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Mechanism of permeability-enhancing effect of EDTA and boric acid on the corneal penetration of 4-[1-hydroxy-1-methylethyl]-2-propyl-1-[4-[2-[tetrazole-5-yl]phenyl]phenyl] methylimidazole-5-carboxylic acid monohydrate (CS-088)

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Abstract

This study was conducted to clarify the penetration 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 ophthalmic agent, and the mechanism of the permeability-enhancing effect of EDTA and boric acid (EDTA/boric acid) on the corneal penetration of CS-088. In the absence of additives, corneal permeability decreased with increasing concentration of CS-088 as CS-088 monomers self-associate to form dimers. Presence of EDTA/boric acid caused no significant changes in the physicochemical properties of CS-088, the apparent partition coefficient or the mean particle size of CS-088. EDTA/boric acid induced only a slight change in the zeta potential of liposomes used as a model of the biological membrane.

On the other hand, EDTA/boric acid significantly increased membrane fluidity of liposomes, whereas other buffering agents tested did not. This effect was synergistic and concentration-dependent for both EDTA and boric acid as was observed in in vitro corneal penetration of CS-088. In accordance with the result, the rate of CS-088 permeation into the liposomes significantly increased by the addition of EDTA/boric acid. Therefore, it was demonstrated that EDTA/boric acid promotes corneal penetration

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of CS-088 through the transcellular pathway by increasing membrane fluidity. Conversely, other buffering agents decreased corneal permeability of CS-088 by inducing further self-association of CS-088 aggregates.

Keywords: CS-088; Penetration enhancer; EDTA; Boric acid; Membrane fluidity; Ophthalmology

1. Introduction

Corneal penetration of an ophthalmic agent is of great importance in topical instillation. In order to increase the bioavailability of drugs, pharmaceutical approaches such as sustained-release formulation with water-soluble polymer (Bourlais et al., 1998; Balasubramaniam and Pandit, 2003) and hydrophobic ion pair formation between a drug and additives (Wilson et al., 1981; Neubert, 1989; Lengsfeld et al., 2002), have been developed. Although these techniques have been used in several drug products, which are commercially available on the market, whether these techniques are applicable to a new drug or not depends on the physicochemical properties of the drug and compatibility with the drug formula. On the other hand, penetration enhancers are of wide application and various types of penetration enhancers have been added to drug formulas to improve the efficacy of the drugs (Marsh and Maurice, 1971; Camber and Edman, 1987; Sasaki et al., 1995; Saettone et al., 1996). However, penetration enhancers generally exhibit their effects by inducing morphological changes in the corneal membrane, which in large doses occasionally leads to adverse effects such as irritation (Rojanasakul et al., 1990; De Saint Jean et al., 2000; Monti et al., 2002). Therefore, the amount of penetration enhancers added to a drug formula should be minimized. From this standpoint, the synergistic enhancing effect of pharmaceutical additives is advantageous for formula development because the amount of penetration enhancers added in a drug formula could be minimized while maintaining the therapeutic efficacy needed.

CS-088 is a novel type antiglaucoma agent, an angiotensin AT_1 receptor antagonist (Inoue et al., 2001a,b), which is currently undergoing clinical studies (Fig. 1). In a previous study, it was demonstrated that transcellular permeability of CS-088 in rabbit cornea was synergistically promoted in the presence of EDTA and boric acid (Kikuchi et al., 2005).

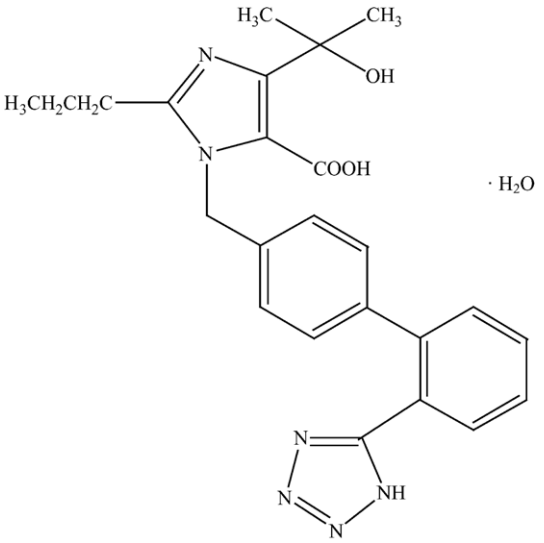

Fig. 1. Chemical structure of CS-088.

This study was conducted to clarify the mechanism of the permeability-enhancing effect of EDTA/boric acid on the corneal penetration of CS-088 by investigating the effect of EDTA/boric acid on the physicochemical properties of CS-088, and the zeta potential and the membrane fluidity of liposomes as a model of the biological membrane.

2. Materials and methods

2.1. Materials

CS-088 was prepared in the Process Development Laboratories, Sankyo Co. Ltd. EDTA and boric acid (pK: 9.3) were purchased from Kanto Chemical Co. Ltd. and Iwai Kagaku Co. Ltd., respectively. KH_2PO_4 (pK_1: 2.1, pK_2: 7.1, pK_3: 12.5), succinic acid (pK_1: 4.2, pK_2: 5.6), tartaric acid (pK_1: 3.0, pK_2: 4.6) and citric acid (pK_1: 3.8, pK_2: 4.8, pK_3:

5.4) were purchased from Kanto Chemical Co. Ltd. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Wako Pure Chemical Industries Ltd. Asolectine from soybeans was purchased from Sigma–Aldrich Corporation. All other chemicals used in this study were of reagent grade or of the highest possible grade.

2.2. In vitro penetration experiment

Male New Zealand White rabbits, weighing about 2.5–3.0 kg each, were sacrificed by administering an overdose of a sodium pentobarbitone solution via the marginal ear vein. All experiments in the present study adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). Then the corneas were dissected and mounted in a penetration chamber (Iwata et al., 1980). Saline solution (2 mL) whose osmolarity was equalized to that of each penetrant solution with sodium chloride was added to the endothelial side (receiver side). Penetrant solution (2 mL, pH 7.0) containing CS-088 with or without additives was added to the epithelial side (donor side) of the penetration chamber. The solutions of each chamber were stirred gently with magnetic stirrers. The apparatus was maintained at 34 °C throughout the experiments. Samples (50 μL) were withdrawn from each receiver solution at specified time points for a period of 2 h, followed by the addition of 50 μL of saline solution to maintain a constant volume of the receiver solution.

Apparent permeability coefficient (P_{app}) was calculated according to the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta_t \times A \times C_0 \times 60} \text{ cm/s}$$

where $\Delta Q/\Delta t$ is the steady state flux (μg/min), A the surface area of membrane (cm^2) and C_0 is the initial concentration in the donor chamber (μg/mL).

2.3. Measurement of octanol/buffer partition coefficient

The apparent partition coefficient (PC) of CS-088 was measured in *n*-octanol/buffer (pH 7.0) system. CS-088 solutions (3 mL) in the absence or presence of additives were equilibrated with *n*-octanol (3 mL) pre-saturated with buffer solution. After centrifugation, the concentration of CS-088 in the aqueous phase was determined by HPLC.

2.4. Preparation of liposomes

Large unilamellar liposomes (LUV) were prepared by the reverse phase evaporation method (Szoka and Papahadjopoulos, 1978). The LUV used in this study had a particle size ranging from 300 to 500 nm as determined using a Particle Sizer. Asolectine (equivalent to 0.9 mmol phospholipid) was dissolved in 5 mL of diethyl ether and 6 mL of a buffer (100 mM D-mannitol, 100 mM potassium chloride and 20 mM Hepes-Tris [pH 7.0]) was added. The mixture was sonicated in a bath-type sonicator under nitrogen for 5 min. Then the organic solvent was gradually removed under vacuum using a rotary evaporator to form liposomes. Following the addition of 15 mL of the above-mentioned buffer, the suspension was further evaporated to remove traces of the organic solvent. Phospholipid concentration was determined according to the method of Bartlett (1959).

2.5. Particle size distribution and zeta potential measurement

Particle size distribution of CS-088 aggregates and the zeta potential of liposomes were measured with a Nicomp Zeta Potential/Particle Sizer, Model 380ZLS equipped with a 50 mW DPPS laser (Nicomp Instr. Corp., USA).

2.6. Membrane fluidity

The membrane fluidity of liposomes was studied by the fluorescence polarization method (Merrill et al., 1987). An aliquot of 0.5 mM DPH solution (5 μL) in tetrahydrofuran was added to the liposome suspension (4 mL) and incubated at 25 °C for 1 h. Fluorescence intensities of DPH were measured with an RF-5300PC spectrophotometer (Shimadzu Corporation) equipped with a Shimadzu fluorescence polarizer, P/N 204-03290-01. The excitation and emission wavelengths were set at 360 and 430 nm, respectively. The degree of fluorescence polarization (P) was calculated from the fluorescence intensities of I_1 and I_2, parallel and perpendicular to the direction of the excitation beam,

according to the following equation:

$$P = \frac{I_1 - I_2}{I_1 + I_2}$$

The value for I_2 was corrected for unequal detection of the light of two polarizations by the fluorometer.

2.7. Permeation into liposomes

CS-088 solutions with or without EDTA/boric acid (20 mL) were mixed with the liposomal suspension (2 mL) and then incubated at 25 °C. Samples (2.5 mL) were withdrawn from the mixture at specified time points for a period of 1 h and untrapped CS-088 was immediately removed by gel filtration with Sephadex G-25 medium (PD-10 Desalting column). The liposomal fraction was collected and the amount of CS-088 trapped in liposomes was determined by HPLC after 10-fold dilution with methanol to dissolve lipsomes. The amount of CS-088 permeated into liposomes was expressed as unit amount of phospholipid. Using a Particle Sizer, it was confirmed that the mean particle size of the liposomes did not change by the addition of CS-088 solution.

2.8. HPLC analysis

After the sample solution was mixed with aqueous solution containing the internal standard (0.008% chloramphenicol), the CS-088 concentration in the sample was measured using an HPLC system (LC-10ADvp, Shimadzu Co. Ltd., Kyoto, Japan) in the reverse phase mode for assay. Chromatographic separation was carried out using an L-column ODS (4.6 mm i.d. × 150 mm length, Chemical Evaluation and Research Institute, Japan). A mixture of 10 mM KH_2PO_4 buffer (pH 7.0) and CH_3CN (82:18, v/v) was used as a mobile phase with a flow rate of 1.0 mL/min. The column effluent was monitored at 225 nm with a UV spectrophotometer (SPD-10Avp, Shimadzu Co. Ltd., Kyoto, Japan).

2.9. Data analysis

All data were statistically evaluated by analysis of variance followed by Student's *t*-test.

3. Results and discussion

3.1. Concentration dependence of CS-088 on corneal penetration

CS-088 is a self-associating compound with critical micelle concentration (c.m.c) of approximately 10 mg/mL. CS-088 predominantly exists as dimers slightly above the c.m.c and further self-associates to pentamers at higher than 60 mg/mL (unpublished data). The mean particle size of CS-088 aggregates and apparent permeability coefficient (P_{app}) were plotted against CS-088 concentrations in Fig. 2. The P_{app} values remained constant at CS-088 concentrations below 20 mg/mL. On the other hand, decreases in P_{app} were observed with increasing CS-088 concentrations at higher than 30 mg/mL, at which CS-088 molecules self-associate to form dimers with mean particle size of approximately 0.71 nm. This result suggested that self-association of CS-088 molecules has a great influence on the corneal permeability of CS-088. In this study, the following experiments were carried out at the CS-088 concentration of 40 mg/mL, equal to that used in a previous study (Kikuchi et al., 2005).

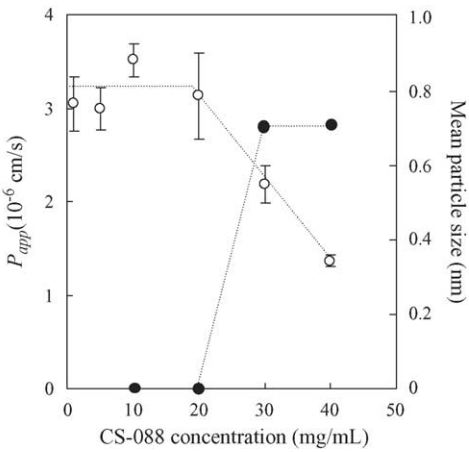

Fig. 2. Concentration-dependence of CS-088 on P_{app} (◯) and mean particle size of CS-088 aggregates (●) in the absence of EDTA/boric acid. Each P_{app} point represents the mean ± S.E. of three experiments. The mean particle size at CS-088 concentrations below 20 mg/mL, at which no particles were detected, is expressed as zero.

3.2. Effects of buffering agents on CS-088 corneal permeability

In previous studies, it was demonstrated that EDTA/boric acid synergistically enhances corneal penetration of CS-088 through the transcellular pathway in a concentration-dependent manner using an in vitro penetration chamber system (Kikuchi et al., 2005). Boric acid was added in the drug formula as a buffering agent. Therefore, in order to investigate whether other buffering agents have such synergistic enhancing effect with EDTA on the corneal penetration of CS-088 or not, P_{app} was determined in the presence of EDTA and other buffering agents equimolar to boric acid. In contrast, it was found that corneal permeability of CS-088 significantly decreased in the case of other buffering agents as shown in Fig. 3. In particular, citric acid markedly decreased the corneal permeability of CS-088 to approximately one third. These results suggest that the synergistic enhancing effect with EDTA on the corneal permeability of CS-088 is specific for boric acid.

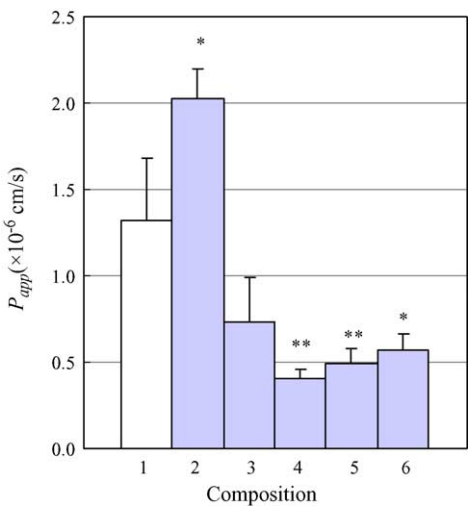

Fig. 3. Effect of various buffering agents on P_{app} of CS-088. Each solution (pH 7.0) contains 4% CS-088 and 0.005% EDTA. The buffer agents are as follows: (1) control, (2) +1% boric acid, (3) +KH_2PO_4, (4) +citric acid, (5) +succinic acid, (6) +tartric acid. Each bar of P_{app} represents the mean ± S.E. of three experiments. Statistically significant differences are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$.

3.3. Effect of EDTA/boric acid on physicochemical properties of CS-088

Hydrophobic ion pair (HIP) formation has been used as useful tool to promote drug penetration across the biological membrane by increasing the lipophilicity of the compounds. In previous studies, it was shown that EDTA/boric acid had no significant effect on the octanol–water partition coefficient (PC) of CS-088 in the absence (-1.80 ± 0.16) or presence (-1.68 ± 0.08) of EDTA/boric acid (Kikuchi et al., 2005). The results indicate that CS-088 molecules do not form HIP complexes with EDTA/boric acid. Similarly, no significant change of the PC was observed in the presence of other buffering agents (Table 1).

As mentioned above, CS-088 molecules predominantly exist as dimers at a CS-088 concentration of 40 mg/mL. Therefore, in order to clarify whether the permeability-enhancing effect of EDTA/boric acid was observed as a result of monomerization of CS-088 dimers or not, mean particle size was determined using a Particle Sizer. The mean particle size of CS-088 aggregates in the presence of additives are summarized in Table 1. No significant differences in mean particle size were observed either in the absence (0.71 nm) or presence (0.70 nm) of EDTA/boric acid, indicating that monomerization of a CS-088 dimer was not induced by addition of EDTA/boric acid. Therefore, it was demonstrated that neither the formation of HIP with EDTA/boric acid nor monomerization of CS-088 dimers by EDTA/boric acid is involved in the promotion of corneal penetration of CS-088.

On the other hand, the mean particle size significantly increased in the presence of other buffering agents, indicating that CS-088 dimers further self-associated to form bigger aggregates. The increase in mean particle size was more remarkable in the presence of citric acid (2.19 nm), succinic acid (1.95 nm) and tartaric acid (1.77 nm) than KH_2PO_4 (1.29 nm). As shown in Table 1, it seems that the P_{app} inversely correlated well with the mean particle size of CS-088.

3.4. Effect of EDTA/boric acid on corneal membrane

It has been reported that electrostatic interaction between an ionized penetrant and the membrane sur-

Table 1
Effect of additives on apparent permeability coefficient (P_{app}), octanol–water partition coefficient, mean particle size of CS-088 and zeta potential of liposomes

Additives		P_{app}[a] ($\times 10^{-6}$ cm/s)	Ratio[b]	Partition coefficient[a]	Mean particle size (nm)	Zeta potential (mV)
EDTA	Buffering agent					
–	–	1.32 ± 0.37	1.00	-1.80 ± 0.16	0.71	−24.1
0.005%	1% Boric acid	2.03 ± 0.17	1.55*	-1.68 ± 0.08	0.70	−20.5
0.005%	KH_2PO_4[c]	0.73 ± 0.26	0.55	-1.81 ± 0.05	1.29	−11.8
0.005%	Citric acid[c]	0.40 ± 0.05	0.31**	-1.57 ± 0.05	2.19	−7.7
0.005%	Succinic acid[c]	0.49 ± 0.09	0.37**	-1.68 ± 0.13	1.95	−6.8
0.005%	Tartric acid[c]	0.57 ± 0.09	0.43*	-1.70 ± 0.07	1.77	−6.0

Each solution (pH 7.0) contains 4% CS-088. Statistically significant at $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with the control.
[a] Values represent means ± S.E. of three experiments.
[b] Ratio of P_{app} to control.
[c] Equimolar to 1% boric acid.

face is an important factor in the penetration process (Sugawara et al., 1995). In this study, the effect of EDTA/boric acid on membrane surface charge was investigated using large unilamellar liposomes (LUV) as a model of the biological membrane. Table 1 shows the zeta potential of the liposomes in the presence of the additives. The zeta potential in the absence of additives (control) was −24.1 mV. Only a slight change was observed in the presence of EDTA/boric acid (−20.5 mV), suggesting that the change in the membrane surface charge is not the cause of penetration enhancement. On the other hand, it was found that the addition of other buffering agents induced apparent positive shift of the zeta potential to −11.8 to −6.0 mV. As shown in Table 1, there was a good relationship between the zeta potential of liposomes and mean particle size of CS-088 aggregates. The positive shift of the zeta potential was more remarkable in the presence of a buffering agent with high ionic valence. Considering the p*K* values of the buffering agents, it would appear that boric acid exists mainly as a monovalent anion in aqueous solution at a pH of 7.0. On the other hand, KH_2PO_4, succinic acid, tartaric acid and citric acid exist as mono and divalent, divalent, divalent and trivalent anions, respectively. The CS-088 molecule has two negatively charged moieties (an ionized carboxyl group and a tertiary amine) at physiological pH. Therefore, it was presumed that buffering agents promote further self-association of CS-088 dimers to bigger aggregates by decreasing the electrostatic repulsion between the CS-088 dimers and the degree of the effects is related to the valence of the buffering agent.

The effect of EDTA/boric acid on the liposomal membrane fluidity was investigated using DPH, a fluorescence probe. The degree of fluorescence polarization (*P*) in the presence of additives is summarized in Table 2. In the presence of either 0.005% EDTA or 1% boric acid, no significant change of fluorescence intensity was observed (0.196 ± 0.009, 0.205 ± 0.006, respectively) compared to the control value (0.200 ± 0.005). On the other hand, the fluores-

Table 2
Effect of additives on membrane fluidity of liposomes

Additives		Fluorescence polarization
EDTA	Buffering agent	
–	–	0.200 ± 0.005
0.005%		0.196 ± 0.009
0.005%	0.1% Boric acid	0.195 ± 0.016
0.005%	0.5% Boric acid	0.186 ± 0.015
0.005%	1% Boric acid	0.176 ± 0.007*
0.005%	2% Boric acid	0.171 ± 0.013*
–	1% Boric acid	0.205 ± 0.006
0.0005%	1% Boric acid	0.188 ± 0.010
0.005%	1% Boric acid	0.176 ± 0.007*
0.05%	1% Boric acid	0.168 ± 0.019*
0.005%	KH_2PO_4[a]	0.198 ± 0.023
0.005%	Citric acid[a]	0.200 ± 0.015
0.005%	Succinic acid[a]	0.206 ± 0.011
0.005%	Tartric acid[a]	0.204 ± 0.019

Each solution (pH 7.0) contains 4% CS-088. Values represent means ± S.E. of three experiments. Statistically significant at $^{*}P < 0.05$ when compared with the control.
[a] Equimolar to 1% boric acid.

cence intensity of DPH significantly decreased, which means an increase in the membrane fluidity, with the simultaneous application of EDTA and boric acid. The value was 0.176 ± 0.007 in the presence of 0.005% EDTA and 1% boric acid. In addition, concentration-dependence was evidently observed for both EDTA and boric acid. While keeping the concentration of EDTA at 0.005%, the value of P was 0.171 ± 0.013 at the highest concentration of boric acid. Similarly, addition of 0.05% EDTA deceased the value of P to 0.168 ± 0.019 while keeping the concentration of boric acid at 1%. From these results, it was shown that the effect of EDTA/boric acid to increase the membrane fluidity of liposomes was synergistic and concentration-dependent for both EDTA and boric acid as was observed in the in vitro corneal penetration of CS-088. Therefore, it was strongly suggested that EDTA/boric acid synergistically promotes corneal penetration of CS-088 by increasing membrane fluidity, leading to a decrease in the barrier through the corneal membrane. On the other hand, no significant changes in membrane fluidity were observed in the presence of other buffering agents. Increase in drug permeability due to the increase of membrane fluidity has been reported in the presence of chlorpromazine (Iseki et al., 1988), salicylic acid (Kajii et al., 1985) and azone (Kai et al., 1993). The decrease in membrane integrity possibly induces adverse effects such as irritation. However, it has been confirmed in a phase I clinical study that CS-088 ophthalmic solution is a safe drug product without any problematic adverse effects. Moreover, in the in vitro penetration experiment, pre-treatment of the corneal membrane by EDTA/boric acid exhibited no significant permeability-enhancing effect on the corneal penetration of CS-088. Therefore, it is suggested that just a slight morphological change of the corneal membrane in the presence of EDTA/boric acid is involved in the promotion of corneal penetration of CS-088 and this effect is reversible.

3.5. Permeation of CS-088 into liposomes

Fig. 4 shows the amount of CS-088 permeated inside liposomes per micromole of lipids against time in the absence and presence of EDTA/boric acid. Permeation of CS-088 into liposomes was significantly promoted in the presence of EDTA/boric acid. This result supported that EDTA/boric acid enhances transcellular permeability of CS-088 in corneal penetration by increasing membrane fluidity.

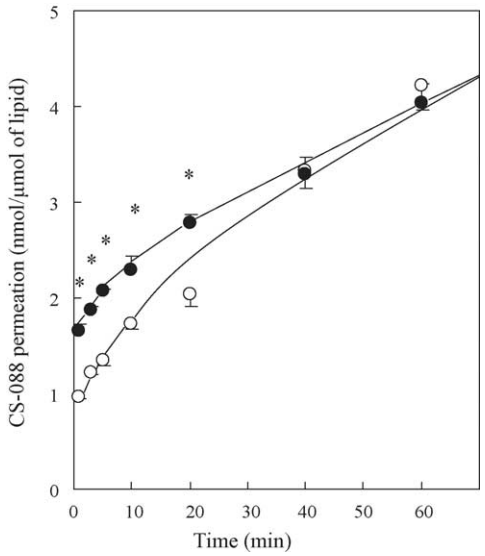

Fig. 4. Effect of EDTA/boric acid on the permeation of 4% CS-088 into liposomes in the absence (○) and presence (●) of EDTA/boric acid. Each point represents the mean ± S.E. of two experiments. Statistically significant differences are indicated as follows: $^{*}P < 0.05$.

Preliminary study showed that corneal penetration of some ophthalmic agents such as β-blockers was similarly enhanced by an addition of EDTA/boric acid (approximately 2.3-fold enhanced in the corneal penetration of timolol). Consequently, permeability-enhancing effect of EDTA/boric acid on the corneal penetration of CS-088 was investigated in this study. It was demonstrated that EDTA/boric acid significantly increased membrane fluidity of liposomes used as a model of the biological membrane and the effect was found to be synergistic and concentration-dependent for both EDTA and boric acid as was observed in the in vitro penetration experiments through cornea. This synergistic increase in membrane fluidity was specifically observed with the combination of EDTA and boric acid, but not with any of the other buffering agents tested. In accordance with the result, the amount of CS-088 permeated into liposomes was significantly increased by the addition of EDTA/boric acid. From these results, it was demonstrated that EDTA/boric acid promotes corneal penetration of CS-088 via the transcellular pathway by increasing membrane fluidity.

These findings suggest that the permeability-enhancing effect of EDTA/boric acid may be applicable to other drug formulations.

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