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RESEARCH PAPER

The Effect of the Membrane Fluidity on Pharmacokinetics for Lipid A Analog E5531

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ABSTRACT

The effect of the dispersing procedure on the aggregate size, membrane fluidity and the pharmacokinetics were evaluated for the lipid A analog E5531. The size of the aggregates prepared by the pH-jump method (pH 11.0 → 7.3) was decreased, reaching 20 nm with increasing dispersing time in 0.003 N NaOH (pH 11.0). The membrane fluidity of the aggregates increased with increasing dispersing time. When prepared by the normal dilution method (pH 7.3 → 7.3), the size of the aggregates remained constant at 150 nm and the membrane fluidity was smaller compared to samples prepared by the pH-jump method. Using samples with different degrees of hydration and different membrane fluidities prepared by the pH-jump method, the pharmacokinetics after intravenous administration into rats were evaluated, and the data obtained confirmed that the membrane fluidity was correlated with the pharmacokinetics in rat. In addition, E5531 vials were stable for 24 months at room temperature when used within 24 hr after reconstitution.
Key Words: *Aggregates; Lipid A; Membrane fluidity; Pharmacokinetics.*

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INTRODUCTION

Lipid A, a lipid anchor in lipopolysaccharide (LPS), is located on the outer membrane of gram-negative bacteria. This is a potent biologically 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).

Numerous attempts have been made to synthesize analogs of lipid A with low toxicity. Christ and coworkers reported that E5531 (Fig. 1), a synthetic disaccharide analog of lipid A, has low toxicity but retains some useful biological activities such as reduction of TNF production (8). This compound has been found to be a specific LPS antagonist in an LPS-binding assay, and it inhibits LPS-induced TNF production in monocytes/macrophages. Thus, the compound has great potential for use for the treatment of septic shock.

An injectable E5531 formulation would be extremely useful, but the dispersion of E5531 in aqueous solution represents a major problem. E5531, like many lipid A analogs, cannot be dispersed at neutral pH and cannot be obtained as a transparent solution. The present study describes the development of a new pH-jump method for dispersing E5531 (9). The advantages of this method are that it is suitable for large-scale production (without mechanical input such as sonication), it can be used at neutral pH, and it gives rise to aggregates that are small in size. The developed method involves the dispersion of E5531 in 0.003 N NaOH solution (pH 11.0, above pK_2) at 50°C (above the phase transition temperature) and subsequent mixing with phosphate-NaOH buffer to adjust the pH to 7.3. E5531 aggregates were vesicles with diameters of approximately 20 nm (10).

In this study, the effect of dispersing procedure on the aggregate size, membrane fluidity, and pharmacokinetic profile was investigated in more detail. E5531 was dispersed using the pH-jump (pH 11.0 → 7.3) and the normal dilution (pH 7.3 → pH 7.3) methods. The pharmacokinetics in rats after an intravenous injection of E5531 aggregates prepared by the various methods were evaluated, and the relationship between the physicochemical properties (such as the aggregate size and membrane fluidity of E5531 aggregates) and the pharmacokinetic profiles in rats are discussed. Finally, the stability of the E5531 lyophilized vials and the reconstituted solutions were investigated.

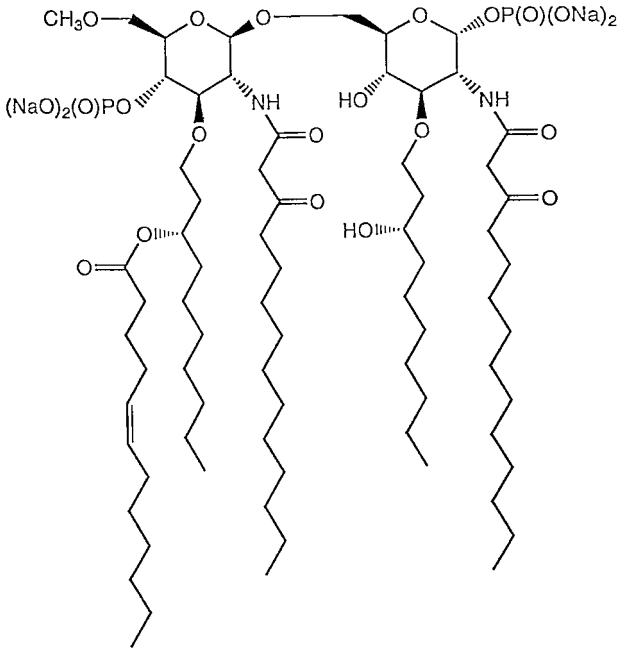

Figure 1. Chemical structure of the synthetic lipid A analog E5531.

EXPERIMENTAL

Materials

E5531 was obtained from Eisai Chemical Company, Limited (Ibaraki, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Wako Pure Chemical Industries, Limited (Osaka, Japan). Lactose hydrate, sodium phosphate monobasic and dibasic, and sodium hydroxide were purchased from Mallinckrodt Company, Limited (Paris, KY).

Methods

Preparation of E5531 Samples

The size of the E5531 aggregates and the fluidity of E5531 membranes prepared using the pH-jump or normal dilution method were determined. For the pH-jump method (pH 11.0 → pH 7.3), 200 mg of E5531 were dispersed in 100 ml of a 0.003 N NaOH solution (pH 11.0) with stirring at 50°C. For the normal dilution method (pH 7.3 → pH 7.3), 200 mg of E5531 were dispersed in 100 ml of buffer solution (4.25 mM phosphate-

NaOH buffer containing 10% lactose, pH 7.3) with stirring at 50°C.

Determination of the Size and Chemical Purity of the E5531 Aggregates

After stirring for 5, 10, 15, 30, and 60 min, 7.5 ml aliquots of the solution sampled from the above E5531 solutions were then mixed with phosphate-NaOH buffer containing lactose. The volume was adjusted to 50 ml by adding water to the formulated solution (E5531: 300 μg/ml, 4.25 mM phosphate-NaOH buffer containing 10% lactose solution, pH 7.3). Then, 5 ml of the solution was placed in glass vials and lyophilized. The lyophilized vials were reconstituted with 5 ml of water (E5531: 300 μg/ml, pH 7.3), and the size distribution of E5531 aggregates was measured at 25°C. The data were analyzed by the histogram method (11), and the weight-averaged sizes were evaluated. Chemical purity of E5531 was also determined by high-performance liquid chromatography (HPLC) (detection wavelength 254 nm).

Measurement of the Membrane Fluidity of E5531 Aggregates

The hydration process and the relationship between dispersing method and dispersing time for E5531 were also evaluated by measurement of the membrane fluidity of E5531 aggregates. Fluorescence anisotropy of DPH for the reconstituted E5531 solutions (300 μg/ml, pH 7.3) were measured as described previously (12) using an H-4500 spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan) at 25°C and 37°C. DPH was added at 1 mol% to the total lipids. The excitation and emission wavelengths used were 360 nm and 428 nm, respectively. The steady-state anisotropy r_s can be defined by the following equation:

$$r_s = (I_{VV} - C_f \cdot I_{VH})/(I_{VV} + C_f \cdot I_{VH}) \quad (1)$$

where I is the fluorescence intensity, and subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analysis (second) polarizers, respectively. C_f ($= I_{HV}/I_{HH}$) represents the grating correction factor. The order parameter S was calculated using the following equation (13):

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

where r_o represents the maximal and limiting fluorescence anisotropy. For DPH, r_o has been estimated to be 0.398 by Heyn (13) using nanosecond time-resolved fluorescence techniques. In this work, Eq. 2 was used to estimate the order parameter S.

Pharmacokinetics in Rats for E5531 Aggregates Prepared by the pH-Jump Method with Different Membrane Fluidity

To investigate the relationship between the membrane fluidity and pharmacokinetics in rats, reconstituted E5531 solutions (300 μg/ml, pH 7.3) prepared by the pH-jump method, dispersed in 0.003 N NaOH solution (pH 11.0) for 5, 10, 15, and 60 min, were administered as a bolus injection (0.3 mg/kg) via a femoral vein. Blood samples were collected before dosing and 2, 10, and 30 min and 1 and 2 hr after dosing. The concentrations of E5531 in plasma (100 μl) were measured by HPLC analysis with fluorescence detection (14). The AUC (0–2 hr) was calculated from the plasma concentration for 2 hr after administration by the trapezoidal method. The

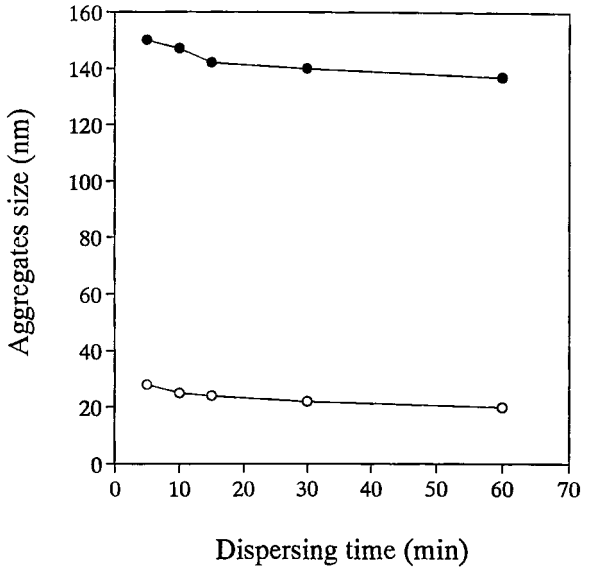

Figure 2. Relationship between dispersing time and size of the E5531 aggregates: ○, E5531 was dispersed at pH 11.0 in the pH-jump method at 50°C (E5531: 2 mg/ml) and after adjustment of the pH from 11.0 to 7.3 by the addition of a phosphate-NaOH buffer containing lactose (E5531: 100 μg/ml, 4.25 mM phosphate NaOH, 10% lactose, pH 7.3), the aggregate sizes were determined. ●, prepared by the normal dilution method. E5531 was dispersed in phosphate-NaOH buffer containing lactose (E5531: 2 mg/ml, 4.25 mM phosphate-NaOH, 10% lactose, pH 7.3) and after dilution with the same buffer (E5531: 100 μg/ml, pH 7.3), the aggregate sizes were determined.

plasma concentration at time zero for AUC calculations was estimated to be equal to the concentration at the first time point measured (2 min).

Effect of the Time After Reconstitution on the Membrane Fluidity and Pharmacokinetics in Rats

The effect of time after reconstitution on membrane fluidity and the pharmacokinetics in rats for E5531 aggregates was evaluated. The vials were produced using the pH-jump method, by which 21 g of E5531 were dispersed in 9 L of 0.003 N NaOH solution for 60 min. The solution was then mixed with phosphate-NaOH buffer containing lactose, and the volume was adjusted to 60 L by adding water to the formulated solution (E5531: 300 μg/ml, 4.25 mM phosphate-NaOH buffer containing 10% lactose solution, pH 7.3). Then, 5 ml of the solution was placed in glass vials and lyophilized. The vials containing 1.5 mg of E5531 were reconstituted with 5 ml of water for injection (E5531: 300 μg/ml) and stored at 4°C in the refrigerator for 24 hr. At 0, 6, and 24 hr, the samples were warmed to room temperature and administrated to rats, and the pharmacokinetic profiles were evaluated. Aggregate size, order parameter, and chemical purity of E5531 were also determined.

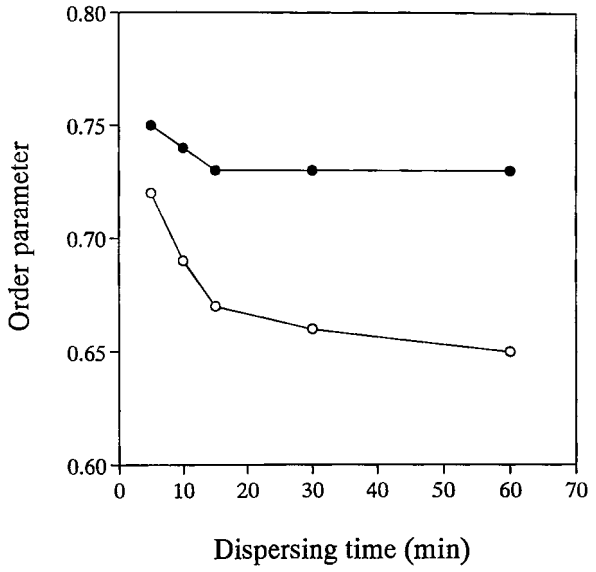

Figure 3. Relationship between dispersing time in 0.003 N NaOH solution and the order parameter of DPH for the E5531 aggregates at 25°C: ○, dispersed at pH 11.0 in the pH-jump method (E5531: 2 mg/ml) and after adjustment of the pH to 7.3 by the addition of phosphate-NaOH buffer containing lactose (E5531: 100 μg/ml, 4.25 mM phosphate-NaOH buffer, 10% lactose, pH 7.3), the order parameters were determined. ●, prepared by the normal dilution method (pH 7.3 → 7.3). E5531 was dispersed in phosphate NaOH buffer containing lactose (E5531: 2 mg/ml, 4.25 mM phosphate NaOH, 10% lactose, pH 7.3) and after dilution with the same buffer (E5531: 100 μg/ml, pH 7.3), the order parameters were determined.

Effect of the Time After Lyophilization on the Membrane Fluidity and Pharmacokinetics in Rats

To confirm the effect of time after lyophilization on membrane fluidity and the pharmacokinetics in rats for E5531 aggregates, the vials containing 1.5 mg of E5531 were stored at room temperature for 24 months. At 0, 6, 12, and 24 months, the samples were reconstituted with 5 ml of water for injection (E5531: 300 μg/ml) and administrated to rats, and the pharmacokinetic profiles were evaluated. Aggregate size, order parameter, and chemical purity of E5531 were also determined.

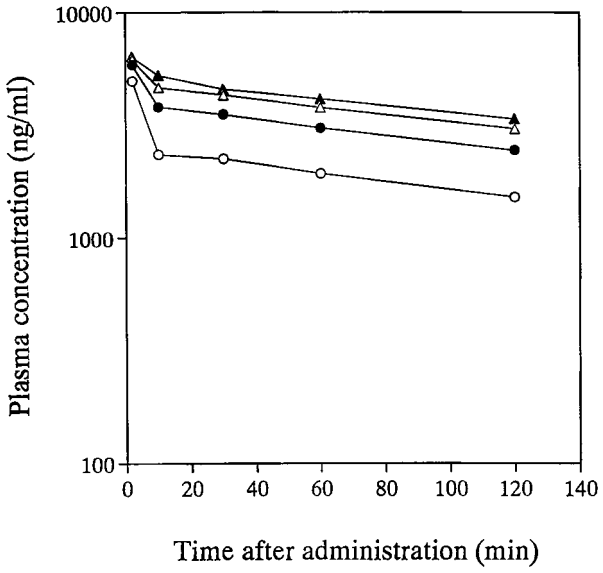

Figure 4. Plasma concentration of E5531 after intravenous administration of E5531 to male rats as a function of the dispersing time in 0.003 N NaOH in the pH-jump method. Each point represents the mean ± SEM of three animals: ○, dispersion for 5 min; ●, dispersion for 10 min; △, dispersion for 15 min; ▲, dispersion for 60 min.

Table 1

Process Parameters for E5531 Dispersion with Different Dispersing Times in the pH-Jump Method

Parameters (After Neutralization to pH 7.3)	Dispersing Time in 0.003 N NaOH (min)				
	5	10	15	30	60
Aggregate size (nm)	28.1 ± 11.2	24.9 ± 6.3	24.2 ± 5.6	22.1 ± 4.9	20.2 ± 4.2
Order parameter for DPH at 37°C	0.61	0.58	0.55	0.54	0.52
Chemical purity by HPLC (%)	99.2	99.4	99.1	99.2	99.1
AUC in rats (0–2 hr) (ng · hr/ml)	4172 ± 182	6450 ± 112	7833 ± 151	Not performed	8526 ± 215

RESULTS AND DISCUSSION

Comparison of the Size of E5531 Aggregates

Figure 2 shows the relationship between dispersing time and the size of E5531 aggregates prepared by the pH-jump method (pH 11.0 → 7.3) and the normal dilution method (pH 7.3 → 7.3). E5531 has two p*K*'s, pK_1 = 6.0 and pK_2 = 9.3 (9). At pH 11.0 in aqueous solution, E5531 was fully ionized and present in the dissociated form. At basic pH above pK_2 (9.3), E5531 is fully ionized, and hydration can be accelerated via the loss of intermolecular hydrogen bonds in the head phosphate group. Just after adjustment of the pH to 7.3, the salt form of E5531 changed to the semi-ionized form, but the size of the aggregates was approximately 20 nm. However, the size of the aggregates prepared by the normal dilution method (pH 7.3 → 7.3) was 150 nm. Because of the intermolecular hydrogen bonds in the head phosphate group, hydration does not directly proceed when E5531 is dispersed at pH 7.3 directly. This indicates that, when E5531 is dispersed in alkaline solution, hydration proceeds, but just after neutralization to pH 7.3, further hydration stops.

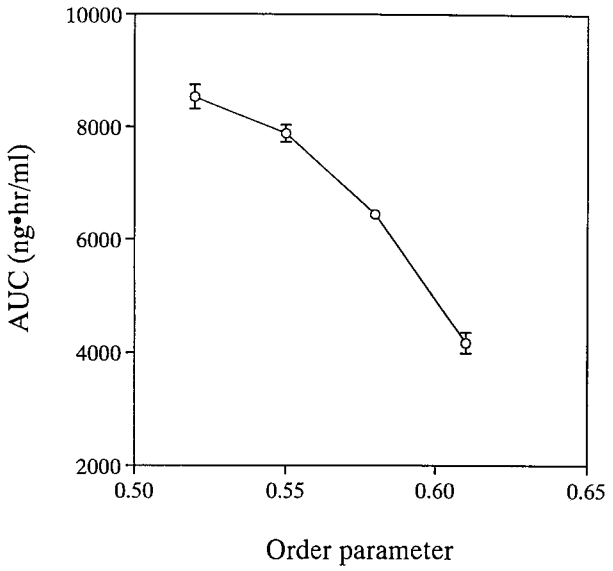

Figure 5. Relationship between the order parameter of DPH for the E5531 aggregates at 37°C (the aggregates were prepared by the pH-jump method with different dispersing times) and AUC (0–2 hr) in rats.

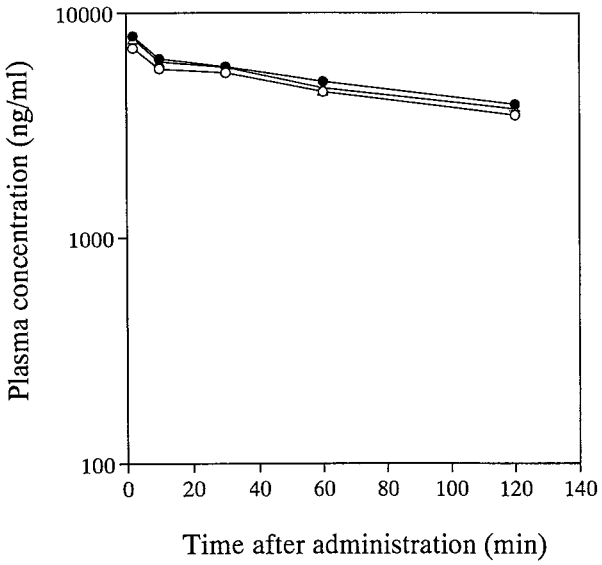

Figure 6. Plasma concentration of E5531 after intravenous administration of E5531 to male rats as a function of the time after reconstitution of the lyophilized vials. Each point represents the mean ± SEM of three animals: ○, immediately after reconstitution; ●, stored at 4°C for 6 hr after reconstitution; △, stored at 4°C for 24 hr after reconstitution.

Table 2

Parameters for E5531 Reconstituted Solution

	Storage Time at 4°C After Reconstitution (hr)		
Parameters	0	6	24
Aggregate size (nm)	20.3 ± 6.2	20.1 ± 4.2	19.4 ± 5.1
Order parameter for DPH at 37°C	0.63	0.61	0.62
Chemical purity by HPLC (%)	99.4	99.2	99.2
AUC in rats (0–2 hr) (ng · hr/ml)	9360 ± 146	10316 ± 506	9926 ± 459

Progress of Hydration and Membrane Fluidity of the E5531 Aggregates

The hydration process and the relationship between the dispersing method and dispersing time were also determined by measurements of the membrane fluidity of E5531 aggregates. Figure 3 shows the relationship between dispersing time and the order parameters of E5531 aggregates at 25°C prepared by the pH-jump method (pH 11.0 → 7.3) and the normal dilution method (pH 7.3 → 7.3). The order parameter of E5531 aggregates prepared by the pH-jump method decreased with increasing dispersing time in 0.003 N NaOH. However, the order parameter of aggregates prepared by the normal dilution method remained unchanged with progress of dispersing time. This indicates that the hydration of the E5531 membrane is increased at the basic pH as a result of dissociation of the head phosphate group, and just after neutralization to pH 7.3, hydration is stopped by the formation of intermolecular hydrogen bonds in the head phosphate group.

Pharmacokinetics in Rats for E5531 Aggregates with Different Membrane Fluidity

To investigate the relationship between the membrane fluidity of E5531 aggregates and the pharmacokinetic profile, a reconstituted E5531 solution (300 μg/ml, pH 7.3) with a different membrane fluidity prepared by changing the dispersing time in the pH-jump method was intravenously injected into rats. As shown in Fig. 4 and Table 1, the plasma concentrations at 2 hr after dosing and AUC (0–2 hr) increased with an increase in the dispersion time. Figure 5 shows the relationship between the order parameter of an E5531 reconstituted solution at 37°C and AUC (0–2 hr), respectively. The order parameter enables changes in AUC to be observed clearly.

Using samples with membrane fluidities that were different, we obtained clearly different pharmacokinetic profiles in rats. This suggests that the fluidity of the E5531 membranes increased as dispersion progressed, and that fluidity affects the pharmacokinetics in rats. In other words, pharmacokinetics in rats can be controlled by changing the dispersing time in the alkaline solution

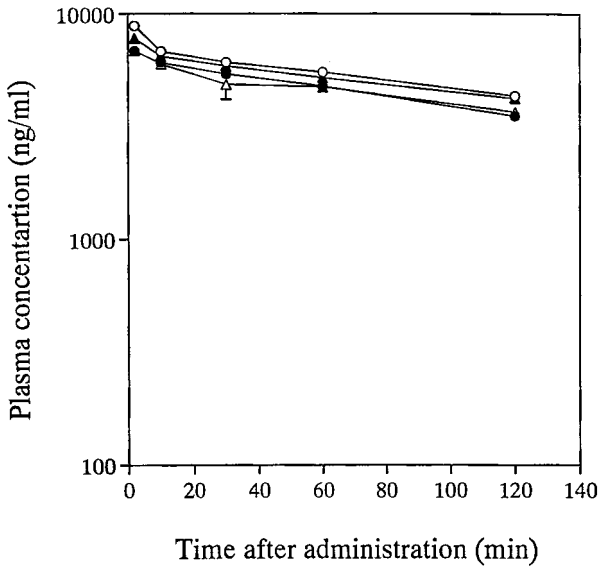

Figure 7. Plasma concentration of E5531 after intravenous administration of E5531 to male rats as a function of the time after lyophilization. Each point represents the mean ± SEM of three animals: ○, immediately after lyophilization; ●, stored at room temperature for 6 months after lyophilization; △, stored at room temperature for 12 months after lyophilization; ▲, stored at room temperature for 24 months after lyophilization.

Table 3

Parameters for E5531 Lyophilized Vials

Parameters	Storage Time at Room Temperature After Lyophilization (M)			
	0	6	12	24
Aggregate size (nm)	19.1 ± 6.3	20.6 ± 5.5	21.4 ± 5.6	20.3 ± 5.3
Order parameter for DPH at 37°C	0.62	0.63	0.62	0.62
Chemical purity by HPLC (%)	99.5	99.4	99.1	99.3
AUC in rats (0–2 hr) (ng · hr/ml)	11324 ± 191	9696 ± 248	9500 ± 366	10764 ± 153

(pH 11.0) and the degree of hydration and membrane fluidity of the aggregates.

Hampton and Raets (15) reported on the interactions between the metabolism of lipid A–like molecules by macrophages and their response to these molecules. They concluded that the uptake of the molecules is mediated by scavenger receptors. At this time, the pathway of the metabolism for E5531 is not known with certainly. It is assumed that, when E5531 molecules are bound to the receptors and incorporated into the macrophages, the more rigid membrane is more easily bound to the receptor, and uptake of the molecules therefore occurs more rapidly.

Effect of the Time After Lyophilization and Reconstitution on the Membrane Fluidity and Pharmacokinetics in Rats

Figure 6 shows the pharmacokinetic profiles of E5531 as a function of the time after reconstitution of the lyophilized vials. As shown in the figure and Table 2, the three dosing solutions stored for 0, 6, and 24 hr at 4°C show no differences in particle sizes, order parameters, chemical purity, and pharmacokinetic profiles and AUC.

Figure 7 shows the pharmacokinetic profiles of E5531 as a function of the time after lyophilization. As shown in the figure and Table 3, the four dosing solutions stored for 0, 6, 12 and 24 months at room temperature show no differences in particle sizes, order parameters, chemical purity, and pharmacokinetic profiles and AUC.

These results indicate that the E5531 vials are stable for 24 months at room temperature when used within 24 hr after reconstitution.

CONCLUSIONS

The effects of the dispersing procedure on the aggregate size, membrane fluidity, and pharmacokinetics were evaluated. The size of the aggregates in the pH-jump method (pH 11.0 → 7.3) was decreased to approximately 20 nm, and the membrane fluidity increased with increasing dispersing time in 0.003 N NaOH (pH 11.0). However, when prepared by the normal dilution method (pH 7.3 → 7.3), the size of the aggregates was 150 nm, and the membrane had less fluidity than that prepared by the pH-jump method and was independent of the dispersing time. Using samples with different membrane fluidities prepared by changing the dispersing times in the pH-jump method, the pharmacokinetics in rats was evaluated, and it was confirmed that the membrane fluidity was correlated with pharmacokinetics in the plasma. In addition, E5531 vials were stable for 24 months at room temperature when used within 24 hr after reconstitution.

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