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RESEARCH PAPER

Preparation of Transparent Injectable Formulation for Lipid A Analog E5531

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ABSTRACT

We developed a ''pH-jump method'' to obtain transparent solutions of E5531, a synthetic lipid A analog, at neutral pH for pharmaceutical injection. E5531 has two pK_a*:* $pK_{a1} = 6.0$ *and* $pK_{a2} = 9.3$*. At pH 11.0, E5531 was dispersed as a dissociated form, and the phase transition temperature* T_c *of E5531 was determined to be 30°C using differential scanning calorimetry (DSC). Based on these results, the pH-jump method procedure involves dispersing E5531 at pH 11.0 (above* pK_{a2}*) at 50°C (above* T_c*) and mixing with a phosphate buffer to neutralize the pH. No degradated products of E5531 were observed at 50°C for 3 hr during dispersing in the alkaline solution. The turbidity of samples prepared with the pH-jump method was similar to that of water and superior to the samples dispersed directly in neutral pH. This method does not need mechanical power and is suitable for large-scale production.*
Key Words: *Aggregates; Dispersing; Lipid A; pH; Turbidity.*

INTRODUCTION

Lipid A is a component of bacterial lipopolysaccharides (LPSs), which are present on the major amphiphilic constituents of the leaflet of gram-negative bacteria. This is a potent biological active site (1,2) and induces the prostaglandins, cytokines such as interferon (3), interleukin (4), and tumor necrosis factor (TNF) (5) in mammalian cells such as macrophages and lymphocytes. This compound also induces undesirable toxic effects such as fever and the Schwartzmann bleeding reactions (6,7).

Many attempts have been made to synthesize the lipid A analog with low toxicity. Christ and coworkers have indicated that E5531, a synthetic disaccharide analog of lipid A (Fig. 1) has low toxicity and retains some useful biological activities of lipid A, such as reduction of TNF

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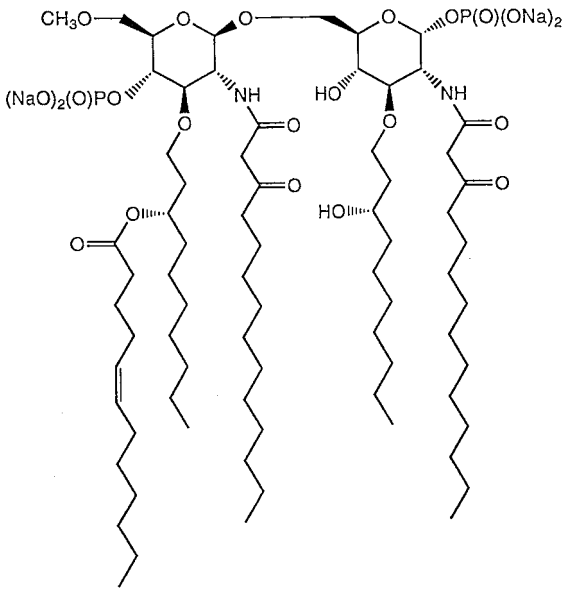

Figure 1. Chemical structure of the synthetic lipid A analog E5531.

production (8). This compound has been found to be a specific LPS antagonist in LPS binding assay and an inhibitor of LPS-induced TNF production in monocytes/macrophages, so it is expected that E5531 will be useful for the treatment of septic shock.

An injectable E5531 formulation will be extremely useful; however, the dispersion of E5531 in aqueous solution is a major problem. E5531, like many lipid A analogs, does not disperse well in neutral pH solutions. Sonication has been used to disperse the neutral lipid A (9,10) and LPS (11) for investigational use. However, sonication has two drawbacks: It is not suitable for large-scale production, and it is difficult to control the power and dispersing time (12,13). Ideally, the pH of E5531 pharmaceutical injections should be neutral since the injection of alkaline or acidic pH formulations is undesirable. It is also important to reduce the size of the aggregates because the size of colloidal particles is correlated with hepatic uptake, and a smaller size (less than 100 nm) is desirable (14).

To overcome these problems, we have developed a new ''pH-jump method'' for dispersing E5531. The advantages of this method include its suitability for large-scale production (without any mechanical input such as sonication) and the preparation of smaller size E5531 aggregates (approximately 20 nm) at neutral pH. In addition, to clarify the behavior of E5531 aggregates, various physicochemical properties (such as the size of the aggregates, membrane fluidity, and micropolarity) were measured.

EXPERIMENTAL

Materials

E5531 was obtained from Eisai Chemical Company, Limited (Ibaraki, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Wako-Chemical Company, Limited (Osaka, Japan). *N*-Dansylhexadecylamine (DSHA) was purchased from Lambda Company, Limited (Graz, Austria). Lactose hydrous, sodium phosphate monobasic and dibasic, and sodium hydroxide were purchased from Mallinckrodt Company, Limited (Paris, KY).

Methods

Determination of pK_a

For determination of pK_a, 3 mg of E5531 were added to 8 ml of water (E5531 0.2 mM) and were dispersed by sonication for 3 min at 50°C. A probe-type sonicator was used (Model UR-200P, Tomy Seiko Co., Ltd., Tokyo, Japan; power setting 100 W). The solution was cooled to room temperature, and the final volume was adjusted to 10 ml by addition of water. The pK_a was determined by titrating the solution with 0.2 mM HCl.

Determination of Phase Transition Temperature

To obtain the dispersing temperature information, the gel-to-liquid-crystal phase transition temperature of E5531 was determined using differential scanning calorimetry (DSC) with a model DSC-100 (Seiko Electronics Co., Ltd., Tokyo, Japan). E5531 (10 mg) in 40 μl of 0.003 N NaOH solution (pH 11.0) were placed in a DSC pan and sealed. An equal volume of 0.003 N NaOH solution (pH 11.0) was placed in the reference pan. Temperature scans were made from 10°C to 70°C at a heating rate of 1.25°C/min. All calorimetric data were obtained from samples during the heating phase. Molar enthalpies were obtained from the molar concentration of E5531.

Determination of Optimum Temperature of E5531 Dispersion in Alkaline Solution

A turbidity method (15) using a model 2100AN turbidimeter (HACH Co., Ltd., Loveland, O) was used to evaluate the dispersing process of E5531. Nepherometry

is a specialized type of turbidity determination that measures the light scattered at an angle of 90° to the incident light beam; turbidity was referred to in terms of nepherometric turbidity units (NTUs). The U.S. Environmental Protection Agency specified the nepherometric method of analysis for turbidity measurement. Formazin was adopted by the American Public Health Association and American Water Works Association as the primary reference standard and was used in this study. E5531 (60 mg) were dispersed in 90 ml of 0.003 N NaOH solution (pH 11.0) at various temperatures (25°C–55°C), and the turbidity was monitored during dispersing at a suitable interval for 3 hr.

Comparison of Turbidity and Size of Aggregates of E5531 Prepared Using pH-Jump and Normal Dilution Methods

The clarity of the solutions prepared using the pH-jump method and normal dilution method was compared by measuring turbidity and the aggregate size. The aggregate size was determined using a DLS 7000DL particle analyzer (Otsuka Electronics Co., Ltd., Osaka, Japan). The preparation procedures for the sample using the pH-jump and normal dilution methods are described below.

pH-Jump Method (pH 11.0–7.3)

E5531 (60 mg) were dispersed in 90 ml of 0.003 N NaOH solution (pH 11.0) with stirring at 50°C (E5531 0.67 mg/ml). Turbidity was measured at 30, 60, 120, and 180 min after stirring. At that time, 7.5 ml of the alkaline solution was sampled and mixed with phosphate-NaOH buffer. Then, the volume was adjusted to 50 ml by addition of water (E5531 100 μg/ml, 4.25 mM phosphate-NaOH, and 10% lactose, pH 7.3). Turbidity and the aggregate size were measured.

Normal Dilution Method (pH 7.3)

E5531 (60 mg) were dispersed in 90 ml of the formulated solution (4.25 mM phosphate-NaOH and 10% lactose, pH 7.3) with stirring at 50°C (E5531 0.67 mg/ml). Turbidity was measured at 30, 60, 120, and 180 min after stirring. At that time, 7.5 ml of the solution was sampled and mixed with phosphate-NaOH buffer. Then, the volume was adjusted to 50 ml by addition of the formulated solution (E5531 100 μg/ml, 4.25 mM phosphate-NaOH, and 10% lactose, pH 7.3). Turbidity and the aggregate size were measured.

Preparation of E5531 Pharmaceutical Injection Using the pH-Jump Method

E5531 (200 mg) were dispersed in 100 ml of 0.003 N NaOH solution (pH 11.0) with stirring at 50°C (E5531 2 mg/ml). Turbidity was measured at 5, 15, 30, 45, 60, 120, and 180 min after stirring. At that time, 2.5 ml of the alkaline solution was sampled and mixed with phosphate-NaOH buffer containing lactose. Then, the volume was adjusted to 50 ml by adding water to the formulated solution (E5531 100 μg/ml, 4.25 mM phosphate-NaOH buffer, and 10% lactose, pH 7.3). The formulated solutions were filtered through a 0.22-μm filter. Turbidity, pH, the aggregate size, and high-performance liquid chromatography (HPLC) purity were determined, and 5 ml of the solutions were filled into vials and lyophilized. Then, the vials were reconstituted with 5 ml of water, and the turbidity, pH, and the aggregate size were measured and compared with those before lyophilization.

Determination of Micropolarity of E5531 Aggregates

To obtain a degree of hydration, micropolarity of E5531 aggregates at pH 11.0 and after neutralized to pH 7.3 was determined using a fluorescence technique (DSHA probe). DSHA has been reported to provide information on the phase transition due to a large increase in fluorescence intensity, mainly as a result of higher partitioning of dye in the head group region of fluid bilayers (16,17). DSHA was added at 1 mol% of the total lipids. The fluorescence spectra were measured on excitation at 330 nm. All fluorescence measurements were carried out using a model F-4500 fluorescence spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan). The micropolarity of DSHA incorporated into E5531 aggregates was evaluated using the wavelength of maximum intensity of emission. DSHA (3.1 ml) were dissolved in 10 ml of methanol, ethanol, propanol, butanol, acetone, tetrahydrofuran, benzene, and hexane. Five μl of each solution were then diluted with 5 ml of the same solvent. The wavelengths at the maximum fluorescence intensity in each solvent were plotted against the polarity of the solvent (18,19).

Determination of the Order Parameter of E5531 Aggregates

The order parameter of E5531 was determined using a fluorescence anisotropy technique (DPH probe) as reported by Iwamoto et al. (20). DPH was added at 1 mol% of total lipids. The steady-state anisotropy r_s was defined by the following equation:

$$r_s = (I_{VV} - C_f' I_{VH})/(I_{VV} + 2C_f' I_{VH})$$

where I is the fluorescence intensity, and the subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analysis (second) polarizers, respectively. C_f ($= I_{HV}/I_{HH}$) is the grating correction factor. The order parameter S was calculated using the following equation (21):

$$S = (r_s/r_o)^{1/2}$$

where r_o represents the maximal and limiting fluorescence anisotropy. For DPH, r_o has been estimated to be 0.398 using nanosecond time-resolved fluorescence techniques (21). In this work, values for the order parameter S of E5531 aggregates in alkaline (pH 11.0) and formulated solutions (pH 7.3) were calculated.

RESULTS

Determination of pK_a

The titration curve of E5531 dispersion with 0.2 mM HCl has two pK_a values (Fig. 2), approximately 6.0 (pK_{a1}) and 9.2 (pK_{a2}). At neutral pH, E5531 exists almost entirely as the bis-salt (2-Na) form. At pH 10 or above, E5531 exists almost entirely as the dissociated form. Therefore, we first dispersed E5531 in alkaline pH (above pH 10) to obtain a dissociated form, and then neutralized the pH to 7.3 to obtain the transparent formulations.

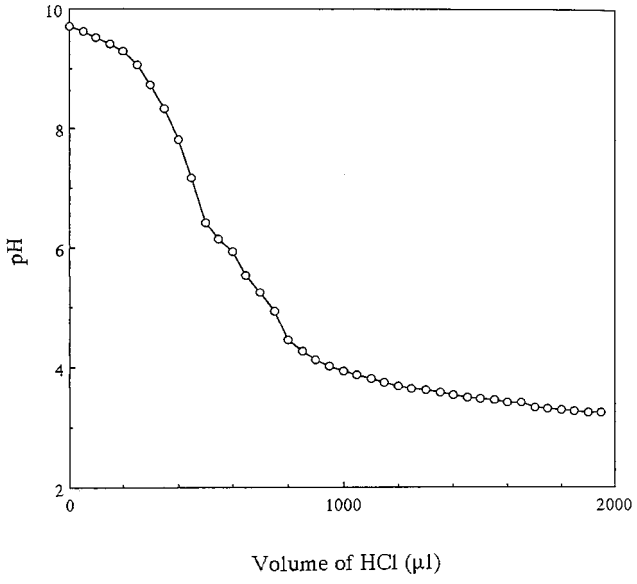

Figure 2. pH titration curve for E5531, with 0.2 mM of E5531 titrated with 0.2 mM of HCl solution. The pH was recorded as a function of the added volume of HCl. pK_{a1} = 6.0 and pK_{a2} = 9.3.

Determination of the Phase Transition Temperature of E5531 Using Differential Scanning Calorimetry

It is well known that lipids such as phospholipids have a phase transition temperature T_c. Dipalmitoylphosphatidylcholine (DPPC) has a T_c at 41°C and dimylstoylphosphatidylcholine (DMPC) at 23°C (22). Above T_c, hydration is accelerated, and dispersing is easier than below T_c. We determined the T_c of E5531 at pH 11.

Figure 3 presents the DSC thermogram of E5531 in 0.003 N NaOH solution (pH 11.0) from the first to third scanning. The T_c and baseline were not stable in the first and second scans because the hydration of E5531 in the

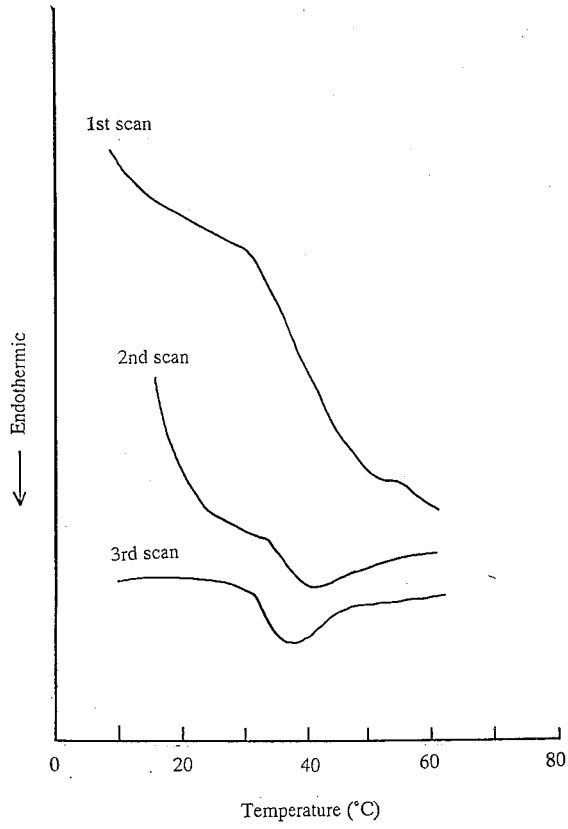

Figure 3. DSC thermogram of E5531 in 0.003 N NaOH solution (pH 11.0). DSC scans were repeated three times. Scan speed was 1.25°C/min, and the temperature range was from 10°C to 70°C.

Table 1

Phase Transition Temperature T_c *and Enthalpy* ΔH

Scan	T_c (°C)	ΔH (kJ/mol)
1	31.7	4.47
2	32.3	7.78
3	31.6	7.56

alkaline solution was not completed. In the third scan, the T_c of E5531 was 31.6°C, and the baseline was stable.

Table 1 presents the T_c and the phase transition enthalpy of E5531. The enthalpy for E5531 at pH 11.0 (7.6 kJ/mol) at the third scan is smaller than those of DMPC (27.3 kJ/mol) and DPPC (36.5 KJ/mol) (22). This indicates that the cooperative interaction between E5531 molecules is smaller than for DMPC and DPPC.

Determination of the Dispersing Temperature for E5531

Turbidity was used for monitoring the E5531 dispersing process. Figure 4 shows the relationship between the turbidity of E5531 dispersions in 0.003 N NaOH solution and the stirring time as a function of dispersing temperature. At 25°C (below T_c), turbidity did not change for 3 hr. However, when the dispersing temperature was above T_c, especially above 40°C, turbidity decreased drastically and reached a constant value (0.2 NTU). When the dispersing temperature range was between 45°C and 55°C, the rate of decrease in turbidity was approximately the same. Therefore, we selected 50°C for a target temperature for dispersing E5531 in alkaline solution.

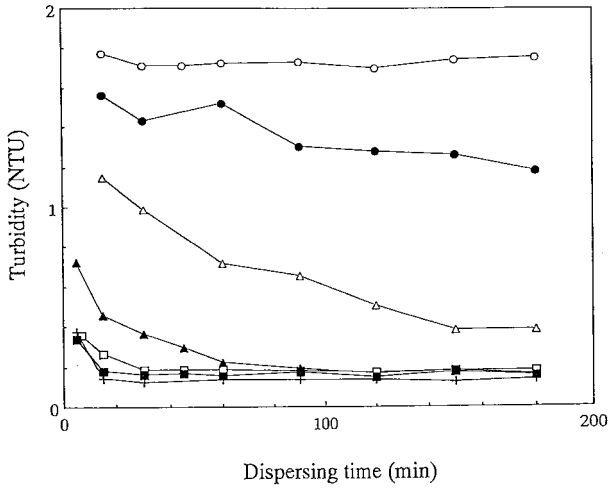

Figure 4. Relationship between dispersing time and turbidity of E5531 in 0.003 N NaOH solution (E5531 0.67 mg/ml) as a function of temperature. Dispersing temperatures were 25°C (○), 30°C (●), 35°C (△), 40°C (▲), 45°C (□), 50°C (■), and 55°C (+).

Comparison of Turbidity Prepared Using the pH-Jump and Normal Dilution Methods

Table 2 presents a comparison of the turbidities obtained using the pH-jump and normal dilution methods. At the stage of E5531 concentrated solution (0.67 mg/ml), turbidity at pH 11.0 was less than 0.2 NTU in 30 min and reached a constant value. This value is similar to the turbidity of water (0.112 NTU). However, turbidity of the solution prepared by the normal dilution method at pH 7.3 directly was approximately 40 NTU and did not change for 3 hr. The turbidity of the solution prepared by the pH-jump method was less than 0.2 NTU after dilution to 100 μg/ml, while that prepared by the normal dilution method was above 10 NTU. A transplant E5531 solution could be obtained only by the pH-jump method.

Preparation of E5531 Injectable Formulation

The formulation prepared by the pH-jump method was lyophilized. The turbidity of the formulated solution (pH 7.3) was approximately 0.2 NTU when the turbidity of the alkaline solution (E5531 2 mg/ml) was about 0.2 NTU (Table 3). The turbidity was not significantly different before and after lyophilization. E5531 aggregate size was also determined and did not change before and after lyophilization (approximately 20 nm). The purity of E5531 was checked using HPLC, and it was found that no degradation occurred when E5531 was dispersed in 0.003 N NaOH (pH 11.0) for 3 hr.

Micropolarity of E5531 Aggregates

The hydration process was evaluated using a fluorescence technique. The micropolarity of the E5531 aggregates was determined at pH 11.0 and after neutralized to pH 7.3. The emission maxima of DSHA embedded in E5531 were monitored as a function of incubation temperature (Fig. 5). It has been reported that the fluorescence characteristics of DSHA depended on the micropolarity around the probe, and the dansyl fluorophore is

Table 2

Comparison of Turbidity and Aggregate Size Prepared by the pH-Jump Method (pH 11.0–7.3) and the Normal Dilution Method (pH 7.3)

Stirring Time (min)	Turbidity of the Solution[a] (NTU)	Turbidity of the Formulated Solution[c] (NTU)	Aggregate Size of the Formulated Solution[c] (nm)
pH-jump method			
30	0.163	0.188	22.4
60	0.154	0.194	19.6
120	0.151	0.204	21.7
180	0.152	0.181	18.3
Stirring Time (min)	Turbidity of the Solution[b] (NTU)	Turbidity of the Formulated Solution[c] (NTU)	Aggregate Size of the Formulated Solution[c] (nm)
Normal dilution method			
30	39.6	14.4	156.9
60	45.7	15.5	148.7
120	44.6	13.5	145.2
180	47.5	13.4	146.8

[a] E5531: 0.67 mg/ml in 0.003 N NaOH solution (pH 11.0).
[b] E5531: 0.67 mg/ml in 4.25 mM phosphate-NaOH buffer containing 10% lactose (pH 7.3).
[c] E5531: 100 μg/ml in 4.25 mM phosphate-NaOH buffer containing 10% lactose (pH 7.3).

located in the glycerol backbone of phospholipid bilayers (17). Therefore, it is expected that the emission maxima of DSHA in E5531 aggregates will provide the information on micropolarity around the surface of the aggregates. The emission maxima for E5531 aggregates at 25°C in alkaline solution (pH 11.0) and neutralized solution (pH 11.0 to 7.3) were 528 and 508 nm, respectively, indicating that the micropolarity around the probe in E5531 aggregates is comparable to that of methanol and butanol, respectively. In this study, the phase transition temperature in alkaline solution was confirmed to be about 30°C; this finding is similar to that from DSC measurement. After neutralizing the pH to 7.3, the phase transition temperature was also confirmed to be about 30°C.

Table 3

Process Parameters During Dispersing of E5531 by the pH-Jump Method

	Alkaline Solution (in 0.003 N NaOH) (E5531 2 mg/ml)		Formulated Solution (Before Lyophilization) (E5531 100 μg/ml)				Formulated Solution (After Lyophilization) (E5531 100 μg/ml)		
Stirring Time (min)	Turbidity (NTU)	pH	Turbidity (NTU)	pH	HPLC Purity (%)	Aggregate Size (nm)	Turbidity (NTU)	pH	Aggregate Size (nm)
5	0.825	11.00	0.198	7.37	99.82	30.8	0.282	7.44	31.0
15	0.277	10.97	0.191	7.34	99.85	17.9	0.235	7.43	15.5
30	0.186	10.97	0.149	7.35	99.74	18.7	0.219	7.48	12.3
45	0.186	10.96	0.146	7.38	NP	NP	NP	NP	NP
60	0.131	10.91	0.167	7.39	99.84	19.6	0.190	7.46	13.8
120	0.154	10.83	0.172	7.36	99.45	21.7	0.178	7.46	15.2
180	0.167	10.68	0.152	7.37	99.84	22.1	0.200	7.46	13.6

NP, not performed.

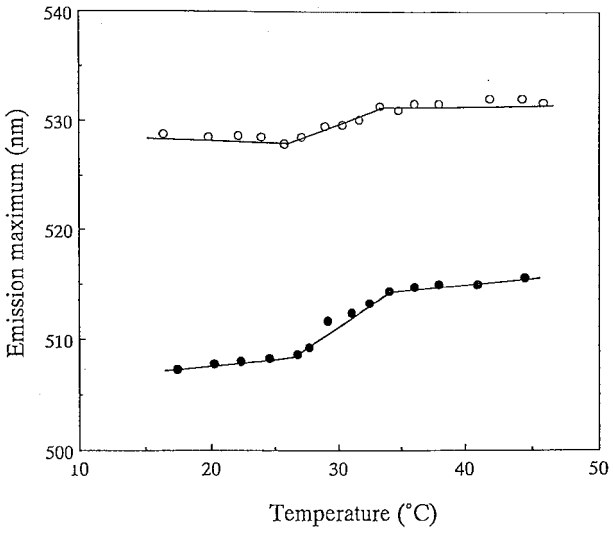

Figure 5. Relationship between temperature and emission maximum of DSHA in E5531 aggregates in 0.003 N NaOH solution (pH 11.0) (○) and in formulated solution (pH 7.3) (●) as a function of temperature.

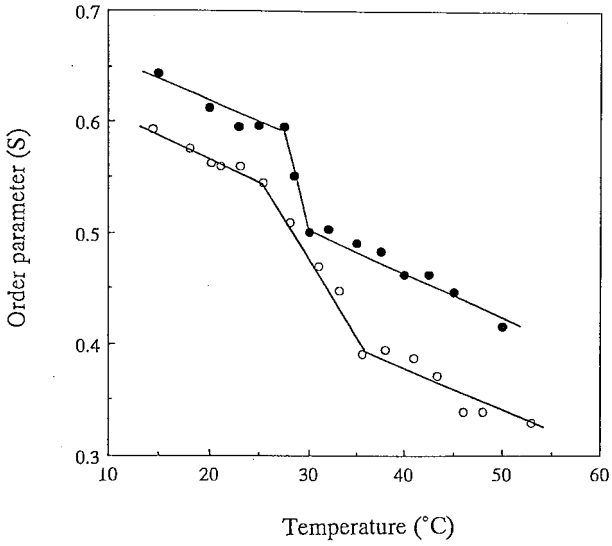

Figure 6. Relationship between temperature and order parameter *S* of E5531 (DPH probe) in 0.003 N NaOH solution (pH 11.0) (○) and in formulated solution (pH 7.3) (●) as a function of temperature.

Order Parameter of E5531 Aggregates

The order parameter of the E5531 aggregates was also determined at pH 11.0 and 7.3 by the fluorescence polarization technique (DPH probe). Figure 6 presents a plot of the order parameter versus incubation temperature for E5531 in 0.003 N NaOH (pH 11.0) and after neutralization to pH 7.3. From this plot, the phase transition temperatures can be estimated (approximately 30°C) at both pH 11.0 and 7.3. This finding is similar to that from DSHA. The order parameter of E5531 in alkaline solution is smaller than that in neutralized solution (pH 7.3). This can be explained by the observation that above pK_{a2}, E5531 exists as a dissociated form, and the degree of hydration will be larger than that with the pH neutralized to 7.3 (below pK_{a2}).

DISCUSSION

We have successfully obtained a transparent formulation of E5531 using the pH-jump method without mechanical input such as sonication. The advantage of the pH-jump method is the suitability for large-scale production of the pharmaceutical injection. At basic pH above pK_{a2} (9.3), E5531 is fully ionized, and hydration will increase in conjunction with the loss of hydrogen bonds in the head group. On the other hand, at neutral pH, E5531 exists as a bis-sodium salt, and E5531 molecules are stabilized by the hydrogen bonds of the head group. Intermolecular hydrogen bonds have been reported to affect both the phase state and, overall, the order of the state in acidic phospholipids (23). Structural stabilization of phosphatidic acid aggregates is the greatest when phosphates are semi-ionized (24). Hydrogen bonding between the head group of the acidic lipids has been shown to rigidify the bilayers and increase the gel-to-liquid crystalline phase transition temperature. Therefore, in the neutral pH solution, E5531 has hydrogen bonds, and a trnsparent formulation cannot be obtained using the normal dilution method (Table 2).

The hydration process was examined using fluorescence techniques. As expected, the micropolarity around the surface of E5531 aggregates estimated from the red shift in emission maximum of DSHA in 0.003 N NaOH solution (pH 11.0) indicates that, above phase transition temperature (30°C), hydration proceeded, resulting in large polarity (Fig. 5). It has been reported that, in phospholipid liposomes, hydration increased greatly above the phase transition temperature (20). Above this temperature, the maximum wavelengths of DSHA increased and exhibited a red shift, indicating that the micropolarity around the surface of E5531 aggregates was large, and the hydration was accelerated. This fact is supported by

the results of the order parameter determined by fluorescence anisotropy (Fig. 6). The order parameters at 25°C were 0.55 and 0.58 in alkaline solution (pH 11.0) and the formulated solution (pH 7.3), respectively, indicating that E5531 aggregates are more fluid in alkaline solution and more hydrated than in the formulated solution.

CONCLUSION

Based on the results obtained in this study, the ideal dispersing conditions for the lipid A analog E5531 in the pH-jump method to prepare the pharmaceutical injection can be summarized as follows:

1. The dispersing pH is set at pH 11.0 (0.003 N NaOH) since above pH 10 (above pK_{a2}) E5531 exists as a dissociated form, and it also is easier to disperse.
2. The dispersing temperature is 50°C in the alkaline solution (pH 11.0) since hydration is accelerated above the phase transition temperature (30°C).
3. The alkaline solution is mixed with phosphate buffer containing lactose and then the pH is neutralized to 7.3. The turbidity of the solution is similar to that of water, and a transparent formulation was obtained. No degradation was observed during 3 hr of dispersal in alkaline solution.
4. The aggregate size was approximately 20 nm and did not change before and after lyophilization. Turbidity also did not change before and after lyophilization.

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