcein permeated the intestinal mucosa *via* a transcellular pathway in rat. This idea is consistent with the finding that phagocytosis, one transcellular pathway, represents the absorption pathway of oil droplets, such as soybean oil, are absorbed by the

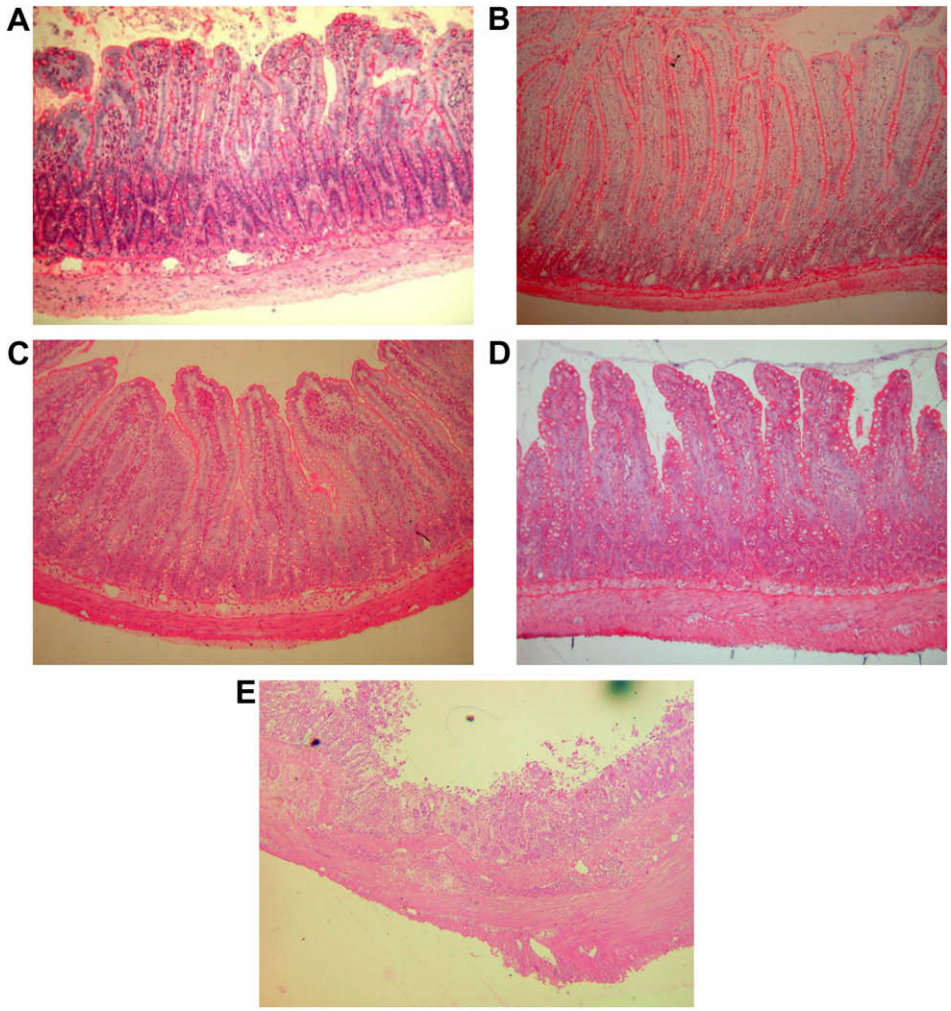

Fig. 7. Representative histological sections of intestinal segments treated according to (A) control, (B) HS-11, (C) Gelucire 44/14, (D) Gelucire 44/14 + PVA, and (E) positive control conditions (H&E stain × 100). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper).

intestinal tract, liver, spleen, abdominal cavity, etc. [39,40]. Although the precise absorption mechanism of the prepared nano-sized w/o/w emulsions from rat small intestine is unknown in the present studies, on the basis of the hydrophobic characteristic of oil droplets, we believe that nano-sized emulsion encapsulating calcein may be mainly absorbed *via* a transcellular pathway. Further investigations are required to prove this concept, and we focus attention on the problem in the next experimental theme.

3.7. Histopathological observations

Gelucire 44/14 is a safe surfactant that conforms to the *2005 European Pharmacopoeia 5th Edition* and that has been used in globally approved pharmaceutical products. HS-11 is also a safe surfactant that has been used as a common food additive. However, we have to consider the mucosal damage by these emulsifiers in the intestinal tract. Some surfactants damage the intestinal epithelium and show enhanced membrane permeability; however, the damage is reversible [24]. The permeability of calcein through damaged intestinal mucosa is higher than that through intact epithelium. Therefore, we examined the damage in epithelial membranes through histopathological analyses.

Fig. 7(A–D) shows images of intestinal segments 8 h after i.d. administration of 5.0% glucose solution (control), and after i.d. administration of w/o/w emulsions containing HS-11, Gelucire 44/14, or Gelucire 44/14 + PVA. As a positive control of mucosal damage, the image of intestinal segment 1 h after i.d. administration of solution containing NAC and Triton X-100 was observed as a decidual alteration in the epithelial membranes (Fig. 7E). On the other hand, the epithelial membranes treated with the three kinds of w/o/w emulsions were free of gross damage, such as abrasion and atrophy of the epithelium, strongly suggesting that the absorption-enhancing effect of w/o/w emulsions containing HS-11 and Gelucire 44/14 + PVA does not cause mucosal damage.

4. Conclusions

The present study investigated the formulation design of w/o/w emulsions with the purpose of enhancing intestinal absorption of calcein, a model BCS class III compound. The oil droplet sizes in the w/o/w emulsions containing different hydrophilic emulsifiers showed remarkable differences when 0.1-μm pore-sized SPG membranes were used to prepare the emulsions. PVA efficiently refined the size of oil droplets in the w/o/w emulsion containing Gelucire 44/14. We performed *in vivo* rat absorption studies and evaluated the resulting serum calcein concentration *vs.* time profiles after i.d. administration of w/o/w emulsions containing HS-11 and Gelucire 44/14 as hydrophilic emulsifiers. The results of these analyses showing the effects of oil droplet size and encapsulation in the w/o/w emulsion system on the intestinal absorption of calcein clearly demonstrated that HS-11 and Gelucire 44/14 + PVA are safe outer water phase additives and that using a 0.1-μm pore-sized SPG membrane is important for preparing w/o/w emulsions capable of enhancing the intestinal absorption of calcein.

Acknowledgements

The authors wish to thank Fuji Yakuhin Co., Ltd. for analyzing particle size. This work was supported in part by the Special Research Fund of Hokuriku University.

References

[1] G.L. Amidon, H. Lennernas, V.P. Shah, J.R. Crison, A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability, Pharm. Res. 12 (1995) 413–420.
[2] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Guidance for Industry, Waiver of in vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System, FDA/CDER, Washington DC, 2000, pp. 1–13.
[3] S. Wang, M. Sun, Q. Ping, Enhancing effect of labrafac lipophile WL 1349 on oral bioavailability of hydroxysafflor yellow A in rats, Int. J. Pharm. 358 (2008) 198–204.
[4] K. Takada, Oral delivery of hematopoietic factors: progress with gastrointestinal mucoadhesive patches, microdevices, and other microfabrication technologies, Am. J. Drug Del. 4 (2006) 65–77.
[5] D.D. Deshmukh, W.R. Ravis, G.V. Betageri, Improved delivery of cromolyn from oral proliposomal beads, Int. J. Pharm. 358 (2008) 128–136.
[6] E. Toorisaka, H. Ono, K. Arimori, N. Kamiya, M. Goto, Hypoglycemic effect of surfactant-coated insulin solubilized in a novel solid-in-oil-in-water (S/O/W) emulsion, Int. J. Pharm. 252 (2003) 271–274.
[7] G.M. Tedajo, S. Bouttier, J. Fourniat, J.-L. Grossiord, J.P. Marty, M. Seiller, Release of antiseptica from the aqueous compartments of a w/o/w multiple emulsion, Int. J. Pharm. 288 (2005) 63–72.
[8] E. Acosta, Bioavailability of nanoparticles in nutrient and nutraceutical delivery, Curr. Opin. Colloid Interf. Sci. 14 (2009) 3–15.
[9] M. Porras, C. Solans, C. Gonzalez, J.M. Gutierrez, Properties of water-in-oil (W/O) nano-emulsions prepared by a low-energy emulsification method, Colloids Surf. A Physicochem. Eng. Asp. 324 (2008) 181–188.
[10] M. Kukizaki, Y. Baba, Effect of surfactant type on microbubble formation behavior using Shirasu porous glass (SPG) membranes, Colloids Surf. A Physicochem. Eng. Asp. 326 (2008) 129–137.
[11] F. Ito, H. Honnami, H. Kawakami, K. Kanamura, K. Makino, Preparation and properties of PLGA microspheres containing hydrophilic drugs by the SPG (shirasu porous glass) membrane emulsification technique, Colloids Surf. B Biointerf. 67 (2008) 20–25.
[12] R. Liu, G. Ma, F. Meng, Z. Su, Preparation of uniform-sized PLA microcapsules by combining Shirasu porous glass membrane emulsification technique and multiple emulsion-solvent evaporation method, J. Control. Release 103 (2005) 31–43.
[13] P.P. Constantinides, G. Welzel, H. Ellens, P.L. Smith, S. Sturgis, S.H. Yiv, A.B. Owen, Water-in-oil microemulsions containing medium-chain fatty acids/salts: formulation and intestinal absorption enhancement evaluation, Pharm. Res. 13 (1996) 210–215.
[14] E.B. Ghartey-Tagoe, J.S. Morgan, A.S. Neish, M.R. Prausnitz, Increased permeability of intestinal epithelial monolayers mediated by electroporation, J. Control. Release 103 (2005) 177–190.
[15] M. Shima, Y. Kobayashi, T. Fujii, M. Tanaka, Y. Kimura, S. Adachi, R. Matsuno, Preparation of fine w/o/w emulsion through membrane filtration of coarse w/o/w emulsion and disappearance of the inclusion of outer phase solution, Food Hydrocolloid. 18 (2004) 61–70.
[16] N. Maurer, E. Prenner, F. Paltauf, O. Glatter, Phase behavior of the antineoplastic ether lipid 1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine, Biochim. Biophys. Acta 1192 (1994) 167–176.
[17] H. Komatsu, S. Okada, Ethanol-induced aggregation and fusion od small phosphatidylcholine liposome: participation of interdigitated membrane formation in their processes, Biochim. Biophys. Acta 1235 (1995) 270–280.
[18] T. Imae, Light scattering investigation for growth and interaction of nonionic micelles in aqueous solution, J. Colloid Interf. Sci. 127 (1989) 256–264.
[19] S. Stanislaw, S. Stanislaw, G. Mariusz, P. Christian, E. Abdelhamid, Tailored synthesis of polyesters by dispersion ring-opening polymerization of epsilon-caprolactone and lactides, Macromol. Symp. 150 (2000) 259–268.
[20] K. Koga, T. Ohyashiki, M. Murakami, S. Kawashima, Modification of ceftibuten transport by the addition of non-ionic surfactants, Eur. J. Pharm. Biopharm. 49 (2000) 17–25.
[21] P. Pirzadeh, A.A. Moosavi-Movahedi, B. Hemmateenejad, F. Ahmad, M. Shamsipur, A.A. Saboury, Chemometric studies of lysozyme upon interaction with sodium dodecyl sulfate and β-cyclodextrin, Colloids Surf. B Biointerf. 52 (2006) 31–38.
[22] E.S. Swenson, W.B. Milisen, W. Curatolo, Intestinal permeability enhancement: structure-activity and structure-toxicity relationships for nonylphenoxypoly-oxyethylene surfactant permeability enhancers, Pharm. Res. 11 (1994) 1501–1504.
[23] M. Miyake, Y. Oka, T. Minami, H. Toguchi, M. Odomi, K.-I. Ogawara, K. Higaki, T. Kimura, Combinatorial use of sodium laurate with taurine or L-glutamine enhances colonic absorption of rebamipide, poorly absorbable antiulcer drug, without any serious histopathological mucosal damages, J. Pharm. Sci. 92 (2003) 911–921.
[24] S. Takatsuka, T. Morita, A. Koguchi, Y. Horikiri, H. Yamahara, H. Yoshino, Synergistic absorption enhancement of salmon calcitonin and reversible mucosal injury by applying a mucolytic agent and a non-ionic surfactant, Int. J. Pharm. 316 (2006) 124–130.
[25] G.T. Vladisavljevic, J. Surh, J.D. McClements, Effect of emulsifier type on droplet distribution in repeated shirasu porous glass membrane homogenization, Langmuir 22 (2006) 4526–4533.
[26] R.P. Gullapalli, B.B. Sheth, Influence of an optimized non-ionic emulsifier blend on properties of oil-in-water emulsions, Eur. J. Pharm. Biopharm. 48 (1999) 233–238.
[27] M. Kukizaki, T. Wada, Effect of the membrane wettability on the size and size distribution of microbubbles formed from Shirasu-porous-glass (SPG) membranes, Colloids Surf. A Physicochem. Eng. Asp. 317 (2008) 146–154.

[28] K. Lindenstruth, B.W. Mueller, Parameters with influence on the droplet size of w/o emulsions, Pharmazie 59 (2004) 187–190.
[29] K.M. Rosenblatt, H. Bunjes, Poly (vinyl alcohol) as emulsifier stabilizes solid triglyceride drug carrier nanoparticles in the alpha-modification, Mol. Pharm. 6 (2009) 105–120.
[30] H. Gao, Y. Wang, Y. Fan, J. Ma, Ethylenediamino bridged bis (beta-cyclodextrin)/poly (DL-lactic-co-glycolic acid) nanoparticles prepared by modified double emulsion method: effect of polyvinyl alcohol on nanoparticle properties, J. Appl. Polym. Sci. 107 (2008) 571–576.
[31] S.C. Lee, J.T. Oh, M.H. Jang, S.I. Chung, Quantitative analysis of polyvinyl alcohol on the surface of poly (dl-lactide-co-glycolide) microparticles prepared by solvent evaporation method: effect of particle size and PVA concentration, J. Control. Release 59 (1999) 123–132.
[32] I. Bravo-Osuna, C. Vauthier, H. Chacun, G. Ponchel, Specific permeability modulation of intestinal paracellular pathway by chitosan-poly (isobutyl-cyanoacrylate) core–shell nanoparticles, Eur. J. Pharm. Biopharm. 69 (2008) 436–444.
[33] Y.H. Lin, C.T. Chen, H.F. Liang, A.R. Kulkarni, P.W. Lee, C.H. Chen, H.W. Sung, Novel nanoparticles for oral insulin delivery via the paracellular pathway, Nanotechnology 18 (2007) 105102/1–105102/11.
[34] A. des Rieux, E.G. E Ragnarsson, E. Gullberg, V. Preat, Y.-J. Schneider, P. Arthusson, Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium, Eur. J. Pharm. Sci. 25 (2005) 455–465.
[35] S.V. Vinogradov, A.D. Zeman, E.V. Batrakova, A.V. Kabanov, Polyplex nanogel formulations for drug delivery of cytotoxic nucleoside analogs, J. Control. Release 107 (2005) 143–157.
[36] M. Shima, M. Tanaka, T. Fujii, K. Egawa, Y. Kimura, S. Adachi, R. Matsuno, Oral administration of insulin included in fine w/o/w emulsions to rats, Food Hydrocolloid. 20 (2006) 523–531.
[37] T. Lindmark, T. Nikkila, P. Artursson, Mechanisms of absorption enhancement by medium chain fatty acids in intestinal epithelial Caco-2 cell monolayers, J. Pharmacol. Exp. Ther. 275 (1995) 958–964.
[38] M.J. Cano-Cebrian, T. Zornoza, L. Granero, A. Polache, Intestinal absorption enhancement via the paracellular route by fatty acid, chitosans and others: a target for drug delivery, Curr. Drug Del. 2 (2005) 9–22.
[39] Wm.M.F. Leat, T. Gillman, Intestinal absorption and clearance of lipids in ruminants and nonruminants, Metab. Physiol. Significance Lipid Proc. Cambridge Engl. 1963 (1964) 257–271.
[40] S.S. Davis, P. Hansrani, The influence of emulsifying agents on the phagocytosis of lipid emulsions by macrophages, Int. J. Pharm. 23 (1985) 69–71.