Self-association properties of 4-[1-hydroxy-1-methylethyl]-2-propyl-1-[4-[2-[tetrazole-5-yl]phenyl]phenyl] methylimidazole-5-carboxylic acid monohydrate (CS-088), an antiglaucoma ophthalmic agent

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Abstract

Self-association properties of CS-088, an antiglaucoma ophthalmic agent, were investigated. Various analytical methods, such as surface tension measurement, demonstrated that CS-088 is a self-associating compound with critical micellar concentration (CMC) of approximately 10 mg/mL. Light scattering analysis revealed that the micellar molecular weight (MMW) of CS-088 aggregates well above the CMC was approximately 2260, corresponding to a pentamer. In addition, the MMW corresponding to a dimer was detected by NMR spectroscopy, indicating that self-association of monomers to pentamers is via the formation of dimers. According to the Stokes–Einstein equation, hydrodynamic radii of the dimer and pentamer were calculated to be 0.87 and 1.16 nm, respectively. The concentration-dependent change in the NMR chemical shift indicated that hydrophobic interaction between biphenyl groups is an important factor in the self-association of CS-088 molecules. Furthermore, measurement of particle size distribution using a Nicomp Submicron Particle-Sizer revealed that the addition of either *n*-propanol or urea to CS-088 solution led to monomerization of the dimers and pentamers, suggesting that not only hydrophobic interaction but also hydrogen bonding is involved in stabilizing CS-088 aggregates. No bigger aggregate than a pentamer was formed in the absence of NaCl, whereas further aggregation was observed with increasing concentrations of NaCl.
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Keywords: CS-088; Self-association; Light-scattering; Particle sizing; Aggregation number; NMR

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1. Introduction

Self-association of various types of pharmacologically active drugs, such as anesthetics, antipsychotics and antibiotics, has been reported (Kahn and Kirschbaum, 1967; Florence and Parfitt, 1970; Kirschbaum et al., 1970; Barthelemy et al., 1974; Carey et al., 1975; Attwood, 1976; Attwood and Agarwal, 1979, 1984; Attwood and Tolley, 1980; Attwood and Fletcher, 1986; Tehrani et al., 2001). These compounds commonly are of amphiphilic nature like detergents. However, the self-association behavior, such as aggregation number, micellar shape and association mode, varies depending on the physicochemical properties of the drugs. In the development of liquid formulation, the solubilizing effect of self-associating drugs upon slightly soluble additives is an important factor. In addition, self-association of drugs possibly influences the pharmacological effects by changing drug–membrane interaction or membrane permeability (Felmeister, 1972; Schreier et al., 2000). Therefore, understanding the self-association characteristics of drugs is important for both formula development and prediction of biological activity.

CS-088 is an antiglaucoma ophthalmic agent, an angiotensin AT_1 receptor antagonist with molecular weight of approximately 446, which is currently undergoing clinical studies (Fig. 1) (Inoue et al., 2001a,b). Previous studies revealed that CS-088 exhibits a solubilizing effect upon insoluble additives as generally seen in detergents. Furthermore, in vitro CS-088 permeability across rabbit corneal membrane decreased at higher concentrations (submitted for publication). Therefore, this study was conducted to investigate the self-association properties of CS-088 by surface tension, NMR and particle size analysis.

2. Materials and methods

2.1. Materials

4-[1-Hydroxy-1-methylethyl]-2-propyl-1-[4-[2-[tetrazole-5-yl]phenyl]phenyl] methylimidazole-5-carboxylic acid monohydrate (CS-088) was prepared in the Process Development Labs, Sankyo Co. Ltd., pinacyanol chloride and urea were purchased from

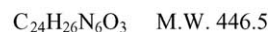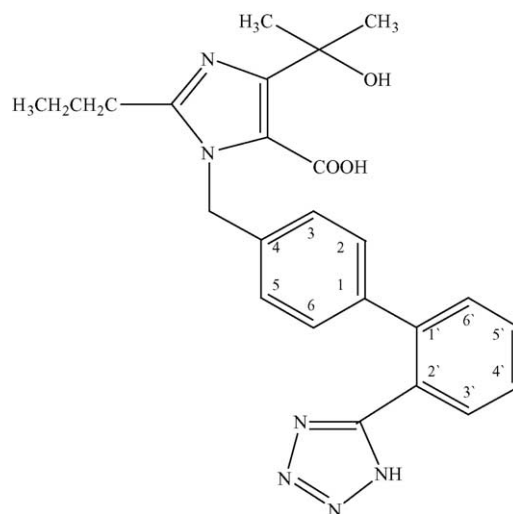$C_{24}H_{26}N_6O_3$ M.W. 446.5

Fig. 1. Chemical structure of CS-088.

Kanto Chemical Co. Ltd. and Wako Pure Chemical Industries Ltd., respectively. All other chemicals used in this study were of reagent grade.

2.2. Sample preparation

Stock solutions at CS-088 concentration of 250 mg/mL (0.56 M) in purified water were prepared and stored below 277 K. The pH of the solution was adjusted to 7.0 by addition of 1 M NaOH. The stock solutions were stepwise diluted with purified water to prepare solutions with various concentrations of CS-088 just before the experiments. The pH of each solution did not change upon dilution.

2.3. Surface tension measurements

Surface tension was measured at 298 K by the Wilhelmy plate method using a FACE Surface Tensiometer CBVP-A3 (Kyowa Kaimenkagaku Co. Ltd., Japan). Measurements were made 10 min after the plate was set on the liquid surface. Critical micellar concentration (CMC) of CS-088 was estimated from the inflection points in the surface tension versus CS-088 concentration curves.

2.4. Spectral change of hydrophobic probe

Pinacyanol chloride, a hydrophobic probe, was used to determine the CMC of CS-088. It has been shown that the absorption spectrum of pinacyanol chloride sharply changes in a hydrophobic environment generated by the formation of micelles (Corrin et al., 1946). Samples were prepared by resolving pinacyanol chloride at a final concentration of 2.5×10^{-5} M in various CS-088 solutions (0–30 mg/mL). The absorption spectra of these samples were measured at various wavelengths between 450 and 650 nm with a UV–vis spectrophotometer, U-1600 PC (Shimadzu, Japan), at 298 K.

2.5. Light scattering measurements

Measurements were made at 298 K with a Dynamic Light Scattering Spectrophotometer, DLS-700 (Otsuka Electronics Co. Ltd., Japan). Sample solutions were sufficiently clarified by ultrafiltration through 0.1 μm membrane filters prior to measurements. The scattered light intensity of each sample was measured at angles between 30° and 140° at intervals of 10° using a wavelength of 633 nm. Micellar molecular weight (MMW) of CS-088 at concentrations well above the CMC was determined by extrapolation of Debye light scattering plots.

2.6. ^{1}H NMR measurements

High-resolution ^{1}H NMR measurements were made at 298 K with a Bruker DMX600 spectrometer (600 MHz). The chemical shifts of the protons of CS-088 were measured at various concentrations with D_2O as an external reference. The hydrodynamic radius (R_H) of CS-088 aggregates was calculated from the diffusion coefficient according to the Stokes–Einstein equation: $R_H = k_B T/6\pi\eta D$, where D is the diffusion coefficient, k_B the Boltzmann constant, T the temperature (Kelvin) and η is the solvent viscosity.

2.7. Particle size analysis

Particle size analysis was conducted using a Nicomp Submicron Particle-Sizer, Model 380ZLS, equipped with a 50 mW DPSS laser (Nicomp Instrument Corp., USA). Mean particle size distribution was determined in volume-weighted Nicomp-fit mode.
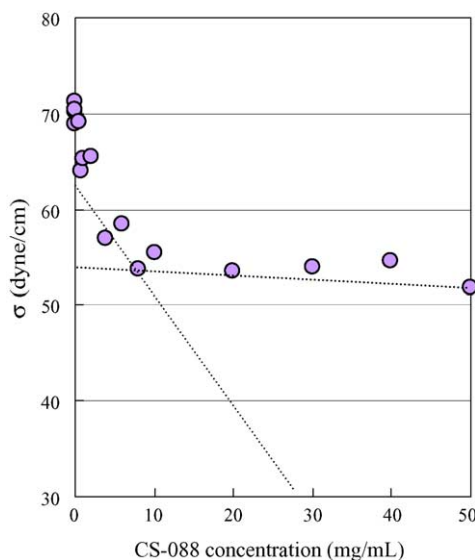

Fig. 2. Surface tension (σ) as a function of CS-088 concentration in aqueous solutions.

3. Results and discussion

3.1. Determination of critical micellar concentration

The plots of the surface tension as a function of CS-088 concentration are shown in Fig. 2. The curve exhibited a pattern typically seen with detergents, where an inflection point is observed around the CMC. The inflection point in the surface tension plots indicates the self-association behavior of CS-088. The CMC value was estimated to be approximately 10 mg/mL.

In order to confirm this result obtained by the surface tension method, absorption intensity of pinacyanol chloride, a hydrophobic dye, was evaluated at increasing concentrations of CS-088. Band maxima in the absorption spectra were observed at 517, 546 and 564 nm in the absence of CS-088. The extinction coefficients at the absorption peaks were plotted against CS-088 concentrations (Fig. 3). Abrupt changes in intensities were observed and the extinction coefficients reached a plateau at higher concentrations, indicating that a hydrophobic environment was formed in the solution around the inflection point. The CMC obtained in this experiment well coincided with that estimated from the surface tension measurement. Therefore, it was
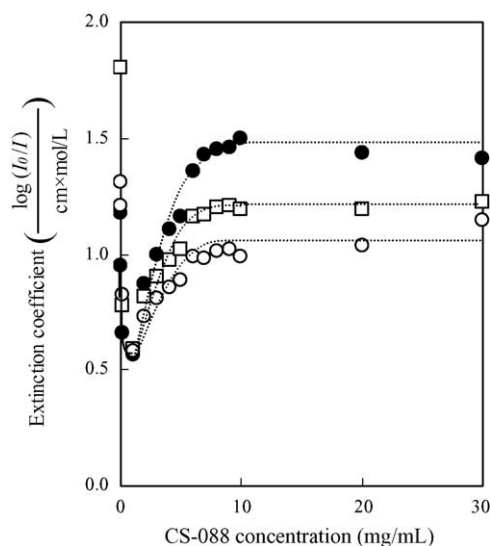
Fig. 3. Plots of extinction coefficients at three absorption peaks versus CS-088 concentration: (○), 564 nm; (□), 546 nm and (●), 517 nm.

demonstrated that CS-088 is a self-associating compound with CMC of approximately 10 mg/mL.

3.2. Determination of micellar molecular weight and hydrodynamic radius

In order to determine the MMW of CS-088 aggregate, light scattering measurements were carried out over a concentration range well above the CMC (50–400 mg/mL). From Debye light scattering analysis, it was found that the MMW of CS-088 aggregate in aqueous solution well above the CMC is approximately 2260, corresponding to a pentamer.

Self-association of CS-088 molecules was further investigated by high-resolution NMR spectroscopy. Table 1 shows the diffusion coefficient, MMW, aggregation number and hydrodynamic radius of CS-088 at different concentrations in the absence of NaCl. The MMWs were calculated from the diffusion coefficient with molecular weight of lysozyme as a reference. As a result, the aggregation number was determined to be five, well above the CMC, and no further association was observed in the absence of NaCl. These results coincided well with those obtained from light scattering analysis. It is worth noting that MMW corresponding to a dimer was observed at 30 mg/mL, suggesting that self-association of monomers to pentamers is via the formation of dimers.

In general, the aggregation number of low molecular weight drugs is small compared to those of detergents. It has been reported that aggregation number is limited to dimers and pentamers for Penicillin G (4) and trimers for methylene blue (Braswell, 1968). Interestingly, Richard (1975) and Hillson and McKay (1965) reported that the self-association of fusidate sodium, a steroid-like antibiotic, and Solway Ultra Blue B, a dye, are limited to pentamers as was observed for CS-088.

The lipophilicity of a compound is an important factor responsible for aggregation number. Although CS-088 has a hydrophobic moiety (biphenyl group) in the molecule, this compound is rather hydrophilic due to two negatively charged moieties (an ionized carboxyl group and tertiary amine) at physiological pH. Therefore, it is thought to be reasonable that self-association of CS-088 molecules is limited to small aggregates, dimers and pentamers. According to the Stokes–Einstein equation for spherical solutes, the hydrodynamic radii of the dimers and pentamers of CS-088 were calculated to be 0.87 and 1.16 nm, respectively.

3.3. Self-association mechanism of CS-088

In order to clarify the self-association process, ^{1}H NMR spectra of CS-088 were measured at various

Table 1
Physicochemical parameters at various CS-088 concentrations obtained from ^{1}H NMR analysis

CS-088 concentration (mg/mL)	Diffusion coefficient ($\times 10^{-6}$ cm^2/s)	MMW	Aggregation number	Hydrodynamic radius (nm)
1.5	3.24	595	1	–
30	2.83	893	2	0.87
60	2.14	2064	5	1.14
80	2.12	2123	5	1.16

Aggregation number was calculated by dividing the MMW by the molecular weight of CS-088 monomer (446.5).
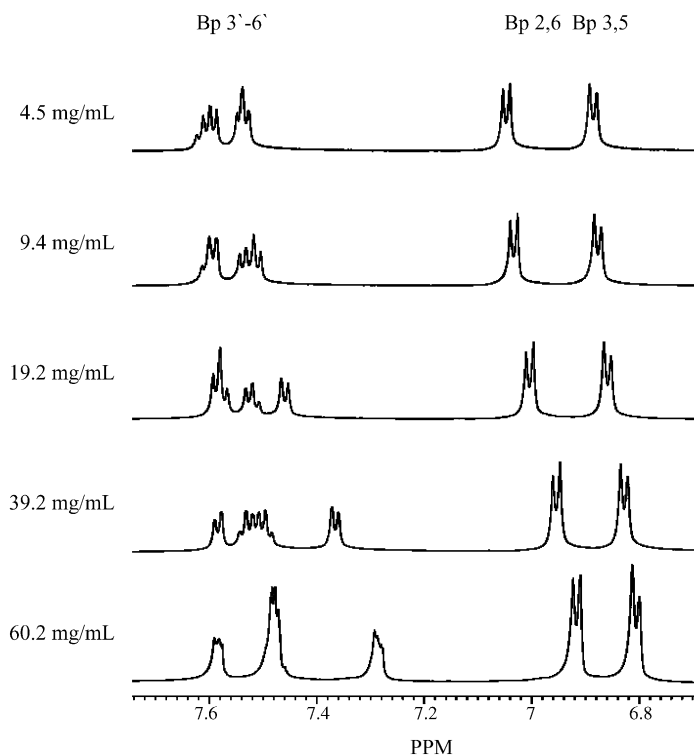
Bp 3`-6`   Bp 2,6 Bp 3,5

Fig. 4. Aromatic proton portions of ^{1}H NMR spectra of CS-088 at concentrations ranging from 4.5 to 60.2 mg/mL. Corresponding positions of biphenyl protons (Bp) are numbered in Fig. 1.

concentrations (4.5–60.2 mg/mL). The biphenyl proton portions (Bp) of ^{1}H NMR spectra, where concentration dependence was particularly notable, are shown in Fig. 4. For the sake of interpretation, protons of the biphenyl group of CS-088 are numbered in Fig. 1. As shown in Fig. 4, it was evident that the aromatic protons shifted to higher frequency in proportion to the CS-088 concentrations. The aromatic protons at $3'–6'$ positions exhibited more remarkable changes in chemical shift than those at 2, 3, 5 and 6 positions, in particular apparent splitting was observed for the $3'–6'$ protons at higher concentrations of CS-088. These results indicate that aromatic protons, particularly those at $3'–6'$ positions, play an important role in the association of CS-088 molecules through the hydrophobic interaction between the biphenyl groups.

For further investigation, particle size analysis was conducted to characterize the self-association process of CS-088. Fig. 5 shows the plots of the volume-weighted mean particle size of CS-088 aggregates against CS-088 concentrations. No particles
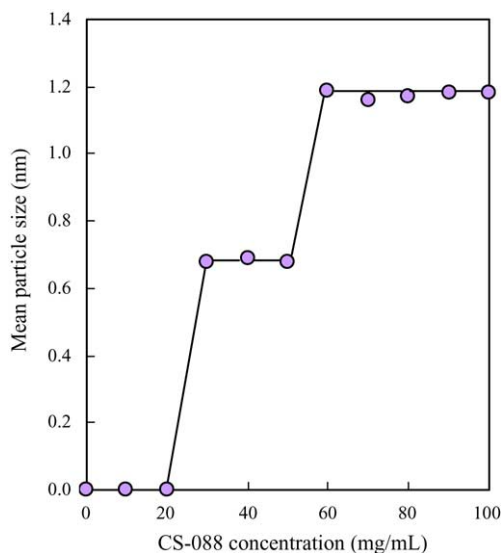

Fig. 5. Mean particle size of CS-088 aggregates in the absence of NaCl. Mean particle size at CS-088 concentrations below 20 mg/mL, where no particles were detected, is expressed as zero.

Table 2
Effect of disaggregating agents and NaCl on the mean particle size (nm) of CS-088 aggregates at various concentrations of CS-088

Additives	CS-088 concentration (mg/mL)		
	10	40	80
Control	ND	0.71	1.26
5 M urea	ND	ND	ND
95% *n*-propanol	ND	ND	ND
0.5 M NaCl	0.75	1.59	1.85
1 M NaCl	0.82	1.96	2.87
2 M NaCl	0.73	2.92	3.41

Control solution contains CS-088 at each concentration (pH 7.0). ND, not detected.

were detected at CS-088 concentrations of not more than 20 mg/mL due to an instrumental limitation. On the other hand, aggregates with mean particle size of 0.71 nm (dimer) and 1.26 nm (pentamer) were observed in the concentration range of 30–50 and 60–100 mg/mL, respectively. Mean particle size determined in this experiment was in good agreement with that obtained from NMR analysis. These results demonstrate that self-association of CS-088 is a phase separation mode, but not a sequential mode as has been reported for small aggregates, where an aggregate grows by stepwise addition of monomers (Attwood, 1976; Chang and Cardinal, 1978; Attwood and Tolley, 1980; Attwood and Fletcher, 1986).

The disaggregating effects of urea and *n*-propanol are shown in Table 2. It was found that the addition of 5 M urea, a disrupting agent of hydrogen bonding, to CS-088 solution led to monomerization of both the pentamers and dimers. A similar transition of pentamers and dimers to monomers was observed in 95% *n*-propanol, which is a sufficient concentration to disrupt hydrophobic interaction. It has been reported that secondary micelles are formed by the association of primary micelles through hydrogen bonding between hydroxyl groups (Small, 1968). CS-088 has a hydrophobic biphenyl group and a non-ionic polar group (hydroxyl group) in the molecule. Therefore, it is highly possible that not only hydrophobic interaction but also hydrogen bonding is involved in stabilizing the dimers and pentamers. In contrast to the disaggregating effects of *n*-propanol and urea, NaCl induces a marked increase in the mean particle size of CS-088 aggregates, indicating further aggregation of pentamers to multimers. With the CS-088 concentra-

tion kept constant at 10 mg/mL, the mean particle size increased to 0.73–0.82 nm, corresponding to a dimer. On the other hand, unlimited increases in mean particle size were observed at CS-088 concentrations of 40 and 80 mg/mL. Multimers with mean particle size of 2.92 and 3.41 nm were formed in the presence of 2 M NaCl at 40 and 80 mg/mL, respectively. Several factors are probably responsible for the increase in aggregate size with increasing NaCl concentration, including decreased electric repulsion, partial dehydration of the hydroxyl groups and salting-out of the hydrocarbon part of the molecule (Small, 1968).

In conclusion, we demonstrated that CS-088 is a self-associating compound with CMC of approximately 10 mg/mL. CS-088 predominantly exists as dimers slightly above the CMC and further self-associates to pentamers at higher concentrations well above the CMC. Hydrodynamic radii of the dimer and pentamer of CS-088 were calculated to be 0.87 and 1.16 nm, respectively. It was thought that not only hydrophobic interaction between biphenyl groups but also hydrogen bonding is involved in stabilizing CS-088 aggregates. Higher aggregation number was observed with increasing concentrations of NaCl. CS-088 produces its pharmacological effect inside a corneal membrane, which reportedly has efficient pore size of 0.8–1.4 nm (Hamalainen et al., 1997). The relationship between mean particle size of CS-088 and transcorneal permeability was previously investigated using an in vitro Ussing chamber system (Kikuchi et al., 2005a,b). The results obtained from penetration experiments showed that the corneal permeability of CS-088 decreased with increasing concentration of CS-088 (submitted for publication). The apparent permeability coefficient (P_{app}) of CS-088 through the corneal membrane was constant at the concentrations of not more than 20 mg/mL, at which concentration no particle was detected using a Particle-Sizer. On the other hand, P_{app} significantly decreased at 30 mg/mL (2.18×10^{-6} cm/s) compared to 20 mg/mL (3.13×10^{-6} cm/s) as CS-088 self-associates to form dimers with a particle size of approximately 0.71 nm. Further decreases in P_{app} were observed at 40 mg/mL (1.37×10^{-6} cm/s) due to the increase in the concentration of the CS-088 dimers. From these results, it is indicated that self-association of drugs is an important factor in the corneal penetration of the drugs themselves.

Acknowledgement

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