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RESEARCH PAPER

Optimization of the Formulation and Characterization of the Physicochemical Properties of the Novel Platelet-Activating Factor Receptor Antagonist E5880

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ABSTRACT

The injectable formulation of E5880, a novel platelet-activating factor (PAF) receptor antagonist, was determined from the study of pH stability, the selection of excipient, and the relationship between moisture and stability. The physicochemical properties of E5880 in the optimized formulation (0.6 mg/ml of E5880, 0.1% [4.8 mM] citric acid, 10% lactose, pH 2.8) were characterized. The critical micelle concentration of E5880 in the buffer was 0.1 mg/ml, and the structure was of spherical micelles. The micellar size was 5.6 nm and did not change before and after lyophilization and storage. The number of the molecules per micelle was 40. The micropolarity around the hydrocarbon region of the micelle was similar to that of butanol.

Key Words: *Injectable formulation; Lyophilization; Micelle; Platelet-activating factor; Structure.*

INTRODUCTION

Platelet-activating factor (PAF) exhibits a variety of biological activities, including activation of platelets (1) and neutrophils (2), bronchoconstriction (3), hypermeability in peripheral veins (4), hypotension (5), and cardiac dysfunction (6). Because these biological activities of PAF are extremely potent, it is generally accepted that PAF is a mediator of inflammation (7) and plays important roles in the pathology of thrombosis, asthma, or hypotension in shock (8–10). Consequently, it is expected that specific PAF receptor antagonists may be beneficial for the treatment of these diseases, and many efforts to develop potent and specific PAF antagonists have been made.

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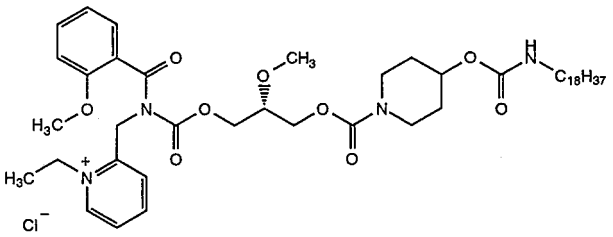

Figure 1. Chemical structure of the platelet-activating factor (PAF) antagonist, E5880.

E5880, a newly synthesized PAF antagonist (Fig. 1), is more potent in PAF receptor binding than PAF (11). This compound is amphiphilic, and it is expected to form the aggregates in aqueous media. For the treatment of the above diseases, an injectable formulation would be extremely useful. To develop the injectable formulation, the clarification of the characteristics of the physicochemical properties for E5880 and its micelle is important.

In this study, the injectable formulation of E5880 was optimized from the study of pH stability, the selection of excipient, and the relationship between moisture and stability. In addition, the physicochemical properties of E5880 micelles (critical micelle concentration, size, the number of the molecules per micelle, the micropolarity around the hydrophobic region of the micelle) in the optimized formulation were determined.

EXPERIMENTAL

Materials

E5880 was obtained from Eisai Chemical Company, Limited (Ibaraki, Japan). Lactose was purchased from Mallinkrodt (Paris, KY), and citric acid was purchased from Kozakai-Seiyaku Company, Limited (Tokyo, Japan). *D*-Mannitol was purchased from Towa-kasei Company, Limited (Tokyo, Japan). Hydrochloride was purchased from Tokai-Seiyaku Company. Sodium phosphate dibasic was purchased from Wako Pure Industrial Limited (Tokyo, Japan). *N*-Phenyl-1-naphtylamine (NPN) was purchased from Tokyo-kasei Company.

Optimization of E5880 Formulation

Determination of the Stability of E5880 in Various pH and Sterilization

For determination of stability, 60 mg of E5880 were dissolved in 100 ml of the buffer solution (for pH 1.0, in 1 N HCl; for pH 2.0, in 0.1 N HCl; for pH 2.8, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0, in 4.8 mM citric acid–sodium phosphate dibasic solution). The sample for control to determine the residual content contained 0.6 mg/ml of E5880 in water. The solution (5 ml) was filled into colorless glass ampoules and sealed. These ampoules were stored at 25°C for 8 hr under light irradiation (1000 lux) at 8 hr and sterilized at 121°C for 20 min. The samples for control were stored in the freezer at −40°C. The residual contents of the samples compared to the control stored in the freezer were determined by comparing the peak area of high-performance liquid chromatography (HPLC) (detection wavelength 266 nm).

Optimization of the Excipient for Lyophilization

For optimization for lyophilization, 300 mg of E5880 were dissolved in 500 ml of the buffer solution (4.8 mM citric acid, 10% lactose, pH 2.8 or 4.8 mM citric acid, 5% *D*-mannitol, pH 2.8) by stirring at room temperature. Then, the solutions were filtered (pore size 0.22 μm), filled into glass vials (1 ml/vial), and lyophilized. These vials were stored in the freezer at −40°C and at 40°C/75% relative humidity (RH) for 6 months. The residual contents of the samples compared to the control stored in the freezer were determined by comparing the peak area of HPLC (detection wavelength 266 nm). In addition, the moisture of the samples was determined.

Effects of Moisture on the Stability of E5880 Lyophilized Vials

The lyophilized vials (0.6 mg E5880, 100 mg lactose, 1 mg citric acid) of various moistures (range 1–5%) were prepared by controlling the lyophilization conditions. These vials were stored in the freezer at −40°C and at 40°C/75% RH for 6 months. The residual contents of the control samples stored in the freezer were determined by comparing the peak area of HPLC (detection wavelength 266 nm). In addition, the moisture of the samples was determined, and the appearance of the sample (20 vials) was observed.

Characterization of E5880 Micelle in the Formulation

Determination of the Size of the E5880 Micelle and the Effect of Storage

The lyophilized vials (0.6 mg/vial) were stored at 40°C/75% RH for 6 months. The size distribution of E5880 micelles in the reconstituted solution (0.6 mg/ml)

was determined by the dynamic light scattering (DLS) technique using a laser particle analyzer equipped with an argon laser (model DLS-7000DL, Ohtsuka Electronics Co., Ltd., Osaka, Japan) at 25°C. The data were analyzed by the histogram method (12), and the weight-averaged size was evaluated.

Determination of the Critical Micelle Concentration by Measurement of Surface Tension

To determine the critical micelle concentration, the surface tension of the E5880 formulated solution (4.8 mM citric acid, 10% lactose, pH 2.8) was measured by Wilhelmy's method using a surface tensiometer (model CBVP-A3, Kyowa Kaimenkagaku Co., Ltd., Tokyo, Japan) at 25°C.

Determination of the Micropolarity Around *N*-Phenyl-1-naphtylamine in the Hydrocarbon Region of the E5880 Micelle

The micropolarity of hydrocarbon regions in the E5880 micelle was determined using a fluorescence technique (NPN probe). NPN exhibits a strong environment-dependent blue shift, a high quantum yield, and low fluorescence in water (14,15). The fluorescence spectra were measured using a fluorescence spectrophotometer (model F-4500, Hitachi Co., Ltd., Tokyo, Japan) on excitation at 340 nm at 25°C. The micropolarity of NPN incorporated into the lipid aggregates was evaluated using the wavelength of maximum intensity of emission. NPN (2.2 mg) was dissolved in 10 ml of acetone (100 μM). Each solution (5 μl) was then diluted with 5 ml of 10 mM of E5880 solutions (600 μg/ml, pH 2.8), methanol, ethanol, propanol, butanol, acetone, tetrahydrofuran, and benzene, respectively. The wavelengths at the maximum fluorescence intensity of each solution were plotted against the polarity of each solvent (16). The micropolarity around the probe was determined using this standard curve.

RESULTS AND DISCUSSION

Optimization of E5880 Formulation

pH Stability

Figure 2 shows the relationship between pH and the residual contents in the freezer as a function of storage and sterilization conditions. Below pH 4.0, the residual contents of E5880 stored at 25°C and 1000 lux irradiation were not decreased. Below pH 4.0, the residual contents after sterilization decreased. Based on these results, we decided to select lyophilization below pH 4.0 for E5880 formulation.

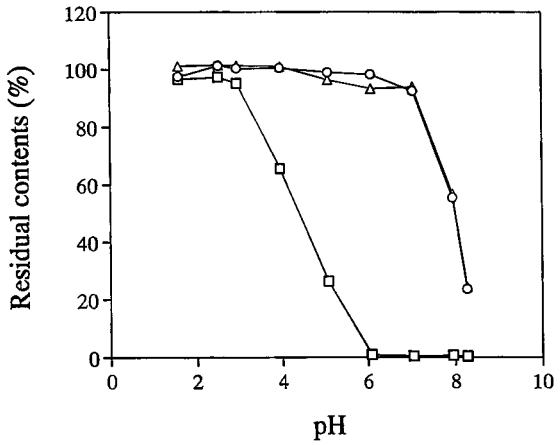

Figure 2. Relationship between pH and residual contents to freezer for E5880 solution (0.6 mg/ml) as a function of storage conditions: ○, 25°C for 8 hr; △, 1000 lux irradiation for 8 hr; □, sterilization at 121°C for 20 min.

Optimization of the Excipient for Lyophilization

Table 1 shows the comparison of the effect of the excipients on the E5880 stability. Apparently, the addition of lactose is more effective on the stabilization of E5880. Lactose was selected as an excipient for E5880 lyophilized vials.

Effect of Moisture on E5880 in the Lyophilized Vials

Table 2 shows the comparison of the stability of E5880 of various moisture contents in the lyophilized vials. The moisture of the vials at all storage conditions was increased during storage. When the moisture at the end of lyophilization (time = 0 in the stability test) was 5%, the residual contents did not decrease at 40°C/75% RH for 6 months. However, the contents of the vials containing moisture above 3.5% appeared to shrink at 40°C/75% RH for 3 months. The contents of the vials containing moisture below 2.6% did not appear to shrink. Based on the results, the target moisture at the end of lyophilization was determined to be below 2.6%.

The optimized formulation of E5880 was the lyophilized vials containing 0.6 mg of E5880, 100 mg lactose, 1 mg citric acid, and moisture below 2.6% at the end of lyophilization.

Table 1

Comparison of the Effects of Lactose and D-Mannitol as an Excipient on E5880 Stability

	Lactose		D-Mannitol	
Excipent	Initial Time (Time = 0)	40°C/75% RH for 6 Months	Initial Time (Time = 0)	40°C/75% RH for 6 months
Residual contents to freezer (%)	100.0	99.7	100.0	83.5
Moisture (%)	0.57	2.36	0.74	0.64

Table 2

Relationship Between Moisture and Stability of the E5880 Vials

	Sample Lot No.					
	01	02	03	04	05	06
Moisture (%)						
Initial time	0.79	1.15	1.59	2.62	3.46	4.82
40°C/75% RH						
For 1 month	1.98	2.66	3.08	3.57	4.47	5.83
For 3 months	2.13	2.36	2.84	3.43	4.30	5.21
For 6 months	2.52	2.64	3.12	3.75	4.41	5.42
Number of shrinked vials (20 vials were checked in every time point)						
Initial time	0/20	0/20	0/20	0/20	0/20	0/20
40°C/75% RH						
For 1 month	0/20	0/20	0/20	0/20	3/20	0/20
For 3 months	0/20	0/20	0/20	0/20	5/20	2/20
For 6 months	0/20	0/20	0/20	0/20	6/20	20/20
Residual contents to freezer (%)						
Initial time	100.0	100.0	100.0	100.0	100.0	100.0
40°C/75% RH						
For 1 month	99.54	99.88	99.81	99.84	99.61	99.45
For 3 months	99.67	100.1	99.99	99.46	99.33	98.77
For 6 months	100.3	100.2	98.98	100.0	98.56	98.26

Characterization of E5880 Micelle in the Formulation

Size of the E5880 Micelle and the Effect of Storage

Table 3 shows the size of the E5880 micelle before and after lyophilization and in the reconstituted solution at the time of the stability test. The data show that the size of the micelle was approximately 5 nm, and no differences were observed in storage.

Critical Micelle Concentration

Figure 3 shows the surface tension at the E5880-buffer interfaces γ as a function of the E5880 concentrations in

Table 3

Size of E5880 Micelle (0.6 mg/ml)

Storage Conditions	Micellar Size (nm)
Before lyophilization	5.6 ± 2.1
After lyophilization (Time = 0 in stability test)	6.1 ± 1.8
40°C/75% RH in the stability test (reconstituted solution)	
For 1 month	4.8 ± 2.0
For 3 months	5.4 ± 2.7
For 6 months	5.9 ± 1.6

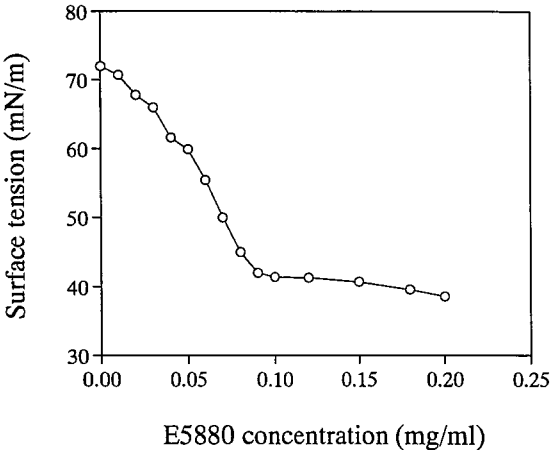

Figure 3. Surface tension of the E5880 at air-buffer (4.8 mM [0.1%] citric acid, 10% lactose, pH 2.8) interface as a function of the E5880 concentration at 25°C.

the buffer C. The γ value at $C = 0$ (γ_0) was 72.0 mN/m at 25°C. The γ value of the E5880 solution falls abruptly in the concentration 0.08–0.1 mg/ml and was stable at concentrations above 0.1 mg/ml. This indicates that the critical concentration for formation of the aggregates was 0.1 mg/ml. The adsorption amount at the surface G (mol/cm^2) is correlated with the change of surface tension ($d\gamma/d \ln C)_{T,P}$ by the Gibbs adsorption equation:

$$G = -(1/2RT) \cdot (d\gamma/d \ln C)_{T,P} \quad (1)$$

Here, R is the gas constant. Figure 4 shows the adsorption amounts G calculated using Eq. 1 as a function of the

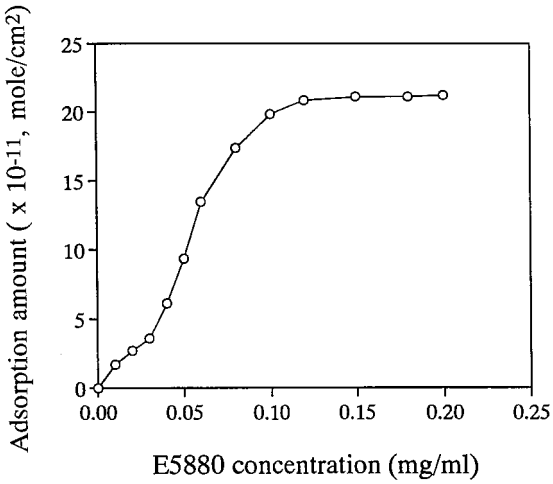

Figure 4. Adsorption isotherms of E5880 at air-buffer (4.8 mM [0.1%] citric acid, 10% lactose, pH 2.8) interface as a function of the E5880 concentration at 25°C.

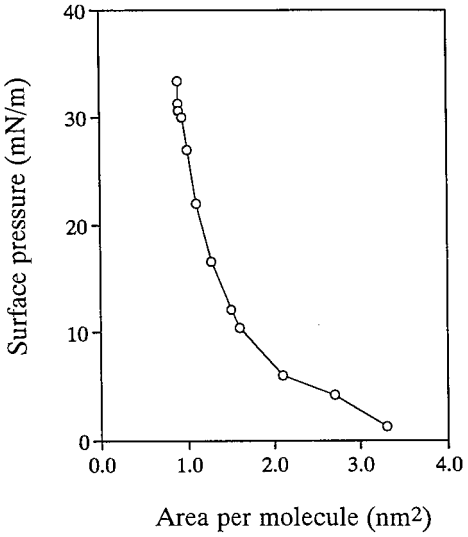

Figure 5. Surface pressure–molecular area curves of E5880 at air-buffer (4.8 mM [0.1%] citric acid, 10% lactose, pH 2.8) interface at 25°C.

concentration of the E5880. The surface pressure of the lipid monolayer F is calculated as

$$F = \gamma_0 - \gamma \quad (2)$$

Surface area per lipid molecule (molecular area) A is evaluated as $A = 1/(N\Gamma)$. Here, N is the Avogadro number. Figure 5 shows the surface pressure–molecular area curves (F-A curves) for the lipids. The limiting area for E5880 was 0.90 nm^2.

Determination of the Micellar Structure by Calculation of the Critical Packing Parameters

The critical packing parameters (18) for E5880 were calculated based on the area per molecule results (Fig. 5), the volume of the hydrophobic part, and the length of the acyl chain. For the formation of closed lamellar bilayer structures, the effective cross section of the hydrocarbon moiety must be lower than that of the hydrophilic head group region. This assumption is confirmed by an estimation of the critical packing parameter according to the formula (17) $x = v/a{\cdot}l$ (v is the volume of the hydrophobic part, a is the area of the hydrophilic head group, and l is the length of the acyl chain). When $v/a{\cdot}l < 1/3$, spherical micelles form; at $1/3 < v/a{\cdot}l < 1/2$, tubular micelles form; at $1/2 < v/a{\cdot}l < 1$, vesicles form; and at $1 < v/a{\cdot}l$, hexagonal H_{II} structures form. The volume of the hydrocarbon domain v and the length of hy-

drocarbon l were calculated using the following equations:

$$v = (27.4 + 26.9n) \times 10^{-}3 \ (\text{nm}^3) \quad (3)$$

$$l = (0.15 + 0.1256n) \ (\text{nm}) \quad (4)$$

where n represents the number of the carbons for hydrocarbon chains. For E5880, $v = 0.51$ (nm^3), $l = 2.4$ (nm), $a = 0.90$ nm^2, and $x = 0.24$. Therefore, it can be expected that these three lipids form spherical micelles structure. The number of molecules in the spherical micelles N can be calculated by the following equations (18):

$$N = 36 \ \pi v^2/a^3 \quad (5)$$

For E5880, micelles in the formulation, $N = 40$.

Micropolarity Around *N*-Phenyl-1-naphtylamine in the E5880 Micelles

The micropolarity around NPN in the E5880 micelles was determined by measuring the emission maxima of NPN embedded in the micelles. It has been reported that the fluorescence characteristics of NPN depend on the micropolarity around the probe, and it is located in a hydrophobic region in the lipid aggregates (14,15). Therefore, it is expected that the emission maxima of NPN in the lipid aggregates will provide information on the micropolarity around the hydrocarbon chains. Figure 6 shows the relationship between solvent polarity and emission maximum of NPN at 25°C. The emission maximum of E5880 micelles was 510 nm, indicating that the micropolarity around the probe in the E5880 micelle is comparable to that of butanol.

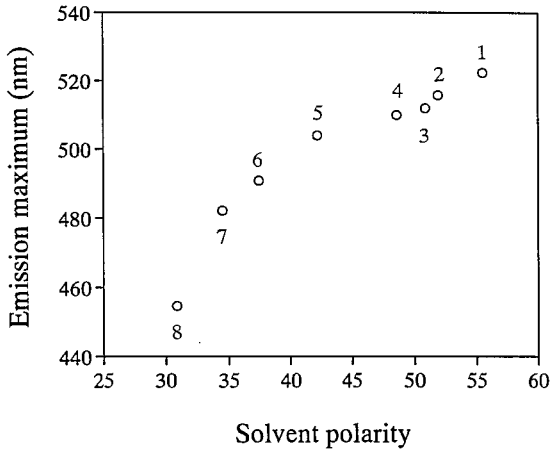

Figure 6. Relationships between solvent polarity and emission maximum of NPN (100 nM) at 25°C: 1, methanol; 2, ethanol; 3, propanol; 4, butanol; 5, acetone; 6, tetrahydrofuran; 7, benzene; 8, hexane.

CONCLUSIONS

The injectable formulation of E5580 was optimized to the lyophilized vials containing 0.6 mg of E5880, 1 mg citric acid, and 100 mg lactose and with the moisture below 2.6% at the end of lyophilization. The physicochemical characteristics of E5880 micelle in the formulation were determined. The critical micelle concentration of E5880 at 25°C was 0.1 mg/ml. The diameter of the micelle was approximately 5 nm, and it did not change before and after lyophilization and storage. The number of molecules per micelle was 40. The hydrocarbon regions in the micelle were similar to butanol.

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