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Ocular delivery of the β-blocker, tilisolol, through the prodrug approach

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Summary

Four lipophilic derivatives of tilisolol, which is a β-blocker to decrease intraocular pressure after instillation, were synthesized. Hydrolytic regeneration of the parent compound, tilisolol, from the derivatives in vitro, in vitro ocular membrane penetration and in vivo ocular absorption were examined in order to evaluate their usefulness as prodrugs of tilisolol in rabbits. All derivatives, including *O*-acetyl, *O*-propionyl, *O*-butyryl and *O*-valeryl esters, showed increased lipophilicity due to the introduction of a lipophilic function. They were gradually hydrolyzed to tilisolol in a pH 7.4 buffer solution. They also showed rapid enzymatic conversion to tilisolol in ocular tissue homogenates. This rapid conversion was reflected in the enhanced penetration of tilisolol in vitro through corneal, conjunctival and scleral membranes. Most of the drug which penetrated through the ocular membranes was detected as tilisolol, not as intact derivative, in the receiver side. Conjunctival membranes showed higher permeability than corneal and scleral membranes. Corneal penetration of lipophilic derivatives was 3–6-fold higher than of tilisolol. The corneal permeability coefficients of drugs demonstrated a linear relationship with their lipophilicities. The lipophilic derivatives did not show significant difference from tilisolol in conjunctival and scleral penetration. The derivatives also increased the concentration of tilisolol in aqueous humor 1 h after instillation to rabbits. The linear relationship between in vivo absorption and in vitro corneal penetration of derivatives suggests that the corneal route, rather than the scleral pathway, contributes to the ocular absorption of drug after instillation. These results indicate the potential utility of lipophilic prodrug as an anti-glaucoma agent.

Introduction

Glaucoma is one of the most serious ocular diseases, which is characterized by high intraocular pressure, hardening of the eyeball and partial or complete loss of vision. This condition has continued to attract much attention in medical treatment. Recently, some β-blockers, which decrease aqueous humor formation by ciliary processes, have become commonly used and are very often indispensable (Görlich, 1987; Katz et al., 1987; Novack, 1987).

Upon instillation of an ophthalmic drug, most

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of the instilled amount is rapidly eliminated from the precorneal area due to drainage by the nasolacrimal duct and dilution by tear turnover, and is readily absorbed into systemic circulation (Chrai et al., 1973; Himmelstein et al., 1978; Lee and Robinson, 1986). Such behaviour results in poor bioavailability and increased severity in the systemic adverse effects of topically applied drugs. In fact, the usefulness of β-blocking agents in glaucoma has been limited by its relatively high incidence of cardiovascular and respiratory side effects (Van Buskirk, 1980; Nelson et al., 1986).

An effective approach to improving the ocular delivery of topically applied drugs and diminishing adverse effects is the use of a polymer vehicles (Saettone et al., 1986; Li and Robinson, 1989), liposomes (Taniguchi et al., 1988; Meisner et al., 1989), nanoparticles (Gurny et al., 1985; Diepold et al., 1989), or polymeric insert (Saettone et al., 1984; Urtti et al., 1990), which resist drug drainage from the precorneal area. Some additives having little toxicity were also reported to improve corneal permeability (Camber and Edman, 1987; Kato and Iwata, 1988). Another promising approach appears to be formulation of transient derivatives (prodrugs) to improve the ability of the drug for corneal penetration and reduce systemic drug load (Wei et al., 1978; Bundgaard et al., 1986; Chang et al., 1987; Bodor et al., 1988; Narurkar and Mitra, 1989; Chien and Schoenwald, 1990; Chien et al., 1991).

Tilisolol was synthesized as a non-selective and hydrophilic β-blocking agent and has been reported to reduce intraocular pressure after instillation (Nakagawa et al., 1984; Tadokoro et al., 1990). In the present study, we prepared four lipophilic derivatives of tilisolol and examined their in vitro conversion to tilisolol, in vitro ocular membrane penetration and in vivo ocular absorption in rabbits to evaluate their usefulness as prodrugs of tilisolol.

Materials and Methods

Materials

Tilisolol hydrochloride was a gift from Nisshin Flour Milling Co., Ltd (Tokyo, Japan). Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories (Richmond, U.S.A.). Salicyl methionine and salicyl tyrosine were prepared according to a routine method in our laboratory (Nakamura et al., 1992). All other chemicals were of reagent grade obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Melting points were determined using a micro-melting point apparatus (no. 80032, Mitamura Riken Kogyo Inc., Tokyo, Japan) and are uncorrected. NMR spectra were taken on a Hitachi R-600 FT-NMR spectrometer (Hitachi Co., Ltd, Tokyo, Japan) and elemental analyses were performed by the Center for Organic Elemental Micro-analysis, Nagasaki University. The analytical results obtained were within $\pm 0.4\%$ of the theoretical values. TLC was carried out on TLC aluminum sheets pre-coated with a 0.2 mm layer of silica gel 60 F_{254} (Merck, Darmstadt, Germany), using the following solvent system: chloroform-methanol-acetic acid (9 : 1 : 0.1, by vol.).

Male Nippon albino rabbits weighing 2.0–3.0 kg were used throughout the study. The animals were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co., Ltd., Tokyo, Japan). The rabbits were starved for 24 h prior to use for experiments but had free access to water.

Synthesis of derivatives

Tilisolol esters were prepared by reacting tilisolol hydrochloride with the fatty acid chloride. Tilisolol hydrochloride (2 g) was suspended in 15 ml of acetonitrile. The appropriate fatty acid chloride (0.7 ml) was added and the mixture was refluxed for 5–20 h. After cooling, the mixture was poured in ether, yielding a white precipitate. The precipitate was filtered and washed several times with ether. The white solid was dried in vacuo, then recrystallized with acetonitrile and ether. The structures of derivatives were confirmed by elemental and ^{1}H-NMR analyses.

Tilisolol: m.p. > 270°C; NMR (DMSO-d_6) δ: 9.10 (1H, br, HCl), 8.60 (1H, br, -CHCH$_2$N$\underline{\text{H}}$-), 7.40–8.40 (4H, m, aromatic protons), 7.25 (1H, s, 3-H), 5.99 (1H, d, $J = 5.0$ Hz, -OH), 4.30 (1H, br, -OCH$_2$C$\underline{\text{H}}$-), 4.01 (2H, d, $J = 5.0$ Hz, -OC$\underline{\text{H}}_2$CH-), 3.50 (3H, s, 2-N-C$\underline{H}_3$), 3.12 (2H, br, -CHC$\underline{H}_2$-NH-), 1.34 (9H, s, -NHC(C$\underline{H}_3$)$_3$).

O-Acetyl tilisolol (**I**): m.p. 123–126°C; NMR (DMSO-d_6) δ: 9.32 (1H, br, HCl), 8.78 (1H, br, -CHCH$_2$N$\underline{H}$-), 7.40–8.40 (4H, m, aromatic protons), 7.24 (1H, s, 3-H), 5.30–5.60 (1H, m, -OCH$_2$C$\underline{H}$-), 4.20 (2H, d, $J = 5.0$ Hz, -OC$\underline{H}_2$-CH-), 3.49 (3H, s, 2-N-C$\underline{H}_3$), 3.23 (2H, br, -CHC$\underline{H}_2$NH-), 2.11 (3H, s, -OCOC$\underline{H}_3$), 1.36 (9H, s, -NHC(C$\underline{H}_3$)$_3$).

O-Propionyl tilisolol (**II**): m.p. 198–200°C; NMR (DMSO-d_6) δ: 9.32 (1H, br, HCl), 8.78 (1H, br, -CHCH$_2$N$\underline{H}$-), 7.40–8.40 (4H, m, aromatic protons), 7.25 (1H, s, 3-H), 5.30–5.60 (1H, m, -OCH$_2$C$\underline{H}$-), 4.21 (2H, d, $J = 5.0$ Hz, -OC$\underline{H}_2$CH-), 3.49 (3H, s, 2-N-C$\underline{H}_3$), 3.23 (2H, br, -CHC$\underline{H}_2$NH-), 2.34 (2H, d, $J = 7.5$ Hz, -OCOC$\underline{H}_2$CH$_3$), 1.36 (9H, s, -NHC(C$\underline{H}_3$)$_3$), 1.05 (3H, t, $J = 7.5$ Hz, -OCOCH$_2$C$\underline{H}_3$).

O-Butyryl tilisolol (**III**): m.p. 173–175°C; NMR (DMSO-d_6) δ: 9.32 (1H, br, HCl), 8.78 (1H, br, -CHCH$_2$N$\underline{H}$-), 7.40–8.40 (4H, m, aromatic protons), 7.24 (1H, s, 3-H), 5.30–5.60 (1H, m, -OCH$_2$C$\underline{H}$-), 4.22 (2H, d, $J = 4.0$ Hz, -OC$\underline{H}_2$-CH-), 3.49 (3H, s, 2-N-C$\underline{H}_3$), 3.23 (2H, br, -CHC$\underline{H}_2$NH-), 2.34 (2H, d, $J = 7.5$ Hz, -OCOC$\underline{H}_2$CH$_2$CH$_3$), 1.20–1.60 (2H, m, -OCOCH$_2$C$\underline{H}_2$CH$_3$), 1.36 (9H, s, -NHC(C$\underline{H}_3$)$_3$), 0.87 (3H, t, $J = 7.0$ Hz, -OCOCH$_2$CH$_2$C$\underline{H}_3$).

O-Valeryl tilisolol (**IV**): m.p. 86–89°C; NMR (DMSO-d_6) δ: 9.36 (1H, br, HCl), 8.84 (1H, br, -CHCH$_2$N$\underline{H}$-), 7.40–8.40 (4H, m, aromatic protons), 7.24 (1H, s, 3-H), 5.30–5.60 (1H, m, -OCH$_2$C$\underline{H}$-), 4.17 (2H, d, $J = 5.0$ Hz, -OC$\underline{H}_2$-CH-), 3.48 (3H, s, 2-N-C$\underline{H}_3$), 3.23 (2H, br, -CH-C$\underline{H}_2$NH-), 2.29 (2H, d, $J = 7.0$ Hz, -OCO-C$\underline{H}_2$(CH$_2$)$_2$CH$_3$), 1.10–1.70 (4H, m, -OCOCH$_2$-(C$\underline{H}_2$)$_2$CH$_3$), 1.36 (9H, s, -NHC(C$\underline{H}_3$)$_3$), 0.81 (3H, t, $J = 6.5$ Hz, -OCOCH$_2$(CH$_2$)$_2$C$\underline{H}_3$).

Measurement of lipophilicity

Apparent partition coefficients of compounds were determined in a 1-octanol and 50 mM phosphate buffer (pH 7.4) system (1:1, v/v) after shaking for 5 min at 22 ± 1°C. Before use, 1-octanol and buffer solution were saturated with the relevant aqueous or organic phase. Apparent partition coefficients were calculated from drug concentrations in the aqueous phase before and after addition of 1-octanol. The initial concentration of compounds was 0.2 or 1.0 mM in buffer.

The lipophilicity indices (log k') were determined by an HPLC system (LC-6A, Shimadzu Co., Ltd, Kyoto, Japan) with a stationary phase of a Cosmosil 5C$_{18}$-P packed column (150 mm length × 4.6 mm i.d., Nacalai Tesque Inc.) and a mobile phase of methanol and 50 mM NaH$_2$PO$_4$ (40:60, v/v) at a flow rate of 1.0 ml/min. Tilisolol and its derivatives were detected fluorometrically (excitation wavelength 315 nm, emission wavelength 420 nm; RF-530, Shimadzu Co., Ltd). The indices were calculated from the equation: $\log k' = \log[(t_r - t_0)/t_0]$, where t_r is the retention time and t_0 denotes the elution time of the solvent.

Hydrolysis experiment

Stock solutions of all compounds were prepared in dimethylsulfoxide at a concentration of 2 mM and an appropriate volume was mixed with biological media for hydrolysis study.

20 rabbits were killed with an overdose of a sodium pentobarbital solution administered via a marginal ear vein to obtain cornea, conjunctiva, iris-ciliary body and aqueous humor. The ocular tissues were homogenized at 0–5°C in a glass-teflon homogenizer to prepare a homogenate with 1.17% KCl, then centrifuged at 1000 × *g* for 20 min, and the supernatant was diluted to 2 mg protein/ml concentration with 1.17% KCl. Intact aqueous humor was used without dilution (0.368 mg protein/ml). The protein concentration was determined by Bio-Rad protein assay. Instead of homogenate, 1.17% KCl solution was used as a control. Aliquots of homogenates were boiled for 5 min and used for hydrolysis experiments as a boiled homogenate. In order to examine hydrolysis in aqueous humor, the aqueous humor sample was separated into high and low molecular weight fractions by ultrafiltration (Centricon-10, molecular weight cut-off size, 10000; Amicon, Lexington, U.S.A.). There was no protein in the low molecular weight fraction. An aliquot of 1.17% KCl was added to the high molecular weight fraction and separated by ultrafiltration again to remove the low molecular weight content. This

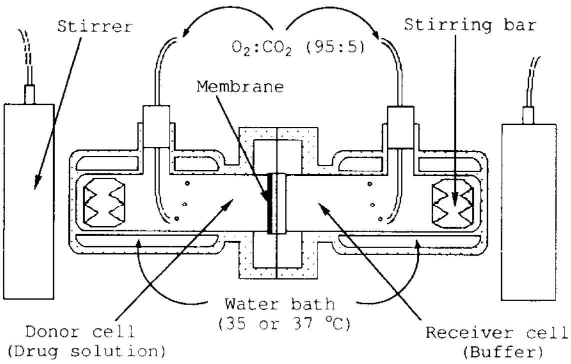


Fig. 1. Diffusion chamber for in vitro penetration experiment.

procedure was carried out three times. Subsequently, the high molecular weight fraction was diluted to 0.368 mg protein/ml with 1.17% KCl.

An equal volume of pH 7.4 isotonic phosphate buffer was mixed with biological samples and used for experiments. Degradation experiments were performed at 37 ± 0.2°C and initiated by adding the stock solution to give a concentration of 0.1 mM. At appropriate time intervals, aliquots of the solution were withdrawn for subjecting to HPLC analysis.

In vitro penetration experiment

The glass apparatus for in vitro diffusion was designed with modifications from an ordinary diffusion chamber (Fig. 1). This apparatus has a pocket for a ring mounted with ocular membranes. The mounting device and corneal ring were the used as same as described by Schoenwald and Huang (1983). Rabbits were killed with an overdose of sodium pentobarbital solution administered via a marginal ear vein. The whole eye was enucleated from its socket and placed on the mounting device. Rabbit corneas were excised and mounted in the ring on the device. The ring with the membrane was mounted in the modified Ussing chambers. Conjunctival and scleral membranes were mounted on the diffusion cell using a similar technique. Fat and connective tissues from the serosal side of the orbital portion of the palpebral conjunctiva were cut off for making a thin conjunctival membrane before mounting in the chambers.

Glutathione bicarbonated Ringer's (GBR) solution was used throughout the diffusion studies (Schoenwald and Huang, 1983). The solution was prepared in two parts: the first part contained sodium chloride (12.4 g/l), potassium chloride (0.716 g/l), monobasic sodium phosphate monohydrate (0.206 g/l), and sodium bicarbonate (4.908 g/l); the second part contained calcium chloride dihydrate (0.230 g/l), magnesium chloride hexahydrate (0.318 g/l), glucose (1.80 g/l), and oxidized glutathione (0.184 g/l). Equal parts of both solutions were mixed prior to use.

4 ml of drug-free GBR solution was added to the endothelial side (receiver side) of the diffusion chamber. 4 ml of the drug-containing solution (5 mM) was then added to the epithelial side (donor side). The contents of each chamber were stirred gently and bubbled with a 95% O_2/5% CO_2 mixture. The diffusion apparatus was jacketed to maintain the ocular membrane, donor solution and receiver solution at 35 ± 0.5°C (cornea) and 37 ± 0.5°C (conjunctiva and sclera). At appropriate time intervals, a sample (50 μl) was withdrawn from the receiver side and immediately replaced by an equal volume of GBR solution. The sample was assayed for tilisolol and its derivatives with HPLC. An epithelial layer of cornea was scraped with a surgical knife and the penetration of tilisolol through the treated cornea was examined. Penetration of tilisolol through the cornea from the endothelium to the epithelium was also studied. The apparent permeability coefficient (P_{app}) and lag time were calculated from the slope and x-intercept of the linear portion of a plot of accumulated drug amount (tilisolol and derivative) in the receiver cell vs time.

After experiments, the water content of the cornea was determined from the weight difference before and after drying overnight in an oven at 60°C. Corneas hydrated more than 83% were considered as damaged (Grass and Robinson, 1988). The procedure followed for preparing ocular membranes was that of Lee et al. (1988).

In vivo ocular absorption experiment

Unanesthetized rabbits were kept in a prone position on a wooden plate. Drug solutions of 25 μl (30 mM) in pH 7.4 phosphate-buffered saline were instilled directly onto the corneas of rabbits. A rabbit was killed by intravenous administration of an overdose of a sodium pentobarbital solution at an appropriate time after instillation and its aqueous humor (150–200 μl) was collected with a syringe. The sample was subjected to HPLC assay.

Analysis

The homogenate sample for the hydrolysis experiments (50 μl), the sample for the in vitro penetration experiment (50 μl) and the aqueous humor for the in vivo absorption experiment (50 μl) were mixed with 0.1 M HCl (50 μl) and methanol (100 μl) including the internal standard (300 μg/ml *O*-ethoxybenzamide for tilisolol, 200 μg/ml salicyl methionine for **I**, **III** and **IV**, 20 μg/ml salicyl tyrosine for **II**). The mixture was centrifuged at 12000 × *g* for 10 min and 50 μl of the supernatant was injected to HPLC.

An HPLC system (LC-6A, Shimadzu Co., Ltd) equipped with a fluorescence spectromonitor (RF-530, Shimadzu Co., Ltd) was used in reverse-phase mode. Excitation wavelength was 315 nm and emission wavelength was monitored at 420 nm. The stationary phase used was Cosmosil 5C_{18}-P packed column (150 mm length × 4.6 mm i.d., Nacalai Tesque Inc., for tilisolol, **I** and **II**) and Cosmosil 5C_8-300 packed column (100 mm length × 4.6 mm i.d., Nacalai Tesque Inc., for **III** and **IV**). A mixture of methanol and 50 mM NaH_2PO_4 (40:60, v/v) was used as the mobile phase at a flow rate of 1.0 ml/min.

Results and Discussion

Physicochemical property of tilisolol derivatives

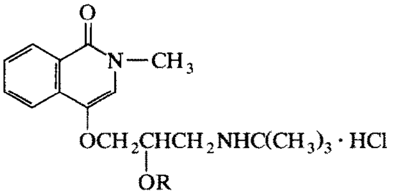
Tilisolol derivatives were prepared by acylation of tilisolol with acyl chloride in acetonitrile. These were *O*-acetyl (**I**), *O*-propionyl (**II**), *O*-butyryl (**III**) and *O*-valeryl tilisolol (**IV**). Their structures and physicochemical properties are summarized in Table 1. All derivatives showed increased lipophilicity, as expected, due to the introduction of a lipophilic function. The logarithmic values of the apparent partition coefficient varied between 1.022 and 2.471. The greater lipophilicity of the derivatives compared to tilisolol was also reflected in the relative lipophilicity index (log *k*′), in HPLC and the R_f value in TLC.

Chemical and enzymatic hydrolysis

The chemical and enzymatic hydrolysis of tilisolol derivatives was examined in pH 7.4 buffer, aqueous humor and ocular tissue homogenates at 37°C. Fig. 2 shows the time course of hydrolysis of **IV** to tilisolol in iris-ciliary body homogenate, as an example of rapid conversion. The simultaneous disappearance of derivatives and appear-

TABLE 1

Structure and physicochemical properties of tilisolol and its derivatives



Compound	R	Mol. Wt	Log PC [a]	Log *k*′ [b]	R_f [c]
Tilisolol	-H	340.8	−0.273	−0.298	0.17
I	$-COCH_3$	382.7	1.022	0.002	0.21
II	$-COCH_2CH_3$	396.7	1.563	0.291	0.25
III	$-COCH_2CH_2CH_3$	410.7	2.024	0.599	0.27
IV	$-COCH_2CH_2CH_2CH_3$	424.8	2.471	0.948	0.29

[a] Logarithmic value of the apparent partition coefficient (PC) between 1-octanol and pH 7.4 phosphate buffer at 22 ± 1°C.

[b] The relative lipophilicity indices (log *k*′) were determined by HPLC (see text).

[c] R_f values were determined by TLC with a solvent of chloroform, methanol and acetic acid (9:1:0.1, by vol.).

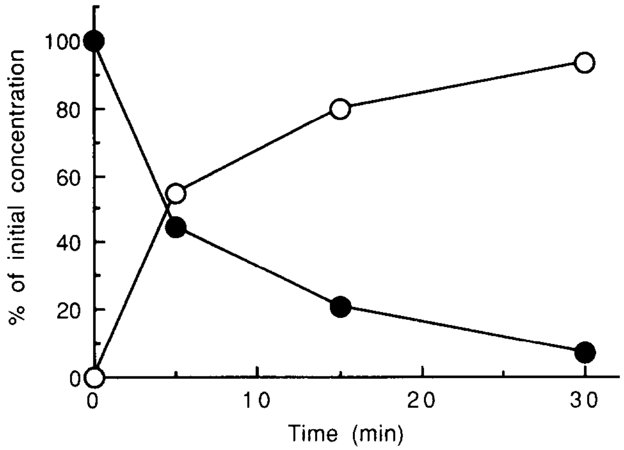


Fig. 2. Hydrolysis of **IV** (●) and appearance of tilisolol (○) in iris-ciliary body homogenate. Each point represents the average of three experiments and S.E. are smaller than the symbols themselves.

ance of tilisolol was observed. Degradation of tilisolol was barely detectable in any case for 0.5 h.

The percentage remaining of derivatives in biological media was plotted vs time on a semilogarithmic scale and the hydrolysis rate constant was calculated from the slope. The results are summarized in Table 2. The derivatives were gradually hydrolyzed to tilisolol in pH 7.4 buffer. Addition of ocular tissue samples significantly accelerated the hydrolysis of most derivatives. In the homogenate of cornea, conjunctiva and iris-ciliary body, **IV** was most sensitive to enzymatic hydrolysis and **I** was most resistant. In compounds **II–IV**, iris-ciliary body homogenate showed the most extensive catalysis. All derivatives were rapidly hydrolyzed to tilisolol in aqueous humor regardless of a low protein concentration. The homogenate boiled for 5 min to denature the enzyme demonstrated a markedly decreased hydrolysis rate constant relative to intact homogenate, except for aqueous humor.

Aqueous humor is a clear, lymph-like fluid that fills the space between the cornea and the lens of the eye. Lee (1983) investigated esterase activities in rabbit eyes and demonstrated a low activity in aqueous humor. In the present study, however, aqueous humor showed high activity on derivative hydrolysis and the activity still remained after boiling of aqueous humor. These results suggest that the hydrolysis activity of aqueous humor on derivatives may not be due to the enzyme. Therefore, aqueous humor was separated into two fractions, high and low molecular weight fractions, using ultrafiltration (molecular weight cut-off size, 10 000). The hydrolysis rate

TABLE 2

Stability of tilisolol and its derivatives in a buffer and ocular tissue homogenates

Compound	Hydrolysis rate constant $(\times 10^{-3})$ (min^{-1}) [a]				
	Buffer [b]	Cornea	Conjunctiva	ICB [c]	Aqueous humor
Tilisolol	– [d]	– [d]	– [d]	– [d]	– [d]
I	6.75 ± 0.13	8.01 ± 0.38 [e]	7.67 ± 0.29 [e]	7.65 ± 0.36	19.32 ± 0.61 [f]
		(5.64 ± 0.27)	(5.63 ± 0.39)	(5.45 ± 0.40)	(19.12 ± 0.81)
II	8.96 ± 0.22	13.22 ± 0.30 [f]	16.83 ± 0.10 [f]	17.26 ± 0.66 [f]	25.40 ± 0.92 [f]
		(6.12 ± 0.67)	(7.78 ± 0.89)	(6.49 ± 0.03)	(19.83 ± 0.06)
III	4.84 ± 0.29	10.65 ± 0.22 [f]	18.71 ± 1.13 [f]	20.28 ± 1.71 [f]	16.38 ± 1.13 [f]
		(4.40 ± 0.50)	(4.04 ± 0.55)	(3.22 ± 0.26)	(13.11 ± 0.63)
IV	3.63 ± 0.51	15.84 ± 0.30 [f]	28.49 ± 1.18 [f]	36.92 ± 0.42 [f]	17.19 ± 1.70 [f]
		(3.18 ± 0.30)	(3.81 ± 0.17)	(3.65 ± 0.16)	(11.43 ± 1.42)

[a] Hydrolysis rate constant was determined in a mixture of homogenate and pH 7.4 isotonic phosphate buffer at 37 ± 0.2°C.

[b] Instead of homogenate, 1.17% KCl solution was used.

[c] Iris-ciliary body.

[d] Degradation of tilisolol was barely detectable for 0.5 h. Significant difference (Student's *t*-test) from buffer: [e] $p < 0.05$, [f] $p < 0.01$. Each value is the mean ± S.E. of at least three experiments. The value in parentheses is the hydrolysis rate constant in a boiled homogenate.

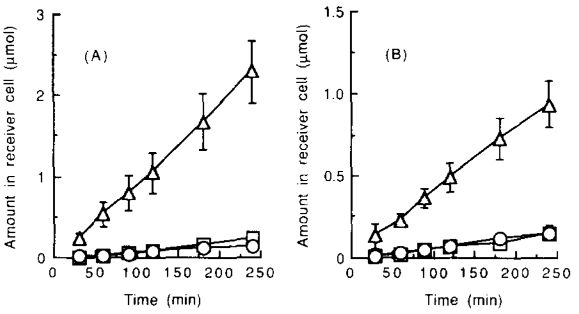


Fig. 3. Penetration of tilisolol through ocular membranes (A) and treated cornea (B). (A) (○) Cornea, (△) conjunctiva, (□) sclera. (B) (○) Cornea, (△) cornea scraping its epithelial layer, (□) tilisolol penetration from endothelium to epithelium when drug solution was added to endothelial side. Each point represents the average of at least three experiments and the vertical bar denotes the S.E.

constants of **I** in the high and low molecular weight fractions were 8.65 ± 0.59 ($\times 10^{-3}$, min^{-1}) and 20.38 ± 0.51 ($\times 10^{-3}$, min^{-1}), respectively. Thus, the hydrolysis of derivatives to tilisolol was accelerated by low molecular weight contents, not enzyme in aqueous humor.

These hydrolysis results indicate that the derivatives are suitable prodrugs of tilisolol, which undergo chemical and enzymatic hydrolysis to tilisolol, in the eye after instillation.

Corneal, conjunctival and scleral penetration of tilisolol derivatives

Penetration of tilisolol through ocular membranes was examined in in vitro experiments and is shown in Fig. 3 (A and B). Tilisolol exhibited considerable penetration through conjunctival membranes and low penetration through corneal and scleral membranes (Fig. 3A). As shown in Fig. 3B, the penetration of tilisolol from the endothelium to epithelium was almost the same as that from the epithelium to endothelium, suggesting a contribution by simple diffusion to the penetration of tilisolol through the cornea. The corneal composite structure is indicated by three primary layers: the epithelium, stroma and endothelium. Among them, epithelium, being lipoidal in nature, is considered to contribute to the corneal penetration barrier of particularly

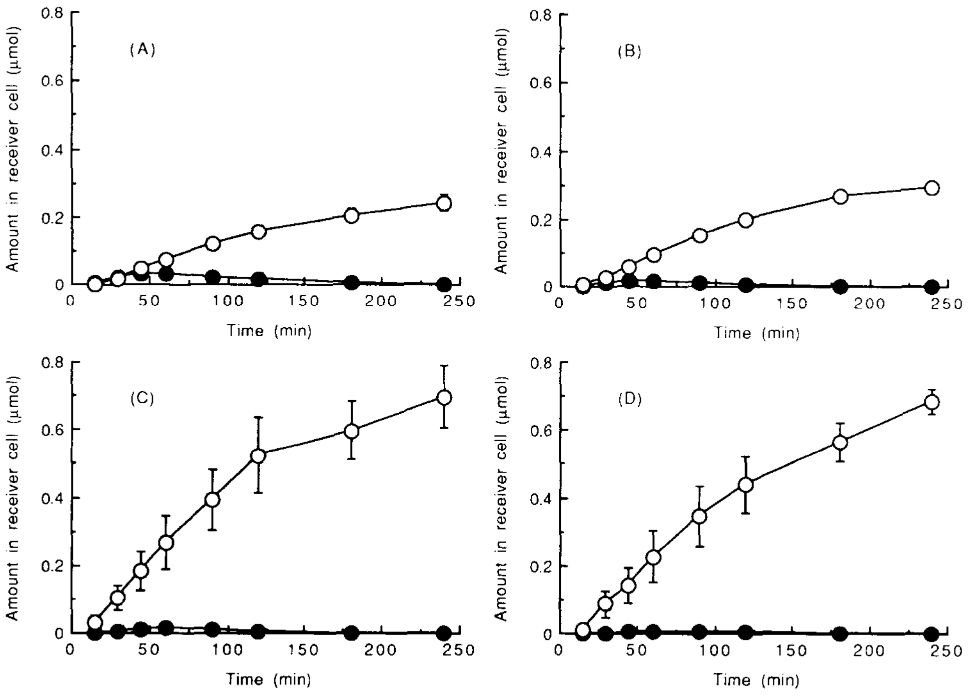


Fig. 4. Corneal penetration of **I** (A), **II** (B), **III** (C) and **IV** (D). (●) Intact derivative, (○) tilisolol. Each point represents the average of four experiments and the vertical bar denotes the S.E.

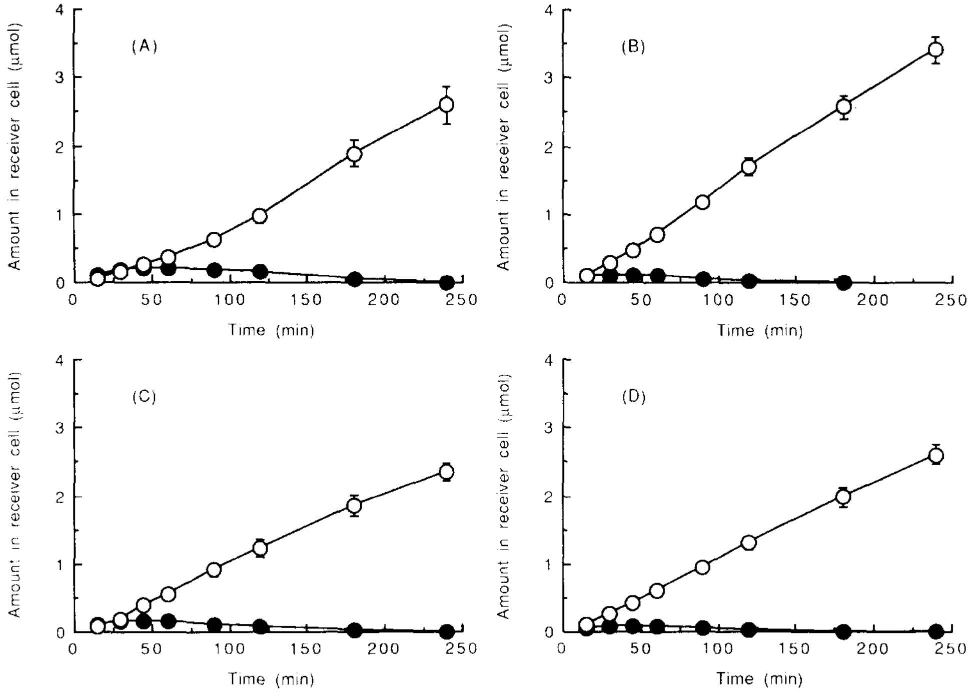


Fig. 5. Conjunctival penetration of **I** (A), **II** (B), **III** (C) and **IV** (D). (●) Intact derivative, (○) tilisolol. Each point represents the average of at least four experiments and the vertical bar denotes the S.E.

hydrophilic drugs (Huang et al., 1983; Chien et al., 1988). In fact, scraping the epithelium markedly increased the corneal penetration of tilisolol (Fig. 3B).

The corneal penetration profiles of **I**–**IV** are shown in Fig. 4A–D. Most of the drug in the receiver cell was detected as tilisolol, not as the intact derivative. These results demonstrate the rapid conversion of derivatives to tilisolol in cornea. All derivatives showed greater penetration than that of tilisolol.

Figs 5A–D and 6A–D depict the conjunctival and scleral penetration of derivatives. Most of drug in the receiver cell was also detected as the parent drug. Conjunctival penetration was much more extensive than corneal and scleral penetration.

The apparent permeability coefficient (P_{app})

TABLE 3

Apparent lag time and permeability coefficient (P_{app}) of tilisolol and its derivatives through ocular membranes

Compound	Cornea			Conjunctiva			Sclera		
	N [a]	Lag time (min)	P_{app} ($\times 10^{-5}$) (cm/s)	N	Lag time (min)	P_{app} ($\times 10^{-5}$) (cm/s)	N	Lag time (min)	P_{app} ($\times 10^{-5}$) (cm/s)
Tilisolol	8	18.0 ± 3.7	0.272 ± 0.037	6	11.5 ± 7.8	4.363 ± 0.884	6	43.4 ± 2.2	0.501 ± 0.035
I	4	12.1 ± 0.5	0.885 ± 0.076 [b]	4	−2.5 ± 1.4	5.106 ± 0.505	6	37.9 ± 3.1	0.455 ± 0.040
II	4	12.6 ± 1.1	0.842 ± 0.068 [b]	4	1.1 ± 1.0	6.596 ± 0.586	4	32.5 ± 6.6	0.540 ± 0.068
III	4	17.3 ± 1.4	1.512 ± 0.067 [b]	4	2.6 ± 0.8	6.559 ± 0.525	4	54.1 ± 11.7	0.477 ± 0.073
IV	4	21.7 ± 1.7	1.324 ± 0.053 [b]	6	−0.1 ± 1.9	5.603 ± 0.481	5	30.2 ± 2.1	0.573 ± 0.004

[a] Number of trials. Significant difference in lag time and P_{app} (Student's t-test) from tilisolol: [b] $p < 0.001$. Each value is the mean ± S.E.

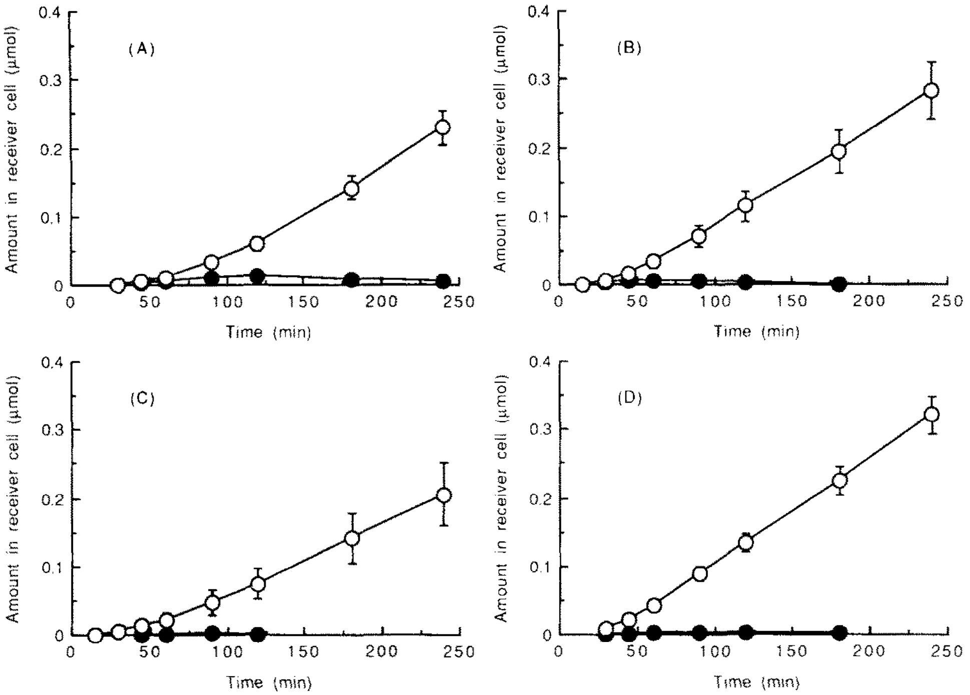


Fig. 6. Scleral penetration of **I** (A), **II** (B), **III** (C) and **IV** (D). (●) Intact derivative, (○) tilisolol. Each point represents the average of at least four experiments and the vertical bar denotes the S.E.

and lag time of tilisolol and its derivatives through ocular membranes were estimated from the slope and x-intercept of the linear portion of plots of accumulated drug amount in the receiver cell vs time. The results are summarized in Table 3. In the case of corneal penetration, **III** resulted in 5.6-fold greater penetration than tilisolol. Other derivatives also showed greater penetration than tilisolol (**IV**, 4.9-fold; **I**, 3.3-fold; **II**, 3.1-fold). On the other hand, conjunctival and scleral penetration of derivatives was not significantly increased.

The lag time for conjunctival penetration was shortest among ocular membranes, reflecting the characteristics of having the greatest permeability. Scleral penetration showed a relatively longer lag time because of its greater thickness.

Fig. 7 shows the relationship between logarithmic values of the apparent permeability and partition coefficients. Corneal permeability demonstrated a linear relationship with lipophilicity. The logarithmic values of compounds (log PC) ranged from -0.273 to 2.471. Over a wide range of lipophilicity, various compounds showed a parabolic relationship between corneal permeability and lipophilicity (Schoenwald and Huang, 1983; Chien et al., 1988; Suhonen et al., 1991). The optimal logarithmic values of their partition coefficients for corneal permeability have been determined to be higher than 2.5.

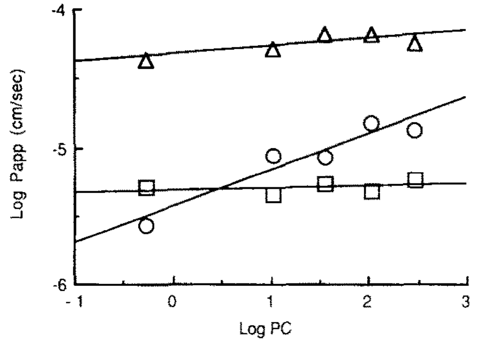


Fig 7. Relationship between logarithmic values of apparent partition coefficient (PC) and permeability coefficient (P_{app}) of tilisolol and its derivatives. (○) Cornea, $\log P_{app} = 0.263 \times \log PC - 5.436$, $r = 0.949$; (△) conjunctiva; (□) sclera. Each point represents the average of at least four experiments.

Chien et al. (1991) also separated a parabolic relationship between the conjunctival permeability and lipophilicity of timolol produgs. However, the conjunctival and scleral penetration of derivatives was not susceptible to the degree of lipophilicity in the present study.

The results of in vitro penetration experiments indicate the different characteristics of the three ocular membranes on drug penetration. The cornea plays the role of a lipophilic barrier against drug penetration, especially a hydrophilic one. The barrier is mainly due to the corneal epithelial layer. Therefore, an increase in drug lipophilicity through the prodrug approach is extremely useful for improving drug permeation.

The conjunctiva is a more leaky membrane than the cornea. Huang et al. (1989) demonstrated an intrinsic difference in paracellular permeability between the conjunctival and corneal tissues. They showed that the conjunctiva was permeable to molecules larger than 20 kDa and that permeation of [^{3}H]mannitol was 55-fold greater than the cornea. The conjunctival paracellular permeability of the present derivatives may explain the extensive penetration without a lag time, regardless of the lipophilicity of the various derivatives.

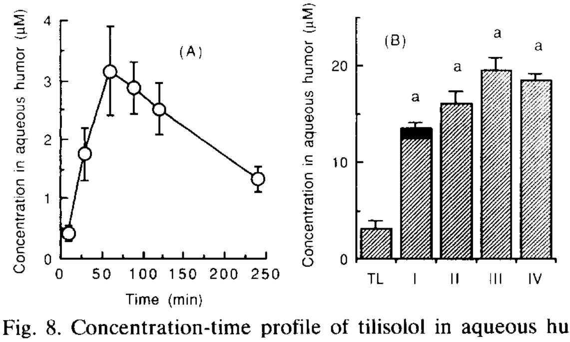


Fig. 8. Concentration-time profile of tilisolol in aqueous humor after its instillation (A) and concentration of drug in aqueous humor 1 h after instillation of tilisolol and its derivatives (B). (A) Each point represents the average of at least three experiments and the vertical bar denotes the S.E. (B) (■) Intact derivative, (▨) tilisolol. TL, tilisolol. Significant difference (Student's t-test) from TL: ^{a} $p < 0.001$. Each value represents the average of at least four experiments and the vertical bar denotes the S.E.

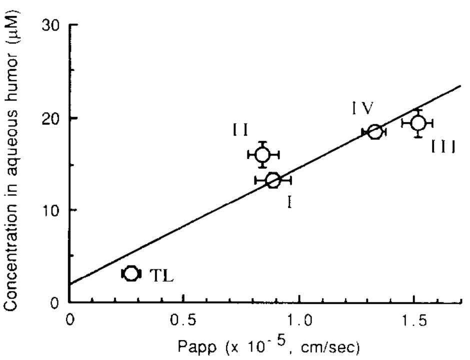


Fig 9. Relationship between apparent corneal permeability coefficient (P_{app}) and drug concentration in aqueous humor. Concentration in aqueous humor = $12.886 \times P_{\mathrm{app}} \times 10^5 + 1.697$, $r = 0.941$. TL, tilisolol. Each point represents the average of at least four experiments, and the vertical and horizontal bars denote the S.E.

The sclera is a tough and thick fibrous tissue. The long lag time for scleral penetration is due to its thickness. Since most derivatives will be hydrolyzed to tilisolol in the donor cell and the scleral tissue during the lengthy lag period, the derivatives may not be able to improve the penetration of tilisolol. Alternatively, most of the drug may permeate through hydrophilic pore routes or vessels in fibrous tissue.

In vivo ocular absorption

The concentration profile of tilisolol in aqueous humor after instillation is illustrated in Fig. 8A. Tilisolol showed a maximum concentration of 3.141 μM at 1 h and gradually decreased. The aqueous humor concentration of tilisolol and its derivatives 1 h after instillation is shown in Fig. 8B. Most of the drug was detected as tilisolol, not as the intact derivative. All derivatives significantly increased the concentration of tilisolol in the aqueous humor (**III**, 6.2-fold; **IV**, 5.9-fold; **II**, 5.1-fold; **I**, 4.0-fold).

Chang et al. (1987) and Luo et al. (1991) demonstrated a close correlation between corneal penetration and drug concentration in the aqueous humor after instillation. In fact, a close correspondence was observed between the apparent corneal permeability coefficient (in vitro) and aqueous humor concentration of drug (in vivo) (Fig. 9). This result suggests that the corneal route, rather than the scleral route, contributes to the ocular absorption of drug after instillation.

In vitro penetration experiments showed that the corneal tissue sufficed to hydrolyze most of the derivative to tilisolol during permeation (Fig. 4). In Fig. 8B, derivative **I**, which was more resistant to hydrolysis than the other derivatives in corneal tissue, was detected as intact (7%) in aqueous humor. These results suggest that tilisolol in aqueous humor after instillation of derivatives is mainly due to hydrolysis in corneal tissue during permeation.

Thus, from the present study, we conclude that the lipophilicity and hydrolysis of prodrugs are important factors that enhance corneal penetration and improve drug concentration in aqueous humor. Although further work is necessary to determine the pharmacological effects and toxicity, the present derivatives are potentially useful prodrugs of tilisolol in improving ocular drug delivery.

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