Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 290 (2005) 83–89
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Synergistic effect of EDTA and boric acid on corneal penetration of CS-088

Takayuki Kikuchi[a,*], Masahiko Suzuki[a], Akira Kusai[a], Ken Iseki[b], Hitoshi Sasaki[c]

[a] *Pharmaceutical Development Laboratories, Sankyo Co., Ltd. 1-12-1, Shinomiya, Hiratsuka, Kanagawa 254-0014, Japan*
[b] *Department of Clinical Pharmaceutics and Therapeutics, Graduate School of Pharmaceutical Science, Hokkaido University, Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan*
[c] *Department of Hospital Pharmacy, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan*

Received 10 September 2004; received in revised form 18 November 2004; accepted 21 November 2004

Abstract

In order to investigate the effects of EDTA and boric acid (EDTA/boric acid) on the corneal penetration of CS-088, an ophthalmic agent, the apparent permeability coefficient of CS-088 in the presence of EDTA/boric acid across the isolated corneal membranes of rabbits was measured using an in vitro penetration chamber system. FITC-dextran (M.W. 4400) and an electrical method based on membrane resistance were used to provide a quantitative assessment of the enhancing effect of EDTA/boric acid.

The corneal penetration of CS-088 was significantly enhanced in the presence of EDTA/boric acid by approximately 1.6-fold. The permeability-enhancing effect of EDTA/boric acid was apparently synergistic and concentration-dependent on both EDTA and boric acid. The penetration of FITC-dextran, a paracellular marker, and electrical resistance of corneal membranes were not affected in the presence of EDTA/boric acid. Furthermore, no enhancing effect of EDTA/boric acid was observed in de-epithelialized corneas, although de-epithelialized corneas exhibited a markedly higher permeability of CS-088 that was 24-fold greater than that for intact corneas. In conclusion, EDTA/boric acid synergistically enhances the transcellular permeability of CS-088 in the outer layer but not in the inner layers of the corneal membrane.

Keywords: EDTA; Boric acid; Synergistic effect; Penetration enhancers; Corneal penetration

1. Introduction

Topical instillation is the drug application method most commonly used in ophthalmology. To improve the efficacy of drugs, various types of enhancers have

* Corresponding author. Tel.: +81 463 31 6442; fax: +81 463 31 6475.
E-mail address: tkikuc@sankyo.co.jp (T. Kikuchi).

0378-5173/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2004.11.019

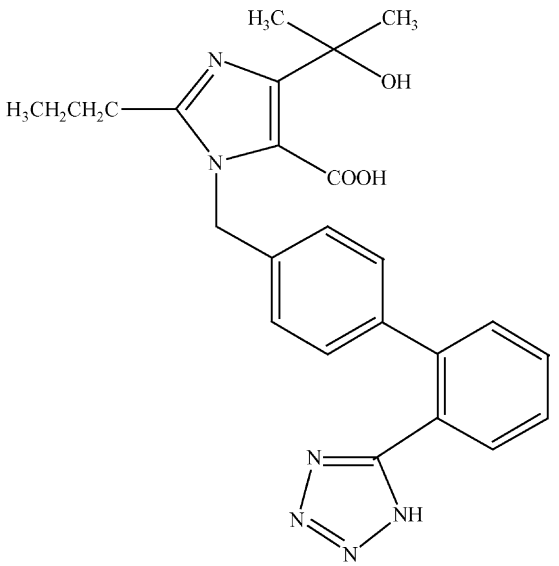

Fig. 1. Chemical structure of CS-088.

been added to drug formulations. Surfactants (Marsh and Maurice, 1971), bile salts (Sasaki et al., 1995a; Saettone et al., 1996), preservatives (Chamber and Edman, 1987; Ashton et al., 1990; Sasaki et al., 1995b; Madhu et al., 1996), or chelating agents (Sasaki et al., 1995c; Madhu et al., 1996; Saettone et al., 1996) are used to promote corneal penetration of the ophthalmic agents. However, these enhancers generally exhibit their effects by inducing morphological changes in the corneal membrane and occasionally lead to adverse effects such as irritation, in large doses (Durand et al., 1989; Rojanasakul et al., 1990; Grant et al., 1992; Jean et al., 2000; Meaney and O'Driscoll, 2000; Monti et al., 2002). Therefore, the amount of penetration enhancers should be minimized to prevent undesirable side effects.

CS-088 is a novel type of anti-glaucoma agent, an angiotensin AT_1 receptor antagonist (Inoue et al., 2001a,b), which is currently undergoing clinical studies (Fig. 1). CS-088 ophthalmic solution contains EDTA and boric acid as a stabilizer and a buffering agent, respectively. It was previously revealed that the in vivo pharmacological activity of CS-088 in rabbits was synergistically enhanced by a simultaneous application of EDTA and boric acid (unpublished data). This synergistic increase is advantageous for formula development because the amount of pharmaceutical additives required in a drug product can be reduced while maintaining the same pharmacological activity. This study was conducted to investigate the effects and mechanism of EDTA/boric acid on the corneal penetration of CS-088 ophthalmic agent using an in vitro penetration chamber system.

2. Materials and methods

2.1. Materials

CS-088 (4-(1-hydroxy-1-methylethyl)-2-propyl-1-1[[2′-[1H-tetrazol-5-yl]biphenyl-4-yl]-methyl] imidazole-5-carboxylic acid) was prepared in the Process Development Laboratories, Sankyo Co., Ltd. FITC-dextran (average molecular weight 4400 Da, FD-4K) was purchased from Sigma Chemical Company (St. Louis, MO). EDTA and boric acid were purchased from Kanto Chemical Co., Ltd. and Iwai Kagaku Co., Ltd., respectively. Methyl parahydroxybenzoate (MP) and propyl parahydroxybenzoate (PP) were purchased from Ueno Pharmaceutical Co., Ltd. All other chemicals used in this study were of reagent grade or of the highest possible grade.

2.2. In vitro penetration experiments

Male New Zealand White rabbits, weighing about 2.5–3.0 kg each, were sacrificed by administering an overdose of a sodium pentobarbital 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). The corneas were dissected and mounted in a penetration chamber (Iwata et al., 1980). For some experiments, the corneas were de-epithelialized by carefully scraping away the corneal epithelium with a scalpel until the stroma was exposed.

Test solution (4% CS-088 or 0.005% FD-4K, 2 mL) containing ophthalmic preservatives (0.033% MP and 0.018% PP) with or without additives (EDTA, boric acid) was added to the epithelial side (donor side) of the penetration chamber. Saline solution (2 mL) whose osmolarity was equalized to that of each test solution with sodium chloride was added to the endothelial side (receiver side). The solutions in each chamber were stirred gently with magnetic stirrers. The apparatus was maintained at 34 °C throughout the experiment. Sam-

ples (50 μL) were withdrawn from each endothelial solution at specified time points for a period of 2 h. After each sampling, 50 μL of saline solution was added to maintain a constant endothelial volume.

2.3. Electrophysiological experiment

After the excised corneal membrane was mounted in the Ussing chamber, modified Ringer's solution (pH 7.0, standard solution) composed of 204 mM NaCl, 5 mM KCl, 3.5 mM $NaHCO_3$, 0.95 mM Na_2HPO_4, 4.85 mM Na_2HPO_4 $2H_2O$, 1.4 mM $MgCl_2$, and 11.1 mM D-glucose was added to both the donor and receiver sides of the Ussing chamber. Modified Ringer's solutions containing EDTA and boric acid (test solutions) with osmolarity equalized to that of the standard solution by adjusting the concentration of sodium chloride, were also prepared. The entire system was preincubated at 34 °C for 20 min until a stable electric potential difference was obtained. Then, the epithelial solution was changed to the test solutions, and incubated at 34 °C for 2 h. The transepithelial electric resistance (TEER) was calculated according to Ohm's law from the potential difference observed when a small external current (0.1 mA) was passed. The resistance of the standard solution without a corneal membrane was below 12.7 Ω cm^2 (not more than 0.5% of the TEER in the presence of a corneal membrane) and was considered to be practically negligible compared to the total resistance.

2.4. HPLC analysis

Each sample of CS-088 (50 μL) was mixed with isotonic saline solution (50 μL) including the internal standard (80 μg/mL chloramphenicol) and the CS-088 concentration in the sample was determined by using an HPLC system (LC-10ADvp, Shimazu Co., Ltd., Kyoto, Japan) in the reverse-phase mode. Chromatographic separation was carried out by using an L-column ODS Waters (4.6 mm i.d. × 150 mm length, Chemical Evaluation and Research Institute, Japan). A mixture of 10 mM NaH_2PO_4 buffer (pH 7.0) and CH_3CN (82:18) 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, Shimazu Co., Ltd., Kyoto, Japan). FD-4K was detected fluorometrically by passing it through an HPLC column packed with a stationary phase (Inertosil ODS-2 (4.6 mm i.d. × 150 mm length), GL Sciences Inc., Tokyo, Japan) and equipped with a fluorescence spectromonitor (RF-10AXL, Shimazu Co., Ltd., Kyoto, Japan). A mixture of 10 mM $NaH_2PO_4{\cdot}2H_2O$ (pH 7.0) and CH_3CN (70:30) was used as a mobile phase, with a flow rate of 1.0 mL/min. The excitation and emission wavelengths were 495 and 514 nm, respectively.

2.5. Data analysis

Apparent permeability coefficients (P_{app}) and the lag time of CS-088 (FD-4 K) through the corneal membranes were estimated from the slope and *x*-intercept of the linear regression line obtained from the graph plotting the amount of drug accumulated in the receiver chamber versus time, between 45 and 120 min. All data were statistically evaluated by analysis of variance followed by Student's *t*-test.

3. Results and discussion

3.1. Synergistic effect of EDTA/boric acid on corneal permeability of CS-088

The penetration profiles of CS-088 across the isolated corneal membrane with and without EDTA/boric

Table 1
Lag time and apparent permeability coefficient (P_{app}) of CS-088 with penetration enhancers

Enhancer	Lag time (min)	P_{app} ($\times 10^{-6}$ cm/s)	Ratio[a]
Control	28.2 ± 1.3	1.32 ± 0.37	1.00
+0.005% EDTA	27.2 ± 3.0	1.30 ± 0.29	0.98
+1% Boric acid	29.9 ± 2.9	1.43 ± 0.40	1.08
+0.005% EDTA/1% boric acid	26.2 ± 4.5	2.03 ± 0.17	1.55*

Control solution (pH 7.0) contains 4% CS-088, 0.033% MP, and 0.018% PP.
[a] Ratio of P_{app} to control. Values represent means ± S.E. of three experiments.
* $P < 0.05$ significantly different from control.

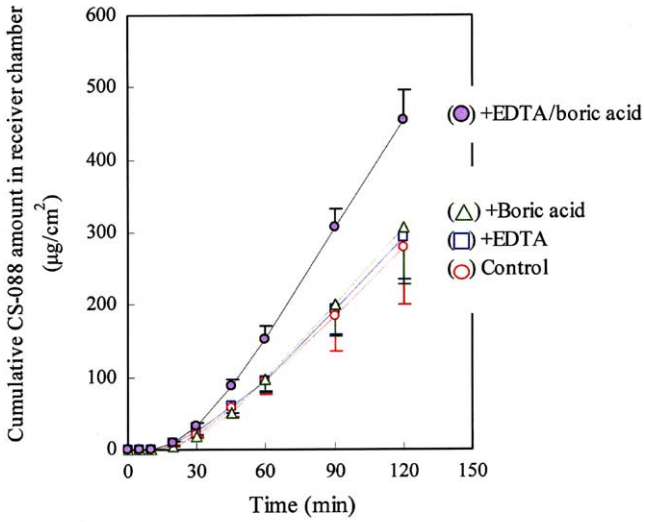

Fig. 2. Penetration profile of CS-088 across a cornea in the presence and absence of EDTA and boric acid. Each point represents the mean ± S.E. of three experiments.

acid are shown in Fig. 2. The lag time, P_{app}, and the ratio of P_{app} (P_{app} in the presence of EDTA/boric acid (test solutions) to that in the absence of EDTA/boric acid (control)) are summarized in Table 1. When EDTA and boric acid were individually administered, neither formulation had significant enhancing effects on the corneal permeability of CS-088. The ratio of P_{app} in the presence of either EDTA or boric acid was approximately 0.98 or 1.08, respectively. In contrast, when EDTA and boric acid were administered simultaneously, the permeability of CS-088 across the cornea was significantly enhanced by approximately 1.6-fold. CS-088 is a highly soluble compound and the solubility of CS-088 is not affected by addition of EDTA/boric acid. There is a good correlation between the corneal permeability of CS-088 and the concentrations of EDTA and boric acid as shown in Table 2. Keeping the EDTA concentration constant at 0.005%, it was clear that the permeability of CS-088 was enhanced in proportion to the concentration of boric acid in the range of 0–2%. At the highest boric acid concentration of 2%, the P_{app} for CS-088 was approximately 1.9-fold higher than that of the control. Similarly, when the boric acid concentration was kept constant at 1%, the ratio of P_{app} with respect to the control significantly increased by approximately 1.8-, 1.4-, and 2.5-fold at the EDTA concentrations of 0.0005, 0.005, and 0.05%, respectively. It is worthy to note that a significant enhancement was obtained even with a low concentration of EDTA (0.0005%) when 1% boric acid was administered simultaneously.

These results clearly indicate that the permeability-enhancing effects of EDTA/boric acid were synergistic. This result is in good agreement with that obtained

Table 2
Effect of EDTA and boric acid concentrations on P_{app} of CS-088

Enhancer	P_{app} ($\times 10^{-6}$ cm/s)	Ratio[a]
0.005% EDTA		
Control + boric acid	1.32 ± 0.25	1.00
0.1%	1.79 ± 0.50	1.36
0.5%	2.09 ± 0.26	1.59
1%	2.06 ± 0.38	1.56*
2%	2.56 ± 0.61	1.94*
1% Boric acid		
Control + EDTA	1.43 ± 0.27	1.00
0.0005%	2.61 ± 0.44	1.82*
0.005%	2.03 ± 0.38	1.42*
0.05%	3.59 ± 0.47	2.51**

Tested solutions (pH 7.0) contain 4% CS-088, 0.033% MP, and 0.018% PP. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from control.

[a] Ratio of P_{app} to control. Values represent means ± S.E. of three experiments.

Table 3
The effect of EDTA and boric acid on P_{app} of FD-4 K and TEER

Enhancer	Lag time (min)	P_{app} ($\times 10^{-6}$ cm/s)	Ratio[a]	TEER[b] (kΩ cm^2)	Ratio[c]
Control	35.2 ± 3.1	5.56 ± 0.57	1.00	2.51 ± 0.20	1.00
+EDTA	35.1 ± 3.1	6.83 ± 1.27	1.23	2.48 ± 0.29	0.99
+Boric acid	39.9 ± 6.2	5.05 ± 1.51	0.91	2.50 ± 0.18	1.00
+EDTA/boric acid	43.0 ± 2.2	6.30 ± 1.40	1.14	2.63 ± 0.23	1.05

Values represent means ± S.E. of three experiments.
[a] Ratio of P_{app} to control.
[b] TEER value at 2 h.
[c] Ratio of TEER to control.

from the in vivo pharmacological experiment in rabbits, where the intra-ocular pressure (IOP) was significantly reduced only with the simultaneous application of EDTA/boric acid (data not shown). The synergistic effect between EDTA and boric acid has not yet been reported.

3.2. Mechanism of the synergistic effect

FD-4K was used as a paracellular marker to estimate the effect of EDTA/boric acid on tight junctions. The results in Table 3 indicated that there were no comparable changes in P_{app} values between the FD-4K solutions containing EDTA/boric acid and those that did not (6.30 ± 1.40 and $5.56 \pm 0.57 \times 10^{-6}$ cm/s, respectively). In addition, to estimate the quantitative effect of EDTA/boric acid on the paracellular pathway, TEER measured in the presence of EDTA/boric acid was compared with that in the absence of EDTA/boric acid. The TEER values at 2 h and the ratio with respect to control values are summarized in Table 3. The TEER value in the absence of EDTA/boric acid was 2.51 ± 0.20 kΩ cm^2 (control). No significant change in TEER values with the addition of EDTA/boric acid was observed (2.48 ± 0.29 for EDTA, 2.50 ± 0.18 for boric acid, and 2.63 ± 0.23 kΩ cm^2 for EDTA/boric acid), suggesting that EDTA/boric acid had no significant effect on tight junctions.

Though EDTA has been generally considered to increase the paracellular permeability by depleting Ca^{2+} in the membrane and loosening tight junctions (Rojanasakul and Robinson, 1991; Harris et al., 1992), no significant change in the corneal penetration of FD-4 K and TEER was observed under the conditions employed here. This could be explained by the fact that the EDTA concentration used in this study (0.0005–0.05%) was extremely low compared with the higher 0.1% (Ashton et al., 1990; Madhu et al., 1996) and 0.5% (Podder et al., 1992; Sasaki et al., 1995a,b; Saettone et al., 1996) used in other reported studies. Thus, it was presumed that loosening of the tight junctions was not induced. On the other hand, the effects of EDTA on the integrity of the corneal membrane have also been investigated. Rojanasakul et al. (1990) demonstrated using confocal microscopic technologies that EDTA affects not only tight junctions but also membrane integrity in rabbits at a concentration of 0.04%. Moreover, Ashton et al. (1990) suggests that even small perturbations in the integrity of the corneal epithelium will result in significant changes in the P_{app} of hydrophilic compounds.

Based on these findings, it is likely that the permeability-enhancing effect of EDTA/boric acid was observed as a result of an improved transcellular permeability of CS-088.

To clarify the relative contribution of EDTA/boric acid on the outer layer (epithelium) and inner layers (stroma, endothelium) of the corneal membrane, the permeability-enhancing effect of EDTA/boric acid was compared between intact and de-epithelialized cornea. The TEER values of the corneal membranes markedly decreased from 2.51 ± 0.20 to 1.84 ± 0.16 k(cm^2 after removing the corneal epithelium (data not shown).

As a result, the permeability-enhancing effects of EDTA/boric acid were found to be much lower in the de-epithelialized cornea (1.06 ± 0.05 times) than that in the intact cornea (1.55 ± 0.13 times). Furthermore, de-epithelialization of the corneas produced a greater increase in the permeability of CS-088 than in the absence of EDTA/boric acid, and a P_{app} value 24-fold greater than that for the intact corneas was observed (Table 4). Previously, Huang et al. (1983) demonstrated that permeability-enhancing effects observed after re-

Table 4
Comparison of permeability-enhancing effects of EDTA and boric acid on intact and de-epithelialized cornea

Enhancer	P_{app} ($\times 10^{-6}$ cm/s)	Ratio[a]
Intact cornea		
Control	1.32 ± 0.37	1.00
+EDTA/boric acid	2.03 ± 0.17	1.55*
De-epithelialized cornea		
Control	30.9 ± 1.74	1.00(23.5)
+EDTA/boric acid	32.9 ± 1.65	1.06 (24.9)

Figures in parentheses represent the ratio of P_{app} to the control of the intact cornea.
[a] Ratio of P_{app} to control.
* $P < 0.05$ significantly different from control.

moving the corneal epithelium were much greater for hydrophilic β-blockers than lipophilic ones. CS-088 is a relatively hydrophilic compound with a partition coefficient of approximately −1.80 (logarithmic value between 1-octanol and pH 7.0 aqueous solution). Therefore, the 24-fold increase in permeability by the de-epithelialization is thought to be reasonable.

These results suggest that EDTA/boric acid synergistically enhances the transcellular permeability of CS-088 in the outer layer, but not in the inner layers of the corneal membrane.

The formation of a hydrophobic ion pair (HIP) could increase the partition coefficient of the drugs, possibly leading to the promotion of drug penetration. Therefore, the octanol-water partition coefficient of CS-088 was determined in the absence and presence of EDTA/boric acid. According to the results, no significant difference in the partition coefficient was observed between the absence (-1.80 ± 0.16) and presence of EDTA/boric acid (-1.69 ± 0.12 for EDTA, -1.61 ± 0.07 for boric acid, -1.68 ± 0.08 for EDTA/boric acid). From these results, it was presumed that EDTA/boric acid may induce synergistic enhancement on the corneal penetration of CS-088 by acting on the corneal membrane. However, the actual mechanism by which EDTA/boric acid synergistically enhances CS-088 penetration across a corneal membrane remains unclear. Further studies are now under way to investigate the cause.

In conclusion, through an in vitro permeability experiment, we demonstrated that EDTA/boric acid synergistically enhances the corneal penetration of CS-088 via the transcellular pathway. The advantage of the synergistic increase is that the amount of pharmaceutical additives required in a drug product as penetration enhancers can be reduced while maintaining the pharmacological activity, decreasing the risk of inducing adverse effects. Therefore, EDTA/boric acid is considered to be a safe enhancer and is expected to show similar effects in other drug formulations.

Acknowledgements

The authors wish to thank Yumiko Mori, Tatsuya Inoue, and Tomihisa Yokoyama of the Pharmacology and Molecular Biology Research Laboratories in our company for their technical assistance.

References

Ashton, P., Diepold, R., Platzer, A., Lee, V.H.L., 1990. The effect of chlorhexidine acetate on the corneal penetration of sorbitol from an arnolol formulation in the albino rabbit. J. Ocul. Pharmacol. 6, 37–42.

Chamber, O., Edman, P., 1987. Influence of some preservatives on the corneal permeability of pilocarpine and dexamethasone. In vitro Int. J. Pharm. 39, 29–234.

Durand, C.G., Duprat, P., Molon, N.S., Delort, P., Rozier, A., 1989. Corneal endothelial changes with azone, a penetration enhancer. Lens Eye Toxic. Res. 6, 109–117.

Grant, R.L., Yao, C., Gabaldon, D., Acosta, D., 1992. Evaluation of surfactant cytotoxicity potential by primary cultures of ocular tissues: I. Characterization of rabbit corneal epithelial cells and initial injury and delayed toxicity studies. Toxicology 76, 153–176.

Harris, D., Liaw, J.H., Robinson, J.R., 1992. Routes of delivery: case studies. (7) Ocular Delivery of Peptide and Protein Drugs. Adv. Drug Del. Rev. 8, 331–339.

Huang, H.S., Schoenwald, R.D., Lach, J.L., 1983. Corneal penetration behavior of β-blocking agents, II. Assessment of barrier contributions. J. Pharm. Sci. 72, 1272–1278.

Inoue, T., Yokoyama, T., Koike, H., 2001a. The effect of angiotensin II on uveoscleral outflow in rabbits. Curr. Eye Res. 23, 139–143.

Inoue, T., Yokoyama, T., Mori, Y., Sasaki, Y., Hosokawa, T., Yanagisawa, H., Koike, H., 2001b. The effect of topical CS-088, an angiotensin AT_1 receptor antagonist, on intraocular pressure and aqueous humor dynamics in rabbits. Curr. Eye Res. 23, 133–138.

Iwata, S., Ohtani, Y., Osada, E., Ogino, H., 1980. Aspect on corneal permeability of bupranolol. YAKUGAKU ZASSHI 100, 402–406.

Jean, M.D.S., Debbasch, C., Brignole, F., Rat, P., Warnet, J.-M., Baudouin, C., 2000. Toxicity of preserved and unpreserved antiglaucoma topical drugs in an in vitro model of conjunctival cells. Curr. Eye Res. 20, 85–94.

Madhu, C., Rix, P.J., Shackleton, M.J., Nguyen, T.G., Tang-liu, D., 1996. Effect of benzalkonium chloride/EDTA on the ocular bioavailability of Ketorolac tromethamine following ocular instillation to normal and de-epithelialized corneas of rabbits. J. Pharm. Sci. 85, 415–418.

Marsh, R.J., Maurice, D.M., 1971. The influence of non-ionic detergents and other surfactants on human corneal permeability. Exp. Eye Res. 11, 43–48.

Meaney, C.M., O'Driscoll, C.M., 2000. A comparison of the permeation enhancement potential of simple bile salt and mixed bile salt: fatty acid micellar systems using the CaCo-2 cell culture model. Int. J. Pharm. 207, 21–30.

Monti, D., Chetoni, P., Burgalassi, S., Najarro, M., Saettone, M.F., 2002. Increased corneal hydration induced by potential ocular penetration enhancers: assessment by differential scanning calorimetry (DSC) and by desiccation. Int. J. Pharm. 232, 139–147.

Podder, S.K., Moy, K.C., Lee, V.H.L., 1992. Improving the safety of topically applied timolol in the pigmented rabbit through manipulation of formulation composition. Exp. Eye Res. 54, 747–757.

Rojanasakul, Y., Liaw, J., Robinson, J.R., 1990. Mechanisms of action of some penetration enhancers in the cornea: laser scanning confocal microscopic and electrophysiology studies. Int. J. Pharm. 66, 131–142.

Rojanasakul, Y., Robinson, J.R., 1991. The cytoskeleton of the cornea and its role in tight junction permeability. Int. J. Pharm. 68, 135–149.

Saettone, M.F., Chetoni, P., Cerbai, R., Mazzanti, G., Braghiroli, L., 1996. Evaluation of ocular permeation enhancers: In vitro effects on corneal transport of four β-blockers, and in vitro/in vivo toxic activity. Int. J. Pharm. 142, 103–113.

Sasaki, H., Igarashi, Y., Nagano, T., Nishida, K., 1995a. Different effects of absorption promoters on corneal and conjunctival penetration of ophthalmic beta-blockers. Pharm. Res. 12, 1146–1150.

Sasaki, H., Nagano, T., Yamamura, K., Nishida, K., Nakamura, J., 1995b. Ophthalmic preservatives as absorption promoters for ocular drug delivery. J. Pharm. Pharmacol. 47, 703–707.

Sasaki, H., Yamamura, K., Tei, C., Nishida, K., Nakamura, J., 1995c. Ocular permeability of FITC-dextran with absorption promoter for ocular delivery of peptide drug. J. Drug Target 3, 129–135.