Chem. Pharm. Bull. 30(4)1408—1415(1982)

Prolonged Release of Bleomycin from Parenteral Gelatin Sphere-in-Oil-in-Water Multiple Emulsion[1]

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(Received September 24, 1981)

A gelatin sphere-in-oil-in-water (S/O/W) multiple emulsion was prepared by dispersing primary sphere-in-oil (S/O) emulsion into an external aqueous phase and its potential for prolonging parenteral absorption of bleomycin was studied in rats and rabbits. Intramuscular injection of bleomycin in an S/O/W emulsion formulation provided a sustained plasma concentration which was almost equal to those obtained after injection of S/O emulsion and oily suspension, while immediate appearance of bleomycin in plasma and subsequent rapid clearance were observed following intravenous or intramuscular injection of aqueous drug solution. The prolonged release of bleomycin from the multiple emulsion was confirmed by observation of the retarded disappearance of the drug from the injection site. S/O/W emulsion also exhibited enhanced lymphatic transfer of bleomycin following injection into the appendix of rabbits. These results show the potential of S/O/W emulsion as a parenteral dosage form in addition to its superior injectability. The possibility that the instability of multiple emulsions during storage could be at least partly overcome by employing lyophilization processing was demonstrated.

Keywords——S/O/W emulsion; bleomycin; prolonged release; lymphatic transfer; oily suspension; S/O emulsion; lyophilized emulsion; multiple emulsion; intramuscular injection; appendix

The ideal dosage form in cancer chemotherapy is one that provides a tumor site with a sufficient amount of anticancer agents over a long period of time without considerable interaction with normal tissues. In the past, considerable efforts have been directed toward the development of a timed-release device which could be implanted in the closest possible proximity to a malignant tissue[2–4] or a carrier system which could deliver anticancer agents selectively to malignant cells thriving far from the injection site.[5]

In our series of investigations on the utility of various types of emulsions as drug delivery systems, we have demonstrated an increased transport and prolonged supply of antineoplastic agents to the lymphatics by water-in-oil (W/O) emulsion.[6] The greatest enhancement of drug delivery and successful prevention of lymphatic metastasis were obtained with gelatin sphere-in-oil (S/O) emulsion in which W/O emulsion was improved through replacement of its water droplets by gelled gelatin microspheres.[7,8] Detailed examination of physicochemical and biopharmaceutical characteristics of this formulation suggested that the stable incorporation of the drug into the emulsion droplets might be responsible for this superior efficiency.[9,10] However, S/O emulsion is intrinsically difficult to inject because of its high viscosity, and it is also known that sphere-in-oil-in-water (S/O/W) multiple emulsion formed in the interstitial space shortly after injection really acts as the lymphotropic carrier.

In the present investigation, therefore, we prepared gelled sphere-in-oil-in-water multiple emulsion and examined its potential as a prolonged release and lymphotropic carrier system for parenteral administration of bleomycin. Further, lyophilization of S/O/W emulsion was carried out with the aim of developing a stable form of multiple emulsion, since such emulsion is normally unstable under most storage conditions.

Experimental

Material——Bleomycin was supplied by Nihon Kayaku Co. Sesame oil and gelatin were obtained from Nakarai Chemicals Co. Nonionic surfactants, a polyoxyethylene derivative of hydrogenated castor oil (HCO-60) and sorbitan sesquioleate (SO-15) were supplied by Nikko Chemicals Co. All other chemicals were reagent grade products obtained commercially.

Preparation of Parenteral Formulations——The compositions of six formulations tested in the present investigation are shown in Table I. The contents of bleomycin were 15 mg (potency)/ml for all dosage forms. For preparing S/O emulsion, an oily phase incorporating surfactants and an aqueous phase (20% gelatin solution) were emulsified by ultrasonification (Ohtake Sonicator 150). Sonification was carried out in a water bath maintained at 70—80°C followed by rapid cooling to about 0°C.

Table I. Compositions of Dosage Forms examined in the Present Investigation

S/O emulsion		S/O/W emulsion	
Water phase	0.15 ml	S/O emulsion	1.0 ml
Bleomycin	15 mg	(Bleomycin	37.5 mg)
Gelatin	30 mg	External aqueous phase	1.5 ml
Distilled water	q.s.	Gelatin	15 mg
Oil phase	0.85 ml	Distilled water	q.s.
Sesame oil	0.779 ml	Total volume	2.5 ml
SO-15	0.057 ml	O/W emulsion	
HCO-60	0.014 ml	Oily suspension	1.0 ml
Total volume	1.0 ml	(Bleomycin	37.5 mg)
Oily suspension		External aqueous phase	1.5 ml
Bleomycin	15 mg	Gelatin	15 mg
Aluminium monostearate	10 mg	Distilled water	q.s.
Sesame oil	q.s.	Total volume	2.5 ml
Total volume	1.0 ml	Lyophilized S/O/W Emulsion	
Aqueous solution		S/O emulsion	1.0 ml
Bleomycin	15 mg	(Bleomycin	37.5 mg)
Saline	q.s.	Emulsifier (Gelatin)	15 mg
Total volume	1.0 ml	Dispersing medium (Distilled water)	1.5 ml
		Total volume	2.5 ml

S/O/W emulsion was prepared by dispersing S/O emulsion into distilled water containing dissolved gelatin (1%) as an emulsifier. The lyophilization of S/O/W emulsion was carried out using ATMO-VAC freeze dryer (RFS#5000) after adequate dilution with distilled water. The product was kept in a glass tube filled with nitrogen gas and stored in the refrigerator. S/O/W emulsion was regenerated by dispersing the lyophilized product into a suitable amount of distilled water.

The oily suspension of bleomycin was supplied by Nihon Kayaku Co., and had been prepared by suspending bleomycin in sesame oil using aluminium monostearate as a dispersing agent. The oily suspension of bleomycin was also emulsified into 1% gelatin solution to obtain O/W emulsion which might contain bleomycin either as solid fragments in the oily droplets or in solution in the outer aqueous phase.

Procedure for Animal Experiments——Male Wistar albino rats weighing between 200 and 220 g and male domestic rabbits weighing 2.0 and 3.2 kg were used in the present investigation. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital and fixed on their backs during the course of the experiment. Intramuscular injection was undertaken into the center of the thigh muscle using a microliter syringe. For intermittent sampling of blood, a polyethylene catheter (I.D. 0.5 mm, O.D. 0.8 mm) was cannulated into the carotid artery and a blood sample (1 ml) was withdrawn periodically. Three or four blood samples were obtained at intervals from each rat and more than ten rats were used for each dosage form in combination. The dose of bleomycin was 12 mg (potency)/kg and the total dose was injected dividedly into the thigh muscles of both sides. Intravenous injection was performed into the femoral vein. The muscle clearance experiment was carried out according to the procedure of Kakemi *et al.*[11] To avoid problems concerning emulsion instability, emulsion formulations were employed for injection within 1 h after preparation.

Rabbits were anesthetized by intravenous injection of sodium pentobarbital and placed on their backs during the course of the operation. The appendix of the rabbit was exposed through a midline incision of the abdominal wall and various formulations were injected into the sub-serosal layer of the appendix.[12] The dose of bleomycin was 3 mg/kg. At various periods after injection, rabbits were sacrificed, and the appendix and regional lymph nodes were excised. Blood samples were obtained simultaneously.

Analytical Method——The concentration of bleomycin was determined by microbiological assay using *Bacillus subtilis* PCI-219 as a test organism.[13] For the measurement of plasma levels, blood was centrifuged at 3000 rpm for 2 min and the plasma obtained was stored in a freezer until assay. The excised tissue was homogenized in a glass homogenizer after being weighed, and diluted with isotonic phosphate buffer (pH 7.4). After centrifugation at 2800 rpm for 5 min, the supernatant was used for microbiological assay. All assays were undertaken by a disc-plate method and the results were calculated using a standard curve. The minimum sensitivities were 0.5 μg/ml and 1 μg/g for plasma and the tissue, respectively.

Results

Preparation of S/O/W Emulsion

Fig. 1 shows photomicrographs of S/O emulsion and S/O/W multiple emulsion. In S/O emulsion, a large number of particles formed with gelatin gel can be seen dispersed uniformly in the oily phase. S/O/W emulsion provided a fairly uniform multiple emulsion retaining a large number of fine microspheres in the oil droplets. The size of the oil droplets was distributed mostly between 5 to 40 μm. Small amounts of gelatin spheres that had escaped from the oil droplets to the external aqueous phase while still retaining their shape and size were also seen.

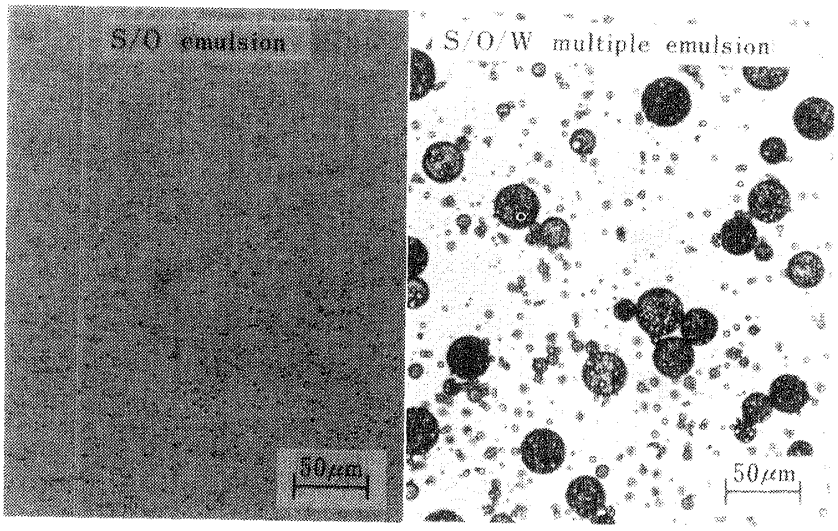

Fig. 1. Photomicrographs of S/O Emulsion and S/O/W Emulsion

Plasma Concentration of Bleomycin following Intramuscular Injection with Various Formulations

Before examining the prolonged release potential of emulsions, the plasma concentrations of bleomycin after intravenous and intramuscular injection of aqueous drug solution were determined. The results are shown in Fig. 2 in arithmetic (a) and semilogarithmic (b) plots. Immediately after intravenous injection of bleomycin, the plasma concentration reached the highest level of 70 μg/ml but it decreased rapidly and the fall-off curve appears to be roughly monoexponential with a half-life of about 30 min. Intramuscular injection of aqueous drug solution resulted in rapid appearance of the drug in the plasma within 30 min after injection, followed by a rapid concentration decrease pharmacokinetically similar to that after the intravenous injection.

Fig. 3 represents the concentration-time course of bleomycin in plasma following injection of S/O/W emulsion and O/W emulsion prepared by dispersing an oily suspension of bleomycin into 1% gelatin solution. Compared with the results for aqueous drug solution, S/O/W emulsion showed a significant delay of appearance of bleomycin in the plasma and a more gradual plasma level decrease. The maximum plasma level of 13 μg/ml was observed at 30 min after

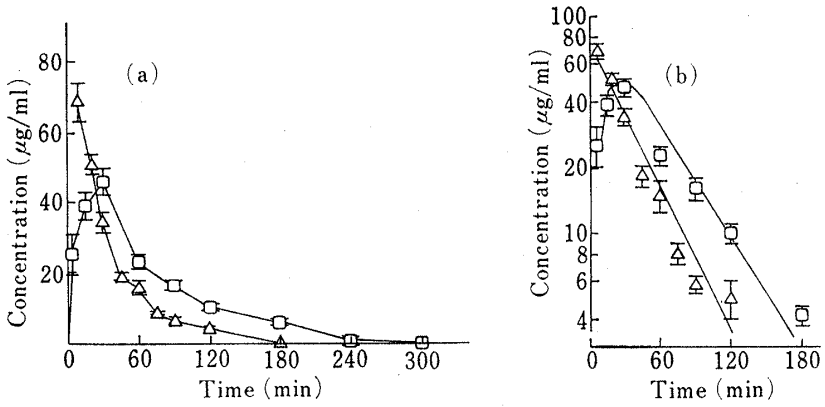

Fig. 2. Plasma Concentration of Bleomycin on Arithmetic (a) and Semilogarithmic (b) Scales after Intravenous or Intramuscular Injection of Aqueous Drug Solution

△, intravenous injection; □, intramuscular injection.
Results are expressed as the means ± S.E. of at least five rats.

injection. In contrast O/W emulsion resulted in rapid appearance of bleomycin in the plasma, and the concentration reached 37 μg/ml at 30 min after injection. The concentration then decreased rapidly.

In Fig. 4, the plasma bleomycin levels after intramuscular injection of S/O emulsion and oily suspension are shown for comparison. Both formulations showed fairly low concentrations throughout the experimental period and in no instance, did the concentration exceed 15 μg/ml.

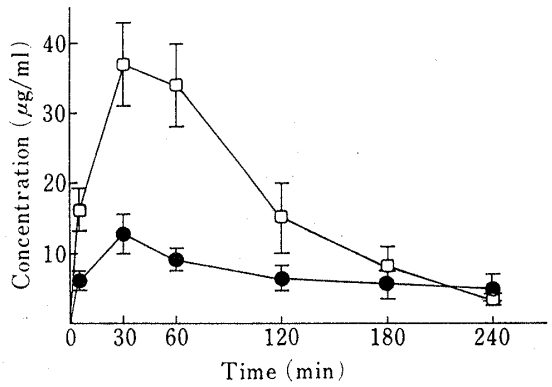

Fig. 3. Plasma Concentration of Bleomycin after Intramuscular Injection of S/O/W Emulsion (●) or O/W Emulsion (□) prepared by Dispersing Bleomycin Oily Suspension in Aqueous Phase

Results are expressed as the means ± S.E. of at least five rats.

In order to clarify the mechanism of prolonged absorption of bleomycin from parenteral S/O/W emulsion, S/O/W emulsion containing bleomycin in the external aqueous phase was prepared and compared with the ordinary emulsion. As shown in Fig. 5, S/O/W emulsion containing bleomycin dissolved in the external phase resulted in rapid plasma appearance and subsequent clearance of the drug. The course resembled that after aqueous solution injection but was different from that after injection of ordinary S/O/W emulsion entrapping bleomycin in the internal aqueous phase.

Disappearance of Bleomycin from the Injection Site after Intramuscular Injection

In order to elucidate the release phenomenon of bleomycin from S/O/W emulsion at the injection site in detail, the disappearance of bleomycin from the thigh muscle was determined periodically, and the results are shown in Fig. 6. The modes of disappearance of bleomycin injected in the forms of S/O emulsion and aqueous solution are also represented for comparison. Bleomycin disappeared rapidly following injection of aqueous drug solution, and the disappearance process appears to be essentially monoexponential with a half-life of about 15 min. S/O/W emulsion and S/O emulsion resulted in relatively slower disappearance, and even after 60 min, 30% and 40% of the dose remained in the thigh muscle, respectively.

Preparation and Potential of Lyophilized S/O/W Multiple Emulsion

The lyophilization products of S/O/W emulsion were obtained as bulky piles of oil droplets

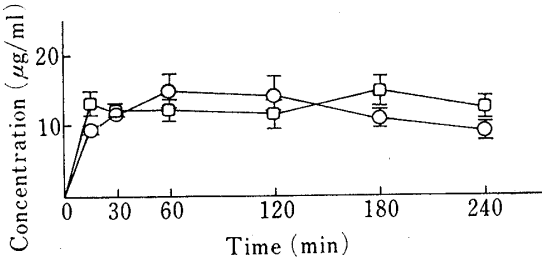

Fig. 4. Plasma Concentration of Bleomycin after Intramuscular Injection of S/O Emulsion (○) or Oily Suspension (□)

Results are expressed as the means±S.E. of at least five rats.

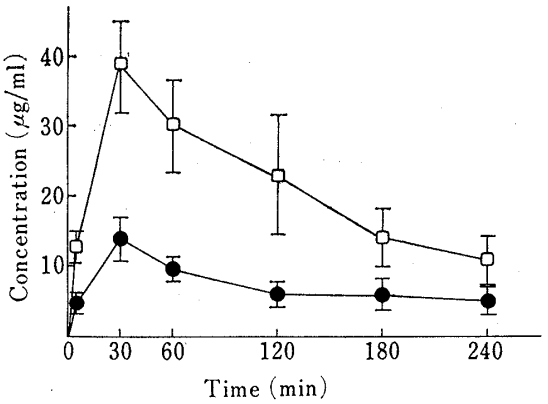

Fig. 5. Plasma Concentration of Bleomycin after Intramuscular Injection of the Original S/O/W Emulsion (●) or S/O/W Emulsion containing the Drug in the External Aqueous Phase (□)

Results are expressed as the means±S.E. of at least five rats.

and were stored in a refrigerator. Immediately before the experiment, the freeze-dried emulsion was resuspended in a corresponding amount of distilled water. The reconstituted S/O/W emulsion was proved to retain its original multiple construction by microscopic observation.

Fig. 7 shows plasma bleomycin concentration following intramuscular injection of the regenerated emulsion in comparison with that after the original emulsion. Regenerated emulsion resulted in a slightly higher plasma concentration during the initial 1 h but the level then decreased to that of the original S/O/W emulsion. However, the increased plasma level was still significantly lower than those of aqueous solution and S/O/W emulsion containing the drug in the external aqueous phase, shown in Figs. 2 and 5.

Lymphatic Transport of Bleomycin after Injection of S/O/W Emulsion into the Appendix of Rabbits

In order to assess the feasibility of S/O/W multiple emulsion as a carrier for delivering

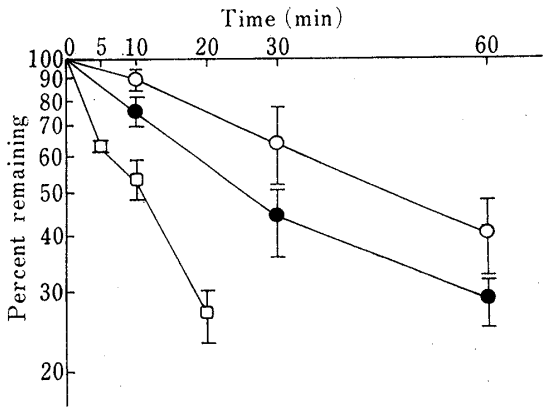

Fig. 6. Disappearance of Bleomycin from the Thigh Muscle after Intramuscular Injection of Various Formulations

□, aqueous solution; ○, S/O emulsion; ●, S/O/W emulsion.

Results are expressed as the means±S.E. of five experiments.

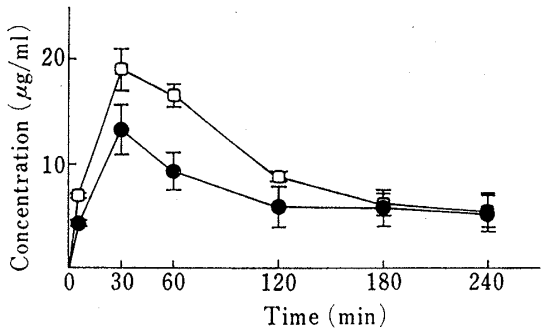

Fig. 7. Plasma Concentration of Bleomycin after Intramuscular Injection of the Original S/O/W Emulsion (●) or S/O/W Emulsion regenerated from the Lyophilized Form (□)

Results are expressed as the means±S.E. of at least five rats.

Table II. Tissue Concentrations of Bleomycin after Injection in the Rabbit

Injection route	Dosage form	Concentration (μg/g or ml)					
		Plasma		Appendix		Lymph node	
		30 min[a]	24 h	30 min	24 h	30 min	24 h
Intravenous	Aqueous solution	7.2[b]	N.D.[c]	N.D.	N.D.	0.77	N.D.
Appendix wall	Aqueous solution	6.7	N.D.	N.D.	N.D.	2.94	N.D.
	S/O emulsion	4.8	N.D.	36.0	11.85	240.42	80.29
	S/O/W emulsion	5.6	N.D.	15.86	3.84	186.41	84.37

a) Time after administration.
b) Each result is the mean value of three experiments.
c) N.D.; not detected.

anticancer agents to the lymphatics, the transport of bleomycin after injection with S/O/W emulsion into the appendix of rabbits was traced, and the results are summarized in Table II together with those of various other formulations for comparison. The plasma, appendix, and lymph node concentrations at 30 min and 24 h after administration are summarized in this table. Topical injection of S/O emulsion gave the highest bleomycin concentration of more than 200 μg/g wet tissue, and S/O/W emulsion gave the next highest value. Topical and intravenous injection of aqueous solution resulted in low concentrations in the lymph node but no antimicrobial activity at the injection site. At 24 h after injection, both emulsions gave significant amounts of bleomycin in the appendix and lymph node, while the aqueous formulation did not. Plasma concentrations after administration by the four modes showed only slight differences.

Discussion

Water-in-oil-in-water (W/O/W) type emulsions were first suggested as a method for producing prolonged antibody response instead of the use of Freund's adjuvant, because of the marked advantages of low viscosity and consequent ease of injection.[14] This multiple emulsion was not only easier to inject but also gave rise to an improved antibody response. On the other hand, the use of a number of drugs in the form of multiple emulsions has been developed by Benoy *et al.*[15] A wide variety of chemotherapeutic systems have been examined and sustained action has been reported. However, the experimental systems tended to be unstable and the final system had to be prepared immediately before use as mentioned by Davis.[16]

In previous investigations,[8–10] S/O emulsion has been proved to be stable, retaining the drug firmly in the innermost aqueous phase, and to provide a superior multiple emulsion. In the present work, we evaluated the utility of S/O/W emulsion as a drug delivery system for anticancer agents in relation to two important pharmaceutical characteristics, *i.e.*, sustained release and lymphotropicity.

As shown in Fig. 3, S/O/W emulsion produced a greatly reduced but prolonged plasma concentration of bleomycin. These effects were almost equal to those of S/O emulsion and oily suspension (Fig. 4), which have been reported to be effective sustained release dosage forms in animals and clinical application.[12,17] In these cases, the original pharmacokinetic characteristics such as distribution volume and excretion rate can be considered to be unchanged from those of intravenous injection (Fig. 2). Consequently, these plasma concentrations appear to demonstrate prolonged absorption of bleomycin from the injection site into the central circulation.

Following intramuscular injection of S/O/W emulsion, bleomycin was retained in the injection site for a long time compared with the case of aqueous solution. This result is in

good agreement with the plasma concentration data, though these results can not be analyzed by a combined kinetic model because of differences in the experimental conditions.

An O/W type formulation prepared by emulsifying the oily suspension of bleomycin in the aqueous phase showed no retardation of drug appearance in the plasma, giving rapid and almost complete migration of bleomycin into the continuous aqueous phase. S/O/W emulsion retaining the drug in the outer aqueous phase also failed to exhibit sustained absorption. For sustained release, therefore, the drug must be incorporated in the innermost aqueous globules which are surrounded by the oil phase, the release-controlling barrier. The increase of viscosity of the injection vehicle may also affect the absorption process of the drug, but no conclusive result was obtained in the present experiments.

In previous work,[12] an experiment was carried out to evaluate lymphotropic carrier systems using the appendix of rabbits as a model injection site, and S/O emulsion was selected as the most efficacious for delivering the antineoplastic agent to the lymph nodes and preventing lymphatic metastasis. In the present work, S/O/W emulsion also resulted in enhanced lymphatic accumulation of bleomycin (Fig. 7) after interstitial injection, suggesting that it may be applicable in cancer chemotherapy.

S/O/W emulsion was lyophilized in order to obtain a stabler form for storage. The regenerated S/O/W emulsion prepared by redispersing the lyophilized emulsion in water exhibited moderately prolonged release of the drug. The degree of stability and regenerability of the lyophilized emulsion presumably depends on the experimental conditions such as emulsion composition, processing method, and storage conditions, and further examination is necessary to clarify these points. In any case, the present approach of using the lyophilization technique seems to be promising for improving the storage stability of multiple emulsions.

On the basis of the evidence presented in this report, it can be concluded that application of S/O/W multiple emulsions as a delivery system for bleomycin would be advantageous, because sustained plasma concentration and enhanced lymphatic accumulation can be obtained after interstitial injection. The biodegradability of major emulsion components, sesame oil and gelatin, would also be advantageous for the use of this dosage form in cancer chemotherapy.

Acknowledgement This work was supported in part by a Grant-in-Aid for Cancer Research (56-10) from the Ministry of Health and Welfare, and a Grant-in-Aid for Scientific Research (No. 56870115) from the Ministry of Education, Science and Culture, Japan.

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