We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists



186,000

200M



Our authors are among the

TOP 1% most cited scientists





WEB OF SCIENCE

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us? Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected. For more information visit www.intechopen.com



Approach for Dispersing a Hydrophilic Compound as Nanoparticles Into Soybean Oil Using Evaporation Technique

Kenjiro Koga

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/52016

1. Introduction

Most popular formulation dispersing a hydrophilic compound into oil phase such as soybean oil is emulsions. An emulsion is a dispersed system that consists of water, oil, and surfactant. In general, apparatuses of an emulsifier, a homogenizer, etc. are used for the preparation. As the pharmaceutical trial to disperse water-soluble compounds in an oil phase, the form of the emulsion is very important. Namely, for pharmaceutical preparations containing a hydrophilic drug dispersed uniformly into the oil phase, water-in-oil (w/o) and water-in-oil-in-water (w/o/w) emulsions are preferred. In these cases, hydrophilic drug molecules must retain a high-density in the dispersed water phase of the emulsion; doing so depends on the oil-to-water partition coefficient of the drug. Furthermore, decreasing the particle size in the dispersed water phase is necessary. Much pharmaceutical technical information about adjusting the size of particles is now available: for example, rotating membrane emulsification [1, 2], shirasu porous glass membrane emulsification [3, 4], electrocapillary emulsification [5, 6]. These methods adjust particle size on the basis of membrane pore size and shearing force, which depends on the flow of dispersion medium or on contact-surface dielectric constant differences between the dispersion medium and the dispersion phase. Therefore, these technologies are advantageous in that they can produce uniform particle sizes. In this chapter, a simple method of preparing w/o emulsions with a narrow range of polydispersity is described. In this method, a Polytron homogenizer and an evaporator are used as apparatuses. Namely, specific and expensive apparatuses were not used. Glycyrrhizin monoammonium (GZ) and indocyanine green (ICG) were used as a hydrophilic compound. Here, the phase behavior, stability in terms of particle size of w/o emulsions prepared using the novel method and the sustained release characteristics drug from nano-sized w/o emulsions were investigated [7,8].



© 2013 Koga; licensee InTech. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

2. Selection of emulsifier for the preparation of stable w/o emulsion

The choice of ideal emulsifier is an important to prepare physicochemically stable w/o emulsion. Furthermore, the emulsifier must be safe in human. Therefore, mainly non-ionic surfactants added in foods or medicines were chosen. The list used in this experiment was shown in Table 1.

Surfactants	Product name	HLB
condensed ricinoleic acid tetraglycerin ester	CR-310 ¹⁾	2.5
polyethyleneglycol distearate ester	CDS-400 ²⁾	8.5
hexaglycerin sesquistearate ester	SS-500 ¹⁾	10.1
tetraglycerin monostearate ester	MS-310 ¹⁾	10.2
tetraglycerin monooleate ester	MO-310 ¹⁾	10.2
tetraglycerin monolaurate ester	ML-310 ¹⁾	10.3
polyethyleneglycol(10EO) monostearate ester	MYS-10 ²⁾	11
hexaglycerin monostearate ester	MS-500 ¹⁾	12.2
hexaglycerin monooleate ester	MO-500 ¹⁾	12.2
polyethyleneglycol(10EO) monolaurate ester	MYL-10 ²⁾	12.5
stearyl macrogol glycerides	GELUCIRE 50/13 ³⁾	13
hexaglycerin monolaurate ester	ML-500 ¹⁾	13.5
lauroyl macrogol-32 glycerides	GELUCIRE 44/14 ³⁾	14
decaglycerin monooleate ester	MO-750 ¹⁾	14.5
Polyethyleneglycol (25EO) monostearate ester	MYS-25 ²⁾	15
decaglycerin monolaurate ester	DECAGLYN 1-L ²⁾	15.5
decaglycerin monolaurate ester	ML-750 ¹⁾	15.7
polyoxyethylene(20EO) oleylether	BO-20 ²⁾	17
polyethyleneglycol(40EO) monostearate ester	MYS-40 ²⁾	17.5
polyoxyethylene(30EO) phytosterol	BPS-30 ²⁾	18
polyoxyethylene(21EO) laurylether	BL-21 ²⁾	19
polyoxyethylene(18EO)nonylphenylether	NP-18TX ²⁾	19
polyoxyethylene(25EO)laurylether	BL-25 ²⁾	19.5
polyoxyethylene(30EO)octylphenylether	OP-30 ²⁾	20
polyoxyethylene(20EO)nonylphenylether	NP-20 ²⁾	20
	Surfactants condensed ricinoleic acid tetraglycerin ester polyethyleneglycol distearate ester hexaglycerin sesquistearate ester tetraglycerin monostearate ester tetraglycerin monolaurate ester polyethyleneglycol(10EO) monostearate ester hexaglycerin monooleate ester polyethyleneglycol(10EO) monolaurate ester hexaglycerin monooleate ester polyethyleneglycol(10EO) monolaurate ester stearyl macrogol glycerides hexaglycerin monolaurate ester lauroyl macrogol-32 glycerides decaglycerin monolaurate ester Polyethyleneglycol (25EO) monostearate ester decaglycerin monolaurate ester polyoxyethylene(20EO) oleylether polyoxyethylene(20EO) nonostearate ester polyoxyethylene(21EO) laurylether polyoxyethylene(25EO)laurylether polyoxyethylene(30EO)octylphenylether polyoxyethylene(20EO)nonylphenylether polyoxyethylene(20EO)nonylphenylether	SurfactantsProduct namecondensed ricinoleic acid tetraglycerin esterCR-310 ¹¹ polyethyleneglycol distearate esterCDS-400 ²³ hexaglycerin sesquistearate esterSS-500 ¹¹ tetraglycerin monostearate esterMS-310 ¹¹ tetraglycerin monostearate esterMO-310 ¹¹ tetraglycerin monolaurate esterML-310 ¹¹ polyethyleneglycol(10EO) monostearate esterMYS-10 ²¹ hexaglycerin monolaurate esterMYS-10 ²¹ hexaglycerin monolaet esterMS-500 ¹¹ hexaglycerin monolaet esterMYS-10 ²¹ hexaglycerin monolaet esterMYL-10 ²¹ stearyl macrogol glyceridesGELUCIRE 50/13 ³¹ hexaglycerin monolaurate esterML-500 ¹¹ lauroyl macrogol-32 glyceridesGELUCIRE 44/14 ³¹ decaglycerin monolaurate esterMYS-25 ²¹ decaglycerin monolaurate esterMYS-25 ²² decaglycerin monolaurate esterMYS-25 ²¹ decaglycerin monolaurate esterMYS-40 ²¹ polyoxyethylene(20EO) oleyletherBD-20 ²² polyoxyethylene(30EO) phytosterolBPS-30 ²³ polyoxyethylene(21EO) lauryletherBL-21 ²¹ polyoxyethylene(25EO)lauryletherNP-18TX ²¹ polyoxyethylene(25EO)lauryletherNP-30 ²² polyoxyethylene(20EO)oncylphenyletherNP-20 ²³

Number 1), 2), and 3) indicated in product name were gifts from Sakamoto Yakuhin Kogyo Co.Ltd., Nikko Chemicals Co. Ltd., and Gattefossé, respectively.

Table 1. Surfactants used for the preparation of w/o emulsions.

The following examination was carried out in order to estimate the stability of w/o emulsion. Each surfactant (0.75 g) and soybean oil (6.75 g) were put in a glass tube. The mixture was dissolved or dispersed uniformly at 60°C for 15 min. GZ solution (2.25 mL of 40 mg/mL in dissolved with 100 mM phosphate buffered solution, pH7.4) was added into the glass tube, then immediately, the solution with oil, water phase, and surfactant was emulsified using a Polytron homogenizer (PT-MR 3100, Kinematica AG, Littau/Luzern, Switzerland) at 20,000 rpm for 3 min. The state of the w/o emulsions was observed at 24 h after the preparation. The stability of the w/o emulsions was estimated by objective evaluation scale (OES; 0, 1, 2, 3, 4, and 5). Namely, OES 0 is a completely discrete state without emulsifying. OES 1 is a biphasic separate state with wispy cloud in bottom phase, OES 2 is a biphasic separate state with weakly white turbidity in bottom phase, OES 3 is a biphasic separate state with strongly white turbidity in bottom phase, and OES 5 is a stable state without phase separation.



Figure 1. Observed properties of emulsions with 24 kinds of emulsifiers at 24 h after the preparation using a Polytron homogenizer. The photo of emulsion with CR-310 was referred in Figure 3A. The number under each photo is a product number shown in Table 1.

The results in the observed state after preparation of w/o emulsions are shown in Figure 1. Moreover, Figure 2 shows the relationship between HLB number of emulsifiers and OES.

CR-310 and CDS-400 among 25 kinds of surfactants were convenient for the preparation of stable w/o emulsion. The results were identified with the theory that surfactant with low HLB is suitable for the preparation of w/o emulsions. A difference of viscosity as physicochemical properties was observed between CR-310 and CDS-400. Namely, the viscosity of the w/o emulsion with CDS-400 was high like cream, that of the emulsion with CR-310 was the same as the viscosity of soybean oil. Therefore, it was clear that CR-310 among used surfactants was most convenient emulsifier for the preparation of w/o emulsions when soybean oil was used as an oil phase.



Figure 2. The relationship between HLB number of surfactants and objective evaluation scale. The number of each symbol is a product number shown in Table 1.

3. Preparation of nano-sized w/o emulsions

GZ solution (400 mg/mL) was prepared by dissolving GZ powder at 60°C in 100 mM phosphate-buffered solution (pH7.4) containing 8.0% (w/v) L-arginine. L-arginine was used to inhibit the gelation of GZ [9]. Soybean oil (4.50 g) and CR-310 (0.50 g) were mixed in a glass tube, and then the mixture was heated for 15 min at 60°C in order to blend uniformly. The GZ solution and the mixture of oil and emulsifier were cooled down at room temperature, and 400 mg/mL GZ solution (2.2, 3.3, or 4.4 g) was added to glass tubes containing the mixture. First, the w/o emulsions were prepared by agitating the mixture with a Polytron homogenizer (PT-MR 3100, Kinematica AG) at 20,000 rpm for 3 min. Second, the w/o emulsions were placed into a 50-mL round-bottom flask, which was then placed into a rotary evaporator (R-210, Buchi Labortechnik AG, Flawil, Switzerland) equipped with a vacuum controller (V-850) and a vacuum pump (V-700). The vacuum was initially set to 120 hPa, and then decreased at a rate of 10 hPa per minute until 20 hPa was reached. The mixture was then subjected to these vacuum conditions at 40°C for 90 min. The prepared emulsions were separated into either glass vials or 10-mL centrifuge tubes, depending on the analyses to be done. For phase behavior comparisons, w/o emulsions without GZ was also prepared using distilled water (3.3 g) instead of GZ solution [7].

4. Phase behavior during the preparation of w/o emulsions

The w/o emulsions prepared by adding GZ solution slowly changed in turbidity to pale white, and after 23-24, 26-27, and 31-32 min for GZ solutions of 2.2, 3.3, and 4.4 g, respectively, to clear or slightly turbidity. The samples remained clear only for approximately 2 min. When subjected to prolonged evaporation, the emulsions rapidly changed in turbidity to white as solid dispersion. Figure 3A shows photographs of the emulsions prepared with GZ before evaporation (0 min) and 10, 15, 26, and 90 min after evaporation. To confirm the relationship between evaporation time and phase behavior in w/o emulsions with or without GZ, similar experiments on w/o emulsions lacking GZ were carried out (Figure 3B). The turbidity of w/o emulsions without GZ remained white up to 15 min after evaporation, before gradually changing to pale white. The w/o emulsions finally became transparent 90 min after evaporation. These results suggest that the phase behavior may depend on the water content of the w/o emulsions.

A					
	0 min	10 min	15 min	26 min	90 min
B					
	0 min	10 min	15 min	26 min	90 min

Figure 3. Photographs of w/o emulsions at the indicated times after evaporation. (A) Emulsion prepared with a GZ solution containing 3.3 g GZ. (B) Emulsion prepared without GZ (water phase is distilled water). The times shown below each vial represent the length of time samples underwent evaporation.

5. Particle size of water phase in w/o emulsions

GZ solution (400 mg/mL) was prepared by dissolving GZ powder at 60°C in 100 mM phosphate-buffered solution (pH 7.4) containing 8.0% (w/v) L-arginine and 10 μ g/mL fluorescein sodium. Fluorescein sodium was used as a marker for the fluorescent observation of the dispersion state of water phase in the emulsions. After the GZ solution (3.3 g), soybean oil (4.50 g), and CR-310 (0.50 g) were mixed, w/o emulsions were prepared according to the method described above. The w/o emulsion before dehydration was analyzed with a confocal laser scanning microscope LSM510 (Carl Zeiss GmbH, Jena, Germany) and LMS Image Browser Software (Carl Zeiss GmbH). The excitation and fluorescein wavelengths were set to 405 and 488 nm, respectively.



Figure 4. Fluorescence photomicrograph of a w/o emulsion following the addition of fluorescein sodium to the water phase. The dark gray areas represent the aqueous phase. The position of water phase containing GZ is indicated as green fluorescent particles.

Figure 4 shows the photo by a confocal laser scanning microscope. The water phase was uniformly distributed as small droplets (< 5 μ m), indicating that before evaporation the particle size distribution in the water phase of GZ sample distributed widely after dispersal with a Polytron homogenizer.

Emulsions		Particle	size in relative f	requency (nm)	
	10%	25%	50%	75%	90%
2.2 g GZ sample	135	170	225	287	341
3.3 g GZ sample	219	253	299	349	394
4.4 g GZ sample	310	504	1105	1381	1610

 Table 2. Particle size and size distribution of clear or slightly turbid w/o emulsions.

The particle sizes of w/o emulsions at 23, 26, and 31 min for GZ solutions of 2.2, 3.3, and 4.4 g after evaporation were analyzed using a Particle Size Analyzer (LS 13 320; Beckman Coulter, Inc., Fullerton, CA, United States). Table 2 presents the average particle sizes and size distributions of the clear or slightly turbid w/o emulsions after evaporation. The size distribution of the three kinds of emulsions was narrow, with relative frequency values of 10, 25,

75, and 90%. These results suggest that dehydration proceeded in a way that caused the dispersed phase to approach the narrow distribution. In particular, the particle size distribution of the 2.2 and 3.3 g GZ samples converged toward the nano-size range. This was consistent with our prediction that large water droplets would efficiently evaporate from the surface of the w/o emulsions as their round bottom flasks were rotated and that particle size distribution would narrow as a function of evaporation time.

During the preparation of transparent w/o emulsions containing GZ, the only component removed by evaporation was water. Thus, the components dissolved in the water phase (GZ, L-arginine, and phosphate salts) were gradually concentrated as the water content decreased. The observation that a simple evaporation process changed the turbidity of the w/o emulsions from white to clear within a short time suggests that the particles comprising the dispersed phase became extremely small in size. In fact, the particle size range of sample which prepared with 3.3 g GZ solution was 219-394 nm by dynamic light scattering assay (LS 13 320; Beckman Coulter, Inc.). The state of the nano-sized droplets was observed in transmission electron microscopy (TEM). Namely, a clear w/o emulsion was prepared by adding GZ solution (3.3 g) to the mixture of soybean oil (4.50 g) and CR-310 (0.50 g), and the resulting clear w/o emulsion was passed through a quantitative filter (No. 5B, Advantec; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filter was then hardened with cured acryl resin. After embedding the filter, ultrathin sections were obtained by cutting the surface of the block containing the filter on an ultramicrotome equipped with a diamond knife (EM-Ultracut-s; Leica Microsystems Vertrieb GmbH, Wetzlar, Germany). The ultrathin sections were mounted onto freezing support grids and then stained with ruthenium tetrachloride. Next, the emulsified particles were observed with a transmission electron microscope (TEM; JEM-2100; Jeol Ltd., Tokyo, Japan).



Figure 5. A) TEM photograph of dispersed particles in a clear w/o emulsion prepared by adding GZ solution. (B) Solid GZ particles after 90 min evaporation.

To determine whether the nano-sized droplets exist as a liquid state in the water phase, the shape of particles in the clear w/o emulsion (3.3 g GZ sample) was observed with TEM. As the water content of the emulsion decreases with evaporation, part of the dissolved GZ may pre-

cipitate as a solid state from the dispersed phase. If this hypothesis is accurate, then the particles may be not spherical. However, TEM analysis revealed that the particles were spheres of approximately 200 nm in diameter and were maintained a uniform globe (Figure 5A), strongly suggesting that the nanoparticles in the clear w/o emulsions existed as a liquid phase.

Following additional evaporation, the turbidity of the w/o emulsions again changed to white, indicating a change in phase behavior. This suggests that the hydrophilic components dissolved in the water phase separated as solid states. In fact, microscopic analysis demonstrated that the w/o emulsions containing GZ contained 1-10 µm-diameter solid particles >90 min after evaporation (Figure. 5B) [8].

6. Water contents in w/o emulsions

The water content (%, w/w) of the w/o emulsions was analyzed using a Karl Fischer titration apparatus (870, KF Titrino Plus; Metrohm Shibata Co., Ltd., Tokyo, Japan). The water content (%, w/w) of the 2.2, 3.3, and 4.4 g GZ samples before evaporation was 16.8, 21.9, and 25.4% (w/w), respectively. On the other hand, the water content of the clear or slightly turbid 2.2, 3.3, and 4.4 g GZ samples was 7.8 ± 0.1 , 9.4 ± 0.3 , and $11.9 \pm 0.3\%$ (w/w), respectively. Furthermore after 90 min of evaporation, the water content of the 2.2, 3.3, and 4.4 g GZ samples was all in the range of 1.3-1.8% (w/w). The water content of w/o emulsion lacking GZ was 9.3 ± 0.2 and $0.18 \pm 0.02\%$ (w/w), respectively, at 26 and 90 min after evaporation. These results indicate that the phase behavior of the w/o emulsions changed from white turbid to translucent or transparent when the water content reached approximately 8-12% (w/w). Since the water content of the clear or slightly turbid w/o emulsions correlated well with GZ content (0.8, 1.2, and 1.6 g for 2.2, 3.3, and 4.4 g GZ samples; correlation coefficient was 0.992), it is plausible that the precipitation of hydrophilic components, such as GZ, L-arginine and phosphate salts, was responsible for the increased turbidity resulting after prolonged evaporation.

From these results, it is concluded that the water contents of w/o emulsions changed the phase behavior in the emulsions, that is, from white turbid phase to clear phase when the water content reached to be approximately 9%, and then from clear phase to white turbid phase. The decreasing rate of the water content is affected by the setting of a vacuum controller. In the experiments, although the relationship between the decreasing rate of the water content and maintained interval with clear phase in the w/o emulsions was not investigated, the pressure condition in the evaporation process will be a major problem for the simple preparation of clear w/o emulsions.

7. Stability of w/o emulsions

The stability of the w/o emulsions were evaluated according to two criteria: i) the uniform dispersal of the water phase containing GZ in the emulsion; and ii) the size distribution at steady-state conditions. Clear or slightly turbid w/o emulsions (2.2, 3.3, and 4.4 g GZ solu-

tions) were transferred to 10-mL centrifuge tubes (Figure 6), which remained undisturbed at 20 ± 2 °C for 10 days. The w/o emulsion prepared with 3.3 g GZ solution remained under undisturbed conditions continuously for 65 days. Next, a pipette was inserted 1 cm from the top or bottom of each centrifuge tube and a small amount (50 mg) of emulsion was removed and transferred into two screw vials (one vial for the "top" sample, the other vial for the "bottom" sample). Methanol (30 mL) was added to each vial, and the vials were shaken for 15 min on a vortex mixer. The methanol-containing GZ samples were adequately diluted with 100 mM phosphate-buffered solution (pH 7.4) and were injected into an HPLC system in order to determine the GZ concentration in the samples [10]. The water content (%, w/w) in the w/o emulsions obtained from both samples was analyzed by Karl Fischer titration.



Figure 6. Photograph of clear and slightly turbid w/o emulsions. (A) 2.2 g GZ sample. (B) 3.3 g GZ sample. (C) 4.4 g GZ sample.

7.1. Uniformity of GZ concentration and water content in w/o emulsions

The GZ concentrations of 2.2, 3.3, and 4.4 g GZ samples that were clear or slightly turbid were 11.1, 14.5, and 17.0% (w/w), respectively. The GZ concentrations and water content of clear or slightly turbid w/o emulsions 10 days and 65 days after preparation are shown in Table 3. As the phase behavior changes with time, the aggregation or coalescence of the dispersed phase occurs more frequently after emulsification is achieved, because the emulsion is thermodynamically unstable. If the dispersed stability is not maintained, then the GZ concentration and water content in the emulsion will differ in the parts of the emulsion near the top and bottom of the sample. This is because the dispersed phase containing GZ moves toward the bottom due to specific gravity differences. GZ concentrations in the top and bottom parts of the emulsions were identical in the 2.2 and 3.3 g GZ samples but not in the 4.4 g GZ sample. Furthermore, similar results were obtained in our comparative analysis of water content. These results suggest that the dispersed stability of the 2.2 and 3.3 g GZ w/o emulsions was extremely high. On the other hand, the difference in phase behavior in the top and bottom layers of the 4.4 g GZ sample suggests dis-

persed instability. Specifically, after 10 days the turbidity of the bottom layer became clearer than that of the top layer, indicating that the dispersed phase containing 4.4 g GZ gradually moved toward the bottom of the 10-mL centrifuge tube. The specific gravity of the dispersed phase in the w/o emulsions increased with increasing GZ concentration.

w/o emulsions	GZ concentr	ration (%, w/w)	Water content (%, w/w)		
	upper position	bottom position	upper position	bottom position	
2.2 g GZ sample	11.5 ± 0.7	11.2 ± 0.4	8.2 ± 0.2	8.4 ± 0.2	
3.3 g GZ sample	14.9 ± 0.5	14.7 ± 0.5	10.1 ± 0.2	10.2 ± 0.3	
3.3 g GZ sample**	15.0 ± 0.4	14.8 ± 0.4	9.8 ± 0.3	9.6 ± 0.2	
4.4 g GZ sample	10.2 ± 0.8	17.4 ± 0.7	7.4 ± 0.1	12.0 ± 0.3	

GZ concentration and water content were determined after the sample was stored undisturbed for 10 days at 20 \pm 2°C. *Samples were obtained 1 cm from the top and bottom of the emulsions contained within a 10-mL centrifuge tube. **The sample was stored undisturbed for 65 days at 20 \pm 2°C.

Table 3. GZ concentration and water content in different parts of the w/o emulsions*.

7.2. Uniformity of particle size of dispersed phase in w/o emulsions

The particle size distribution of the GZ sample containing 3.3 g GZ solution at 65 days after preparation was 226-421 nm. This range was similar to that measured during immediate intervals after preparation. In general, refinement of emulsion particle size lowers thermodynamic stability, because the phase behavior of an emulsion-which is a very complicated system of oil, water, and surfactant-is affected by decreases in particle size [11]. Nano-sized emulsions must be stabilized with polymers and excessive amounts of surfactants [12]. In GZ sample, the concentration of surfactant in the oil phase was 10% (w/w); the emulsions did not contain other stabilizers. However, the GZ sample (nano-sized emulsions) was stable at least for 2 months. Moreover, although the particle sizes of the top and bottom layers in the 4.4 g GZ sample were not determined, the aggregation of dispersed particles suggests that the size distribution in this w/o emulsion tended to be on the large side. Taken together, these observations suggest that dispersed stability decreases with increasing GZ content.

8. Sustained release effect by nano-sized w/o emulsions

From the viewpoint of medical treatment, drug release from w/o emulsions is important for the efficiency of controlled release. The pharmacokinetics of GZ by nano-size w/o emulsion, aqueous formulation, o/w emulsion, and w/o emulsion with solid GZ was investigated in order to clarify the degree of the controlled release. Furthermore, the release characteristics of a hydrophilic compound, indocyanine green (ICG), from administered subcutaneous site in rats was observed using a near-infrared fluorescent camera (Photo Dynamic Eye, PDE).

8.1. In vivo experiments in GZ pharmacokinetics

Pharmacokinetic studies of GZ were investigated in detail in human [13, 14], in rat [15, 16], and other species. The elimination half-life of GZ in rats after the intravenous administration (20-50 mg/kg) is approximately 2-4 h in plasma [17, 18]. GZ is rapidly excreted into bile via multidrug resistance-associated protein 2 (MRP2) ATP-binding cassette transporter C2 (ABCC2) transporter [19]. Therefore, the release of GZ from w/o emulsions was estimated as GZ elimination into bile.

The protocol of this study was approved by the Committee of Animal Use of Hokuriku University. All animal experiments were conducted in accordance with the Institutional Guidelines of Care and Use of Laboratory Animals. Male Sprague-Dawley rats (180-200 g) were housed for at least 10 days in a clean room. The rats were given free access to commercial chow and water and were maintained according to the Hokuriku University Animal Guidelines. For in vivo experiments in GZ formulations, the rats (250-280 g) were randomly divided into four treatment groups as four rats per group.

A GZ stock solution (400 mg/mL) was prepared at 60°C in 100 mM phosphate-buffered solution, pH7.4, containing 8.0% (w/v) L-arginine. The GZ stock solution was stored in a refrigerator. An aqueous formulation of GZ (150 mg/mL; Rp-II) was prepared by adding 100 mM phosphate-buffered solution (pH7.4) to the GZ stock solution. Preparation of an oil-in-water (o/w) emulsion of GZ was as follows: soybean oil (1.00 g), HCO-60 (0.12 g), and egg yolk lecithin (0.12 g) were blended uniformly by heating at 90°C for 15 min on a block heater. The mixture was then cooled at room temperature. The o/w emulsion of GZ (150 mg/mL; Rp-III) was prepared by combining the soybean oil mixture (1.0 mL), GZ stock solution (1.16 mL), and 100 mM phosphate-buffered solution, pH7.4 (0.84 mL) and by using a Polytron homogenizer (PT-MR 3100) at 20000 rpm for 3 min for emulsification.

Preparation of an w/o emulsion of GZ was described above. The GZ stock solution (3.3 g) was then added to the lukewarm mixture, which was emulsified using a Polytron homogenizer (PT-MR 3100) at 20000 rpm for 3 min. The w/o emulsion was placed into a 50-mL round-bottom flask, which was then set in a rotary evaporator (R-210, Buchi Labortechnik AG) equipped with a vacuum controller (V-850). The vacuum was initially set to 120 hPa at 40°C; thereafter, the pressure was decreased at a rate of 10 hPa per min until 20 hPa was reached. To prepare Rp-I and Rp-IV, the dehydration was continued for 27 min and 120 min, respectively, and then adjusted the GZ concentration to 150 mg/mL by adding the soybean oil/CR-310 (9 : 1, w/w) mixture. Adminidtration method of four kind formulations and sampling of bile in rats were described in detail in reference [8].

As the characteristics of used formulations, the average particle sizes in the Rp-I and Rp-III formulations were 299 nm and 376 nm, respectively. The 10-90% ranges of size distribution in Rp-I and Rp-III were 208-402 nm and 255-512 nm, respectively. Two peaks in size distribution were observed in the Rp-IV formulation: 312 nm and 5000 nm. Microscopic observations revealed that large-size GZ particles were solid GZ, because they were not spherical. The water content in Rp-IV (1.5%, w/w) was very low compared with that in Rp-I (9.4%,

w/w). Moreover, the small- and large-size particles were not observed after evaporation for 120 min in a w/o emulsion lacking GZ.

Almost all GZ transported to hepatocytes via the blood is eliminated into bile as unchanged GZ (i.e., not metabolized) [20, 21]. Therefore, the elimination rate of GZ in bile reflects the bioavailability of GZ in hepatocytes. Figure 7 shows the cumulative elimination (%) of GZ over time after subcutaneous administration of Rp-I, Rp-II, Rp-III, and Rp-IV in rats. After the administration of Rp-I, cumulative elimination at 8 h, 24 h, and 72 h as a function of administered GZ dose (50 mg/kg) in bile was 11%, 20%, and 47%, respectively. These results indicate that the Rp-I formulation resulted in a sustained release of apparent zero-order kinetics. The average elimination rate of GZ up to 72 h was 84.2 \pm 14.2 µg/h.



Figure 7. Cumulative elimination of GZ in bile after subcutaneous administration of GZ formulations in rats. (A) Nanosized w/o emulsion encapsulating GZ, (B) aqueous solution of GZ, (C) o/w emulsion containing GZ, and (D) w/o emulsion with solid GZ. GZ concentrations were all adjusted to 150 mg/mL. The GZ dose administered to all rats was all 50 mg/kg. Data represent means ± S.D. of four experiments.

With the Rp-II formulation, the cumulative elimination at 4 h and 8 h as a function of administered GZ dose was 75% and 97%, respectively. In intravenous and subcutaneous administration models, no difference in the elimination rate of GZ has been observed after intravenous administration of GZ [10]. These results suggest that GZ dissolved in phosphate buffered solution rapidly diffused in the hyperdermis, before being transferred into the general circulation. With the Rp-III formulation, the cumulative elimination of GZ at 8 h and 30 h was 81% and 96%, respectively. The elimination rate of GZ in Rp-III was faster than that in Rp-I but slower than that in Rp-II, suggesting that the oil phase of the o/w emulsion inhibited the diffusion of the water phase containing GZ in subcutaneous regions, even though GZ was dissolved in the outer water phase of o/w emulsion. On the other hand, the cumulative elimination of GZ in Rp-IV was the lowest among the four formulations: The GZ elimination in bile at 8 h, 24 h, and 72 h was 7.1%, 14%, and 31%, respectively. As with the elimination kinetics of the Rp-I formulation, the elimination kinetics of GZ in Rp-IV showed that GZ was released in a sustained fashion for up to 72 h. Since Rp-IV contained solid GZ in w/o emulsions, it was speculated that the elimination rate of GZ in bile after the administration of Rp-IV would be slower than that of GZ in Rp-I. In fact, the eliminated amount of GZ in bile after the administration of Rp-IV was 0.64-fold compared to that of Rp-I. These results suggest that reduced water content in w/o emulsions delays hydration in the subcutaneous region and that much time is required to dissolve the dispersed solid GZ in Rp-IV.

To determine more precisely the characteristics of sustained GZ release from w/o emulsions, the rates of GZ elimination in bile were recalculated every 24 h after the administration of Rp-I and Rp-IV (Figure 8).



Figure 8. Elimination rate per 24 h of GZ in bile after subcutaneous administration of Rp-I and Rp-IV in Rats. Administration dose of GZ was 50 mg/kg in both formulations. Data represent means ± S.D. of four experiments.

The elimination of GZ in Rp-I occurred at a constant rate for 72 h, i.e., the rate ranged from 1.80 to 2.12 mg/day. On the other hand, elimination of GZ in Rp-IV decreased from 1.40-1.41 mg/day at 48 h to 0.60 mg/day at 48-72 h. As the reason for the decrease in GZ elimination rate at 48-72 h in Rp-IV, it was predicted that the presence of solid dispersed GZ may be involved deeply the transfer rate of GZ from subcutaneous site to liver. Actually, dissolved-state GZ and solid-state GZ exist in Rp-IV. Although it was considered that the dissolved GZ in Rp-IV was transferred to liver as similar to GZ in Rp-I, solid GZ particles must be dissolved to some extent which can be passed vascular system such as vein and lymph capillary in order to transfer GZ from subcutaneous site to liver. Therefore, after 48 h, the proportion of solid GZ for residual GZ in the subcutaneous site will increase certainly. As a result, it was guessed that GZ elimination into bile decreased based on the decrease of transfer rate from subcutaneous site to liver. These results indicate that Rp-I was a substantially superior formulation compared to Rp-IV in

terms of sustained release in bile. It was hypothesized that the small and narrowrange polydispersity (208-402 nm in Rp-I) of the dispersed phase in w/o emulsions may be important for stabilizing the release rate of GZ from these emulsions.

8.2. Tissue distribution of ICG from subcutaneous site in rats

For the experiments of ICG administration, two male Sprague-Dawley rats (250, 255 g) were used. A w/o emulsion encapsulating ICG was prepared as follows: 5 mg/mL ICG solution (3.3 g) was added to the mixture of soybean oil (4.50 g) and CR-310 (0.50 g) heated at 60°C. Next procedure was the same with the preparation step of Rp-I described above. This ICG solution and w/o emulsion encapsulating ICG were used to observe the tissue distribution of drugs from the subdermal injection site. To monitor over time the delayed drug distribution and the diffusion of hydrophobic formulation in the subdermal site, a w/o emulsion encapsulating ICG instead of GZ was prepared. ICG is a hydrophilic fluorescent dye and biocompatibility marker with excitation and emission spectra in the near-infrared wavelength range of 600 to 900 nm, and the maximum emission wavelength of ICG in vivo is 845 nm [22, 23]. The kinetics of ICG in vivo was observed using a non-invasive, near-infrared fluorescent camera (Photo Dynamic Eye; PDE), because the near-infrared-wavelength monitoring barely affects biological molecules such as water and hemoglobin. For the experiments of the microscopic image using a fluorescent probe, ICG, rats were anesthetized with an intraperitoneal administration of sodium pentobarbital (50 mg/kg), and then were kept in a supine position on an operation plate. A PDE was set on the upper position of 20 cm from the rat and the photo image was monitored using a personal computer. Two samples, 5 mg/mL ICG solution (0.05 mL) and w/o emulsion encapsulating ICG (0.05 mL) were administered to right and left hind legs of rat, respectively. The diffusion of ICG was observed for 60 min.



Figure 9. Photographs taken at (A) 15 min and (B) 60 min after the subcutaneous injection of ICG solution into the right leg and w/o emulsion encapsulating ICG into the left leg injection volume of ICG was 50 mL in both formulations.

The purpose of the PDE experiments was to determine whether the diffusion rate of w/o emulsions at the subdermal site is remarkably slower than that of solutions like Rp-II of GZ formulation. Figure 9 shows photographs of rats' hind legs 15 min and 60 min after injecting ICG (right leg) and after injecting w/o emulsion encapsulating ICG (left leg). The ICG solution was rapidly absorbed into capillary blood vessels within 15 min. Furthermore, ICG also reached peripheral lymphatics. On the other hand, ICG in w/o emulsions remained in the vicinity of the administration site for 60 min. These results suggested that the outer oil phase inhibited the diffusion of w/o emulsion encapsulating ICG and/or the release of ICG from the w/o emulsion. The viscosity of lipophilic formulations is generally high. Therefore, one would expect the release of ICG from a w/o emulsion to be delayed compared to that from aqueous formulations of ICG. The expectation will be correspondent with the decrease of diffusion and/or release of GZ from Rp-I of GZ formulation.

9. Conclusion

It was clarified that GZ, a hydrophilic compound, was dispersed as nanoparticles into soybean oil by using the evaporation technique. The prepared oil phase containing GZ was nano-emulsions with low polydispersity, and was stable at least 2 months. This ideal dispersion method had to make water content approximately 9%, and it was clear that the further dehydration became solid dispersion. It is concluded that a hydrophilic compound can be dispersed easily into an oil phase such as soybean oil by utilizing this method. Concretely, the w/o emulsions containing GZ (2.2 g of GZ [11.1%, w/w] and 3.3 g of GZ [14.5%, w/w]) with narrow-ranged polydispersity and high-dispersed stability were easily prepared by the measurable removal of water using a Polytron homogenizer and a rotary evaporator. The water content in 2.2 and 3.3 g GZ samples had to be 7.8 and 9.4% (w/w), respectively, because decreasing the water content beyond these levels caused the phase behavior to change (e.g., white turbid). The particle size distribution (relative frequency values ranging from 10 to 90%) of the clear w/o emulsions was in the range of 135 to 421 nm as the samples remained undisturbed for 65 days at $20 \pm 2^{\circ}$ C. The w/o emulsion preparation method described in the present study provides useful information on the lipophilic formulations of GZ.

A nano-sized w/o emulsion of GZ (Rp-I) showed sustained elimination of GZ in bile at a relatively constant rate for 72 h. The sustained GZ elimination in bile was strongly affected by diffusion of the w/o emulsion and by the release of GZ from the emulsion to the perimeter of the subdermal site, based on the PDE observations with ICG. Indeed, the average elimination rate of GZ in bile was 0.084 mg/h over 72 h, when Rp-I (50 mg/kg as GZ) was administered subcutaneously. If GZ release from Rp-I will be maintained as zeroorder elimination (0.084 mg/h), 6-7 day are needed until the GZ release finishes in the rats. Namely, GZ in Rp-I will slowly transfer from subcutaneous tissue to liver 20-fold periods as compared with GZ in Rp-II, the elimination of almost all GZ finished 8 h. These results indicate that the nanosized w/o emulsion encapsulating GZ, which can be subcutaneously administered, will be useful as a new sustained-release formulation.

Acknowledgements

Author thanks Cokey Co. Ltd. for the gift of glycyrrhizin monoammonium, and thanks Sakamoto Yakuhin Kogyo Co. Ltd. and Nikko Chemicals Co. Ltd. for the gifts of several kinds of surfactants.



Address all correspondence to: k-koga@hokuriku-u.ac.jp

Faculty of Pharmaceutical Sciences, Hokuriku University, Japan

References

- [1] Aryanti, N., Hou, R., & Williams, R. A. (2009). Performance of a rotating membrane emulsifier for production of coarse droplets. *Journal of Membrane Science*, 326(1), 9-18.
- [2] Vladisavljevic, G. T., & Williams, R. A. (2006). Manufacture of large uniform droplets using rotating membrane emulsification. *Journal of Colloid and Interface Science*, 299(1), 396-402.
- [3] Vladisavljevic, G. T., Surh, J., & Mc Clements, J. D. (2006). Effect of emulsifier type on droplet disruption in repeated shirasu porous glass membrane homogenization. *Langmuir: the ACS journal of surfaces and colloids*, 22(10), 4526-4533.
- [4] Hosoya, K., Bendo, M., Tanaka, N., Watabe, Y., Ikegami, T., & Minakuchi, H. (2005). An application of silica-based monolithic membrane emulsification technique for easy and efficient preparation of uniformly sized polymer particles. *Macromolecular Materials and Engineering*, 290(8), 753-758.
- [5] Sakai, H., Tanaka, K., Fukushima, H., Tsuchiya, K., Sakai, K., Kondo, T., & Abe, M. (2008). Preparation of polyurea capsules using electrocapillary emulsification. *Colloids and Surfaces, B: Biointerfaces*, 66(2), 287-290.
- [6] Abe, M., Nakayama, A., Kondo, T., Morishita, H., Tsuchiya, K., Utsumi, S., Ohkubo, T., & Sakai, H. (2007). Preparation of tiny biodegradable capsules using electrocapillary emulsification. *Journal of Microencapsulation*, 24(8), 777-786.
- [7] Koga, K., Liu, H., & Takada, K. (2009). A simple preparation method for dispersing glycyrrhizin solution into oil phase using an evaporation technique. *Journal of Drug Delivery Science and Technology*, 19(6), 431-435.

- [8] Koga, K., Nishimon, Y., Ueta, H., Matsuno, K., & Takada, K. (2011). Utility of Nanosized, water-in-oil emulsion as a sustained release formulation of glycyrrhizin. *Biological & Pharmaceutical Bulletin*, 34(2), 300-305.
- [9] Koga, K., Kawashima, S., Shibata, N., & Takada, K. (2007). Novel formulations of a liver protection drug glycyrrhizin. *Yakugaku Zasshi*, 127(7), 1103-1114.
- [10] Koga, K., Tomoyama, M., Ohyanagi, K., & Takada, K. (2008). Pharmacokinetics of glycyrrhizin in normal and albumin-deficient rats. *Biopharmaceutics & Drug Disposition*, 29(7), 373-381.
- [11] Porras, M., Solans, C., Gonzalez, C., & Gutierrez, J. M. (2008). Properties of water-inoil (W/O) nano-emulsions prepared by a low-energy emulsification method. *Colloids and Surfaces, A: Physicochemical and Engineering Aspects*, 324(1-3), 181-188.
- [12] Sood, A. (2008). Modeling of the particle size distribution in emulsion polymerization. *Journal of Applied Polymer Science*, 109(3), 1403-1419.
- [13] Yamamura, Y., Kawakami, J., Santa, T., Kotaki, H., Uchino, K., Sawada, Y., Tanaka, N., & Iga, T. (1992). Pharmacokinetic profile of glycyrrhizin in healthy volunteers by a new high-performance liquid chromatographic method. *Journal of Pharmaceutical Sciences*, 81(10), 1042-1046.
- [14] Ploeger, B., Mensinga, T., Sips, A., Seinen, W., Meulenbelt, J., & De Jongh, J. (2001). The pharmacokinetics of glycyrrhizic acid evaluated by physiologically based pharmacokinetic modeling. *Drug Metabolism Reviews*, 33(2), 125-147.
- [15] Yamamura, Y., Santa, T., Kotaki, H., Uchino, K., Sawada, Y., & Iga, T. (1995). Administration-route dependency of absorption of glycyrrhizin in rats: intraperitoneal administration dramatically enhanced bioavailability. *Biological & Pharmaceutical Bulletin*, 18(2), 337-341.
- [16] Takahashi, M., Nakano, S., Takeda, I., Kumada, T., Sugiyama, K., Osada, T., Kiriyama, S., Toyoda, H., Shimada, S., & Samori, T. (1995). The pharmacokinetics of the glycyrrhizin and glycyrrhetic acid after intravenous administration of glycyrrhizin for the patients with chronic liver disease caused by type C hepatitis virus. *The Japanese journal of gastro-enterology*, 92(12), 1929-1936.
- [17] Ishida, S., Sakiya, Y., Ichikawa, T., & Taira, Z. (1992). Dose-dependent pharmacokinetics of glycyrrhizin in rats. *Chemical & Pharmaceutical Bulletin*, 40(7), 1917-1920.
- [18] Koga, K., Takekoshi, K., Kaji, A., Hata, Y., Ogura, T., & Fujishita, O. (2006). Basic study on development of glycyrrhizin formulations for self administration. *Iryo Yaku-gaku*, 32(5), 414-419.
- [19] Horikawa, M., Kato, Y., Tyson, C. A., & Sugiyama, Y. (2002). The potential for an interaction between MRP2 (ABCC2) and various therapeutic agents: probenecid as a candidate inhibitor of the biliary excretion of irinotecan metabolites. *Drug Metabolism and Pharmacokinetics*, 17(1), 23-33.

- [20] Ishida, S., Sakiya, Y., Ichikawa, T., Taira, Z., & Awazu, S. (1990). Prediction of glycyrrhizin disposition in rat and man by a physiologically based pharmacokinetic model. *Chemical & Pharmaceutical Bulletin*, 38(1), 212-18.
- [21] Ichikawa, T., Ishida, S., Sakiya, Y., Sawada, Y., & Hanano, M. (1986). Biliary excretion and enterohepatic cycling of glycyrrhizin in rats. *Journal of Pharmaceutical Sciences*, 75(7), 672-675.
- [22] Baker, K. J. (1966). Binding of sulfobromophthalein sodium and indocyanine green by plasma (sub α1)-lipoproteins. *Proceedings of the Society for Experimental Biology and Medicine*, 122(4), 957-963.
- [23] Benson, R. C., & Kues, H. A. (1978). Fluorescence properties of indocyanine green as related to angiography. *Physics in Medicine & Biology*, 23(1), 159-163.

