

Basic Studies on Formulation, Method of Preparation and Characterization of Water-in-Oil-in-Water Type Multiple Emulsions Containing Vancomycin

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A method of preparing a stable water-in-oil-in-water type multiple emulsion (w/o/w emulsion) as a carrier of peptides was sought by studying rotation rate of homogenizer, mixing ratio of each component and concentrations of surfactants. We used "Lipiodol Ultra-Fluid® and isoropyl myristate oil mixture (4.5:5.5)" for an oil phase of the w/o/w emulsion to minimize the difference in specific gravity between this phase and an aqueous phase and to reduce the viscosity of the former. w/o/w emulsions entrapping vancomycin hydrochloride in their internal aqueous phases were prepared by a modified two-stage emulsification procedure and their particle size, entrapment efficiency, viscosity, separation and drug release property studied.

The particle size of the w/o/w emulsion was smaller with an increase in rotation rate at an emulsification stage ($96.3 \pm 31.8 \mu\text{m}$ at 1000 rpm and $2.81 \pm 1.37 \mu\text{m}$ at 20000 rpm), but its entrapment efficiency was slightly reduced with rate increase. The best mixing ratio of internal aqueous phase:oil phase:external aqueous phase was 1:4:5. For surfactants, HCO-40 (5% (w/v)) and Pluronic F-88 (5% (w/v)) were dissolved in the oily and the external aqueous phase, respectively. Drug release from the w/o/w emulsion was extremely slow and sustained, and that property tended to be faster with a decrease in their particle size (0% at $96.3 \mu\text{m}$ and $2.97 \pm 0.41\%$ at $2.81 \mu\text{m}$). The stability of w/o/w emulsion of $10 \mu\text{m}$ diameter was good from observations of optical and scanning electron microphotographs and measurement of w/o/w emulsion diameters. The w/o/w emulsion prepared in this study is viewed as a possible carrier of water-soluble drugs.

Key words w/o/w emulsion; water-soluble drug carrier; Lipiodol Ultra-Fluid® isopropyl myristate oil mixture; poly(oxyethylene)poly(oxypropylene) block co-polymer; homogenizer rotation rate; drug release property

This paper reports basic studies on water-in-oil-in-water (w/o/w) type multiple emulsion (w/o/w emulsion) as a carrier of high molecular weight water-soluble drugs such as peptides. The effects of the oily phase, kinds and concentrations of surfactants and formulations on products were studied, and the characteristics of and drug release from the w/o/w emulsions were evaluated.

Effective drug delivery systems (DDS) must be developed to realize an ideal chemotherapy.^{1,2)} Encapsulation methods using carrier systems or chemical modification methods consisting of macromolecules and so on are now used for DDS; we selected the w/o/w emulsion for this purpose.

w/o/w emulsions are viewed as useful carriers for delivery of many kinds of water-soluble drugs. This was discovered by Seifriz³⁾ in 1925 and Matsumoto *et al.*⁴⁾ developed a two-stage emulsification procedure in 1976. There are many reports on such topics as preparation⁵⁾ and properties⁶⁾ of w/o/w emulsions, prolonged drug release,⁷⁻¹⁰⁾ treatment of drug overdoses,¹¹⁾ stabilization of drugs¹²⁾ and targeting carrier of drugs.¹³⁾ However w/o/w emulsions are known to be thermodynamically unstable systems. Stable w/o/w emulsions were achieved by modifying the internal aqueous phase by gelling using poly(acrylamide),¹⁴⁾ gelatin or amino acid,¹⁵⁾ and making it hypertonic by an increase in the concentration of the solute (glucose or sodium chloride).¹⁶⁾ We believed that the instability of w/o/w emulsions was mainly attributable to difference in their particles size and specific gravities between the water phase and the oil phase. Either because of instability or too large particle size, the use of w/o/w emulsions in *in vivo* studies has been limited.

The purpose of this research was to establish a method of preparation of a stable w/o/w emulsion which showed good drug entrapment efficiency, for use as the carrier of high molecular weight water-soluble drugs such as peptides. We selected vancomycin hydrochloride (VCM), which is a glycopeptide antibiotic and has five peptide-bonds in its structure, as a representative water-soluble drug. To obtain w/o/w emulsion stability we used an oil mixture of Lipiodol Ultra-Fluid® and isopropyl myristate (4.5:5.5) and minimized the differences in specific gravities between each phase of the w/o/w emulsion. The specific gravity of this oil mixture at 20 °C was about 1.01, which was almost equal to the value of normal saline (1.01), the basic solvent of each aqueous phase. We used the derivatives of polyethylene glycol hydrogenated castor oils and the poly(oxyethylene) poly(oxypropylene) block co-polymers for surfactants. Further, we modified the emulsification process of the w/o/w emulsion to achieve good dispersion and stability using a sonicator and a high speed homogenizer. We also evaluated formulations of each phase and kinds and concentrations of surfactants. After preparation of each w/o/w emulsion, the characteristics of w/o/w emulsion such as drug entrapment efficiency, release property, particle size, viscosity, separation and microscopic shape of each w/o/w emulsion were evaluated.

Materials and Methods

Materials VCM was provided by Shionogi Pharmaceutical Co. (Osaka). The derivatives of hydrogenated castor oil; HCO-40 and HCO-60 were supplied by Nikko Chemicals Co. (Tokyo), and the poly(oxyethylene) poly(oxypropylene) block co-polymers, Pluronic F-68 (F-68, M.W. 8350) and Pluronic F-88 (F-88, M.W. 10800), were supplied by Asahidenka Kogyo Co. (Tokyo). Lipiodol Ultra-Fluid® (LPD,

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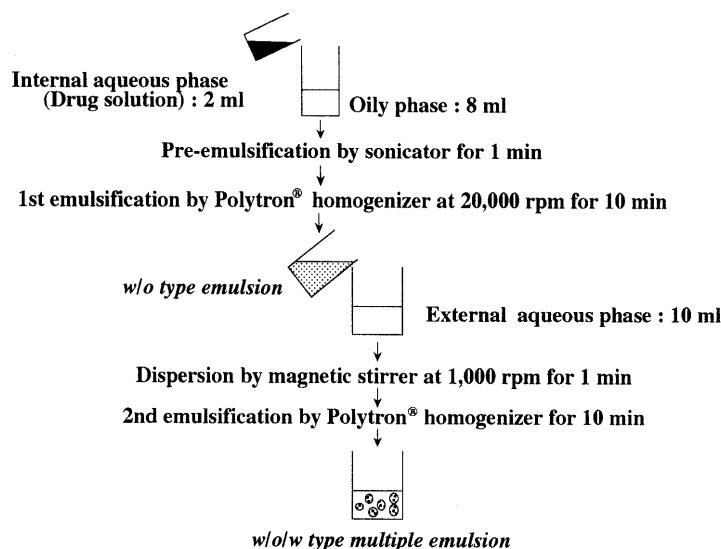


Fig. 1. Schematic Diagram for Preparation of w/o/w Emulsions

specific gravity; 1.28 at 20°C) which is an iodized poppy-seed oil ethyl ester (contains 37% (w/w) iodine) was purchased from Kodama Co. (Tokyo) and isopropyl myristate (IPM, specific gravity 0.89 at 20°C) was a gift from Nippon Oil and Fats Co. (Tokyo). Water was distilled and treated with a Mill-Q reagent-grade water system produced by Millipore Co. (Massachusetts, U.S.A.). All other chemicals were of reagent grade.

Preparation of w/o/w Emulsions We modified the two-stage emulsification procedure reported by Matsumoto *et al.*⁴⁾ and prepared w/o/w emulsions, the components of which were as follows. The internal aqueous phase was a normal saline drug solution. In the oily phase, HCO-40 was dissolved at 5% (w/v) in an LPD-IPM oil mixture (LPD:IPM=4.5:5.5). In the external aqueous phase, either F-68, F-88 or HCO-60 was dissolved in normal saline.

The method of preparation is schematically shown in Fig. 1. The internal aqueous phase was poured into the oily phase and sonicated for 1 min (pre-emulsification). Then, this mixture was stirred by a high speed homogenizer (Polytron®, Kinematica) for 10 min to obtain a water-in-oil type (w/o) emulsion (first emulsification). This w/o emulsion was then poured into the external aqueous phase and agitated by a magnetic stirring bar (10×4 mm) at 1000 rpm for 1 min, then stirred by the homogenizer for 10 min (second emulsification).

Determination of Entrapment Efficiency of VCM into the w/o/w Emulsions Determination of entrapment efficiency was done by the method of Fukushima *et al.*⁷⁾ Two milliliters of w/o/w emulsion was put into a dialysis tube (Spectra/Por® 2, molecular cutoff: 12000—14000, Spectrum Medical) and was dialyzed to its own external aqueous phase solution for 6 h. The dialysis solution was then sampled and the concentration of VCM was measured. Entrapment efficiency of VCM into the w/o/w emulsion was calculated from the following equation:

$$\text{entrapment efficiency in percent} = \frac{V_i \times C_i - C_d(V_d + V_e)}{V_i \times C_i} \times 100$$

where C_i and C_d are the concentration of VCM (mg/ml) in the internal aqueous phase at initial state and the external aqueous phase after dialysis, respectively. V_i , V_d and V_e are the volume (ml) of the internal aqueous phase, the external aqueous phase and the total dialysis solution, respectively.

Drug Release Study After determination of the encapsulation efficiency, each dialysis tube filled with the w/o/w emulsion was put into 100 ml of normal saline at 37°C and dialyzed. During dialysis, 0.5 ml of the dialyzed solution was sampled at 1, 2, 3, 6, 9, 12, 24, 48 and 72 h and the drug concentrations at each point were calculated. Percent of drug released is described by:

$$\text{percent of drug released} = \frac{(C_n - C_d) \times (V_e + V_a - V_b)}{\{V_i \times C_i - C_d \times (V_d + V_e)\} - V_r \times \sum_{n=1}^{n-1} C_n} \times 100$$

where C_n is the drug concentration at n -th sampling (mg/ml), V_a is the

initial w/o/w emulsion volume (ml), V_b is the w/o/w emulsion volume (ml) after dialysis and V_r is the sampling volume (ml). In this calculation, we assumed that the water permeation takes place only from the dialysis solution to the external phase of the w/o/w emulsion through the dialysis tube, and not from the external phase to the internal phase of the w/o/w emulsion.

Determination of Particle Size of the w/o/w Emulsions Particle sizes of the internal aqueous phase of the w/o/w emulsion were measured by an automatic particle analyzer (BI-90AT, Brookhaven Instruments). Particle sizes were measured using a particle dispersion analyzer (Model CIS-1, Galai). We used the volume mean particle diameters as the particle sizes of w/o/w emulsions.

Measurement of Viscosity of the w/o/w Emulsions w/o/w emulsion viscosity was measured using a cone-plate rotary viscometer (E-type, Tokyo Keiki). One and two-tenths milliliters of the w/o/w emulsion was placed on the viscometer and measured at 37°C for 5 min.

Assessment of Stability of the w/o/w Emulsions Two methods were used to determine the w/o/w emulsion stability. The first method was the measurement of particle size of the w/o/w emulsion. One hundred microliters of the emulsion was dispersed into 100 ml of either normal saline, phosphate buffer solution (PBS), pH 7.4 or 10% (w/w) of fetal bovine serum (FBS) at 37°C, and the particle size was measured at 0, 1, 3, 6 and 12 h. The other was the measurement of the separation of the w/o/w emulsion; this was based on the measuring cylinder-method reported by Zhang *et al.*¹⁵⁾ Five milliliters of the emulsion was taken into a syringe and allowed to stand at 4°C, and we measured the elapsed time before separation began and calculated the degree of separation from the equation:

$$\text{percent of separation} = \frac{E_w - S_w}{E_w} \times 100$$

where E_w is the volume of the initial external water of the w/o/w emulsion and S_w is the volume of the water separated from the w/o/w emulsion.

Microscopic Observation of the w/o/w Emulsions Optical photomicrographs of the w/o/w emulsion were taken with an optical microscope (BH-2, Olympus) connected to a photography system (PH-6, Olympus); the magnification was 400 times. Scanning electron photomicrographs were taken using a JSM-5400 model with a cryo system (JEOL).

Determination of Concentrations of Vancomycin Determination of concentrations of VCM was made by the method of Jehl *et al.*¹⁷⁾ High performance liquid chromatography (HPLC) was used (LC-6A, SPD-3A, CR-3A, Shimadzu, Kyoto, Japan) under the following conditions: column, LiChrospher 100 RP-18 (5 μm); mobile phase, acetonitrile: 0.02 M ammonium acetate buffer, pH 5.4 = 9 : 1; flow rate, 1 ml/min; temperature, 50°C; absorbance, 280 nm; and injection volume, 50 μl.

Results

Effects of Rotation Rates of the Polytron® Homogenizer and Kinds and Concentrations of Surfactants on Properties of w/o/w Emulsions Formed To examine the effects of

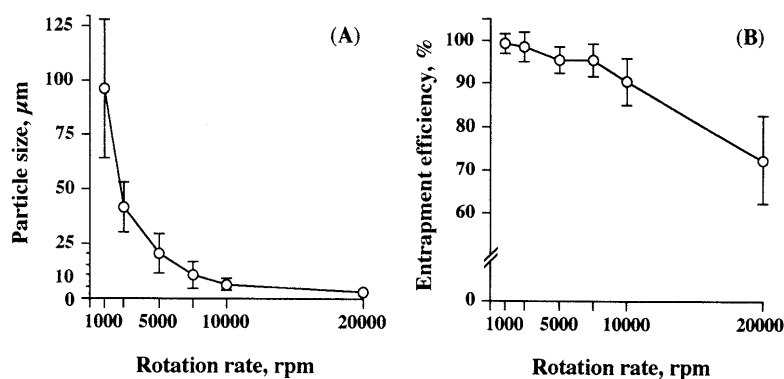


Fig. 2. Relationships between Rotation Rates of Polytron® Homogenizer and Particle Sizes and Entrapment Efficiencies (B) of w/o/w Emulsions^{a)}

a) Each point represents the mean \pm S.E.M. of three experiments.

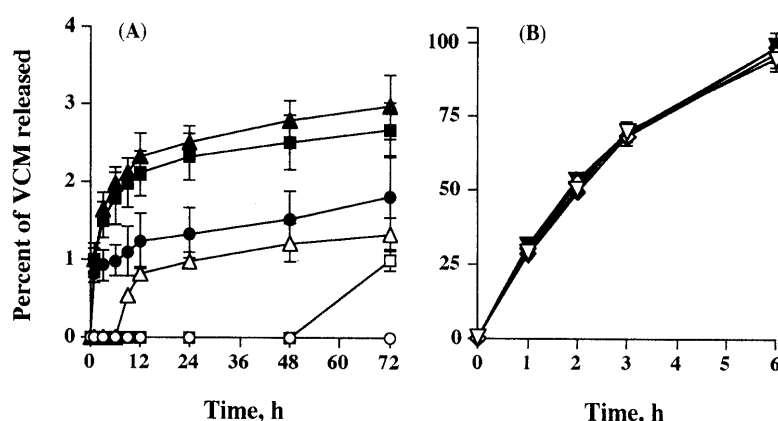


Fig. 3. Release Profiles of Vancomycin from w/o/w Emulsions Containing the Drug (5 mg/ml) in the Internal Aqueous Phase (A) and Its Solution and the Drug (5 mg/ml) Added to Drug-Free Emulsions (B)^{a)}

○, 1000 rpm; □, 2500 rpm; △, 5000 rpm; ●, 7500 rpm; ■, 10000 rpm; ▲, 20000 rpm. a) Each point represents the mean \pm S.E.M. of three experiments.

rotation rates of the Polytron® homogenizer at the second emulsification stage, we prepared w/o/w emulsions at various rotation rates and compared their characteristics. The formulation in this study was so fixed that the ratio of internal aqueous phase:oil phase:external aqueous phase was 1:4:5 and the concentration of VCM in the internal phase was 5 mg/ml. This formulation was the best as found in our previous report.¹⁸⁾ Figure 2 shows the relationship between the rotation rates and particle sizes (A) and entrapment efficiencies (B) of the w/o/w emulsions. As shown in Fig. 2A, particle sizes of the w/o/w emulsions were decreased with an increase in the rotation rate of the homogenizer. Similarly, entrapment efficiency of VCM was decreased with rotation rate increase (Fig. 2B). Figure 3A shows the release profiles of VCM from the w/o/w emulsions shown in Fig. 2. The release rate of VCM entrapped in the internal phase of the w/o/w emulsions was accelerated according to the increase in the rotation rate, but the amount of drug released was less than 3% in 72 h. Figure 3B shows the release profiles of VCM from the VCM solution and 3 types of w/o/w emulsions to which VCM was added after preparation; the profiles of VCM were almost the same.

To determine the best kind and concentration of the surfactants dissolved in the external aqueous phase, we prepared the w/o/w emulsions (the rotation rate at the second emulsification stage was 7500 rpm). The formula-

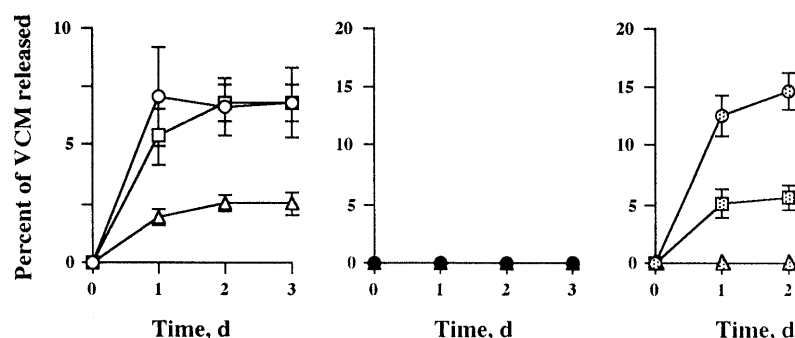
tions and the results of this study are shown in Table 1 and Fig. 4. When we used F-68, the entrapment efficiencies were extremely high at about 100%, but they were easily separated in 3 d. In contrast, when F-88, especially at 5%, was used, the entrapment efficiency was also about 100% but there was little separation. Using HCO-60, separation took place in a day. As shown in Fig. 4, VCM release was suppressed by an increase in concentration of surfactants.

Effects of Formulations on Properties of w/o/w Emulsions To study the w/o/w emulsion formulations which were ratios of internal aqueous phase:oil phase:external aqueous phase and VCM concentrations of the internal phase, we prepared w/o/w emulsions which differed in some parameters. Compositions of each formulation and characteristics of these emulsions are shown in Table 2. Most of them showed extremely high entrapment efficiencies of more than 92%, with the exception of F-10 (81.2%). The particle size was increased with an increase in the ratio of oily phase to the internal phase up to F-17 (the ratio was 1:4); beyond that, the particle size was smaller again. The viscosity was increased with an increase in the amount of the oily phase to the internal phase in a similar manner as the particle size. F-15, 16 and 17 did not separate. VCM releasing profiles are illustrated in Fig. 5. In these w/o/w emulsions, the percentage of VCM release from F-10 (8.3%) and F-11 (2.5%) was relatively large, but in others it was slightly

Table 1. Formulations and Characteristics of w/o/w Emulsions Containing Vancomycin Prepared with Three Different Surfactants^{a)}

Formulation		1	2	3	4	5	6	7	8	9
Internal aqueous phase (Saline solution)	(ml)	2	2	2	2	2	2	2	2	2
Vancomycin	(mg)	10	10	10	10	10	10	10	10	10
Osmolarity	(mOsm/kg H ₂ O)	288	288	288	288	288	288	288	288	288
Oily phase	(ml)	8	8	8	8	8	8	8	8	8
LPD	(g)	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
IPM	(g)	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8
Conc. of HCO-40	(%w/v)	5	5	5	5	5	5	5	5	5
External aqueous phase (Saline solution)	(ml)	10	10	10	10	10	10	10	10	10
Conc. of Pluronic F-88	(%w/v)	0.5	1	5	—	—	—	—	—	—
Conc. of Pluronic F-68	(%w/v)	—	—	—	0.5	1	5	—	—	—
Conc. of HCO-60	(%w/v)	—	—	—	—	—	—	0.5	1	5
Osmolarity	(mOsm/kg H ₂ O)	290	293	293	286	289	290	292	292	290
Encapsulation efficiency	(%)	92.0	97.3	≤100	≤100	≤100	≤100	81.1	96.2	96.4
Mean diameter										
Just after preparation	(μm)	14.8	13.8	9.64	19.0	14.0	13.5	17.7	17.3	19.7
7 d after preparation	(μm)	16.1	15.5	9.17	26.8	21.9	18.4	29.2	25.7	27.9
Viscosity										
Just after preparation	(cP)	9.75	13.1	12.7	26.6	17.6	18.3	10.3	13.8	20.6
7 d after preparation	(cP)	16.2	20.7	14.3	25.3	20.1	14.6	9.75	27.0	25.5
Separation										
Time until separation began	(d)	5	7	—	2	2.5	3	0.5	0.5	1
Degree of separation	(%)	20	15	0	26	24	20	26	26	20

a) Each value represents the mean of three experiments.

Fig. 4. Effect of Formulation Variables on Release Profiles of Vancomycin from w/o/w Emulsions^{a)}

○, F-1; □, F-2; △, F-3; ●, F-4; ■, F-5; ▲, F-6; ⊙, F-7; ⊠, F-8; △, F-9. a) Each point represents the mean ± S.E.M. of three experiments.

Table 2. Characteristics of w/o/w Emulsions Containing Vancomycin Prepared with Different Composition Ratios^{a)}

Formulation number		10	11	12	13	14	15	16	17	18	19
Composition (w : o : w)		1 : 1 : 2	1 : 2 : 3	1 : 3 : 4	1 : 4 : 3	1 : 4 : 4	1 : 4 : 5	1 : 4 : 5	1 : 4 : 5	1 : 6.5 : 7.5	1 : 9 : 10
Concentration of VCM in the whole w/o/w emulsion	(mg/ml)	2.25	1.67	1.25	1.25	1.11	1.0	2.5	5.0	0.67	0.5
Particle size	(μm)	5.09	8.84	9.14	11.9	11.3	11.6	10.4	10.4	6.88	6.95
Viscosity	(cP)	3.48	36.1	54.3	208	208	54.6	88.2	119	58.8	80.4
Entrapment efficiency	(%)	81.2	92.5	96.2	92.9	96.0	96.2	99.3	99.3	97.9	96.8
Time until separation began	(d)	0.5	3	6.5	7	7	—	—	—	6	6

a) Each value represents the mean of three experiments.

less than 0.5% at 72 h.

Evaluation of Stability of w/o/w Emulsions In Fig. 6, we show optical photomicrographs of a stable w/o/w emulsion (F-17). Each particle (oily phase) was made up of small particles, the internal aqueous phase, and we confirmed that w/o/w emulsion was formed. Comparing b) and c), form and particle size of the w/o/w emulsion changed little over 10 h. From a) and b), when this

emulsion was diluted, some particles aggregated, but this phenomenon was not coalescence because this w/o/w emulsion did not separate and its particle size did not change.

The scanning electron photomicrograph (SEM) of the w/o/w emulsion (stable form F-17) is shown in Fig. 7, where a and b were just after preparation and c and d were 7 d afterwards. Each particle was w/o emulsion

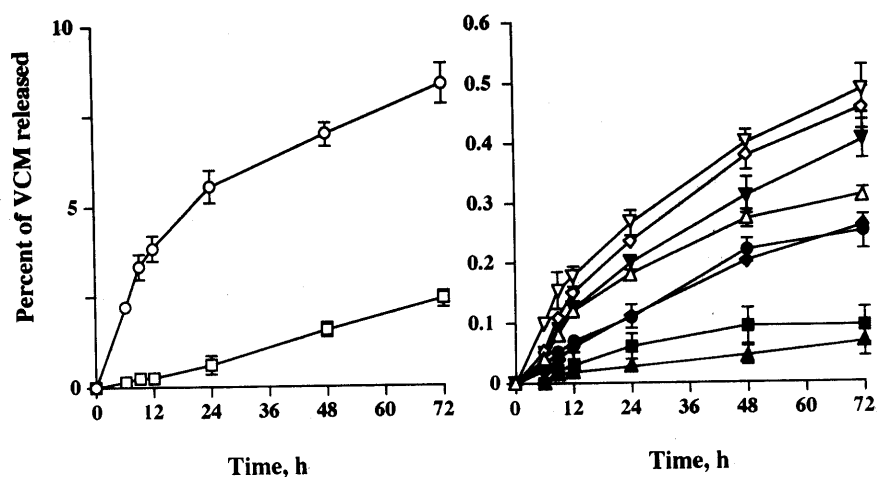


Fig. 5. Release Profiles of w/o/w Emulsions Containing Vancomycin Prepared with Different Rotation Rates^{a)}

○, F-10; □, F-11; △, F-12; ▽, F-13; ◇, F-14; ●, F-15; ■, F-16; ▲, F-17; ▼, F-18; ◆, F-19. a) Each point represents the mean \pm S.E.M. of three experiments.

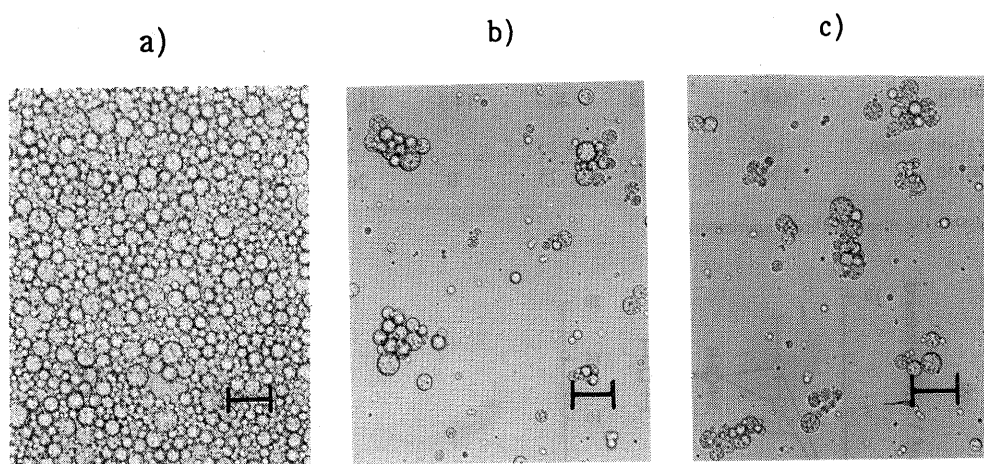


Fig. 6. Optical Photomicrographs of w/o/w Emulsions

a) Undiluted, b) just after 10-fold dilution, c) 10 h after 10-fold dilution. Each bar represents 30 μ m.

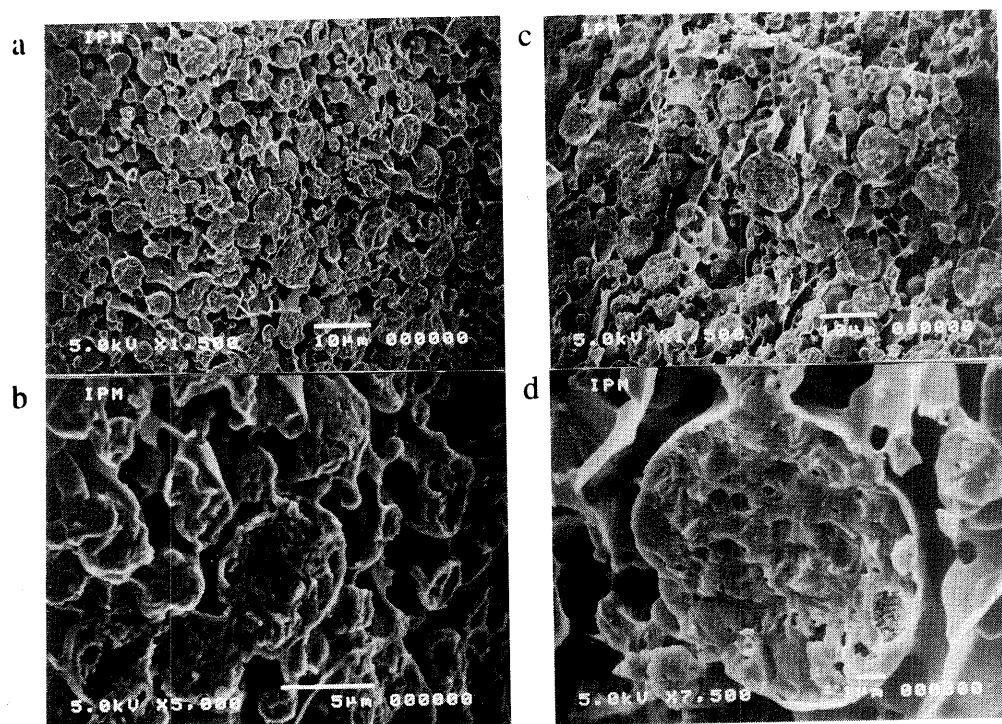


Fig. 7. Scanning Electron Photomicrographs of w/o/w Emulsions with a Cryo System

a and b: just after preparation. c and d: 7 d after preparation. The bars in c and d read 10 μ m and 1 μ m, respectively.

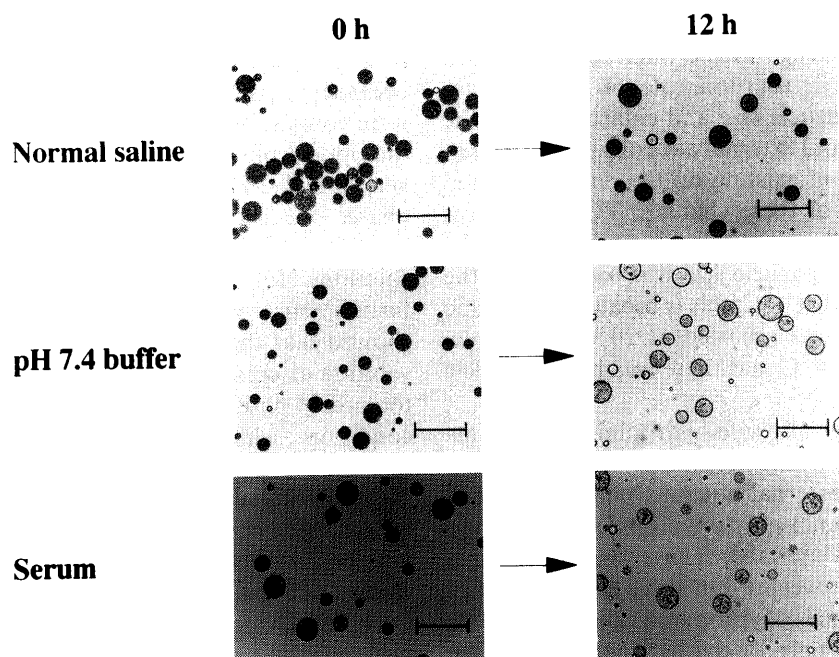


Fig. 8. Effect of Some Physiological Solutions on Stability of w/o/w Emulsions
Each bar represents 30 μm .

particles and was dispersed relatively homogeneously; each particle had many cavities and these cavities were thought to be internal aqueous phases. These cavities are clearer in photograph b, which is a magnification of a. The mean diameter of these internal aqueous phases was about 256 ± 112 nm (measured by particle analyzer). Comparison of a with c or b with d shows that the particle size and dispersion state were preserved 7 d after preparation. Moreover, it was clear that the w/o/w emulsion had internal phase in its structure for at least 7 d.

Changes in the optical structures and particle sizes of the w/o/w emulsion were determined to evaluate stability. We used F-17 as a representative of the w/o/w emulsions. From the optical photomicrographs in Fig. 8 and measurement of particle sizes, the particle structures and sizes of each w/o/w emulsion did not change very much under the physiological conditions (normal saline, PBS and FBS at 37°C) for 12 h.

Discussion

Knowledge of the stability, drug entrapment efficiency and drug releasing property of w/o/w emulsion is of value. Here, we studied the method of preparation and the formulation of a stable w/o/w emulsion as a possible carrier of peptides.

The Stokes formula, which describes the precipitation velocity at free gravity states, shows that

$$v = \frac{d^2 \times (\rho_s - \rho_o) \times g}{18 \times \eta_o}$$

the velocity (v cm/s) of precipitation depends on the particle size (d cm), the density difference between the dispersion phase (ρ_s) and the continuous medium (ρ_o) and the viscosity (η_o) of the continuous medium. In this formula, g is acceleration of gravity. According to the Stokes formula, when the particle size or the density

difference is made small, the velocity will be small. We thus focussed on particle size and density (as a specific gravity).

Nakamura *et al.*¹⁹⁾ prepared a stable doxorubicin-in-oil emulsion by mixing Urografin®, which is a hydrophilic contrast medium, with purified water to adjust the specific gravities of oily and aqueous phases. In a previous study,¹⁸⁾ we used an LPD-soybean oil mixture for the oily phase to minimize the specific gravity difference between water and oily phase and studied the characteristics of w/o/w emulsions. This oil mixture, however, had high viscosity and w/o/w emulsions prepared with this oil had large particle sizes (minimum 30 μm). There are limitations on the uses of these emulsions for intravascular treatment because of their large particle size and rapid separation.

In this study, we used the LPD-IPM oil mixture and studied the preparation method of w/o/w emulsion using a sonicator and a homogenizer. IPM is a low viscosity oil and an ester, so it is expected to be easily mixed with LPD, ethyl ester of iodized poppyseed oil. In preparation, VCM was degraded by sonication for more than 90 s, but was stable for sheer stress; we therefore used the method shown in Fig. 1 to achieve good dispersion of w/o and w/o/w emulsion. The LPD-IPM oil mixture had smaller viscosity compared with the LPD-soybean mixture, so the particle sizes tended to be smaller (2.81 ± 1.37 μm) and could be controlled to between 3 and 100 μm (Fig. 2A).

The particle size and entrapment efficiency decreased with an increase in rotation rate (Fig. 2). The release profiles of Fig. 3B show that the release of VCM in the external phase of w/o/w emulsions was fast and completed within 6 h. In Fig. 3A, on the other hand, the release of VCM entrapped in the internal phase was extremely slow and most of the release curves were two-phase forms which showed burst phenomena. Percent and rate of drug released at burst were higher with a decrease in the particle

size of the w/o/w emulsion. The cause of this phenomenon is thought to be the change in the interface of the w/o droplet. The increase in rotation rate of homogenizer caused a decrease in particle size and enlargement of the interface between oily and external aqueous phase, so that the amount of surfactant existing on the interface would be decreased. Because of these changes, VCM release is likely to be accelerated. Although we do not show the detailed data, when the particle size was over 10 μm , the separation was observed at 4 °C; this is because the specific gravity of each phase was adjusted at 20 °C but w/o/w emulsion was stored at 4 °C and there was broad particle distribution.

We used three biologically safe hydrophilic surfactants (Table 1 and Fig. 4) to find a suitable surfactant and concentration for the external aqueous phase. F-68 and F-88 are poly(oxyethylene) poly(oxypropylene) block copolymers (shown in Materials). These two surfactants differ in their molecular weight, but there is no difference in the ratio of oxyethylene : oxypropylene and HLB value. They are absorbed strongly to the hydrophobic surface and are not displaced by dilution with aqueous buffers.²⁰⁾ In contrast, HCO-60 is not absorbed, but adheres to the hydrophobic surface. It is therefore believed that F-68 and F-88 can make a stable surface of the emulsion. With respect to the entrapment efficiency and VCM release curve, the w/o/w emulsion using 5% F-88 was the best of all, because entrapment was about 100%, without separation or release of VCM. Except for F-68, VCM release was prolonged by increase in concentration of surfactants. This prolongation may be rationalized as follows: although the concentrations of surfactant were increased, there was little change in particle size. Therefore, the amount of surfactant absorbed on the interface is expected to be increased with an increase in the concentration of surfactant dissolved in the external phase, and those surfactants can prolong VCM release.

The ratio of 1 : 4 : 5 was the best composition of w/o/w emulsion, showing a high entrapment efficiency (over 96%) and no separation for more than 7 d (Table 2). In Table 2, F-15, 16 and 17 were different in drug concentration in the internal phase, but showed almost the same entrapment efficiency. It is thus believed that we could prepare a w/o/w emulsion which contains a much greater quantity of drug. The viscosity of w/o/w emulsion was affected by the particle size, ratio of oily phase to external aqueous phase and the viscosity of the internal aqueous phase, that is, the concentration of VCM in the internal phase. However, we are unable to explain the effect of viscosity on the drug release property shown in Table 2 and Fig. 5. Further study on this is needed. Considering all things, except F-10 and 11, our method of preparation was useful for making w/o/w emulsion that entraps a drug effectively and releases it slowly.

We used photographs and particle sizes primarily to evaluate the stability of the w/o/w emulsion; optical microphotographs,^{7,9)} SEM,¹⁶⁾ etc. were used to evaluate the forms or stabilities. The photographs of Figs. 6 and 8 confirmed that each particle of w/o emulsion has many internal phases. The dispersion of the emulsion did not

change for 10 h, but some particles joined to aggregate (Fig. 6). This aggregation did not shift to coalescence, because the particle sizes measured by the particle analyzer were compatible with those measured by observations of the optical photographs and their values did not change very rapidly. From the SEM, we can see not only oily phases but also each internal phase (Fig. 7); the shapes and particle sizes showed little change in physiological solutions for at least 12 h (Fig. 8). Furthermore, turbidimetry, which is a stability test of emulsion,²¹⁾ showed that there was little change in each physiological solution dispersing w/o/w emulsion (data not shown). We took SEM photographs by the cryo-system under freezing conditions, which will allow us to research new aspects of the dispersion system such as emulsions.

Our results suggest that use of the LPD-IPM oil mixture whose specific gravity is adjusted to water phase for the oily phase makes it possible to prepare stable w/o/w emulsions with high drug entrapment efficiencies. Emulsion particle sizes can be adjusted and drug release prolonged. Further studies on preparations of smaller w/o/w emulsion, entrapment of other drugs or application to *in vivo* systems are desired.

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