Role of Enzymatic Lability in the Corneal and Conjunctival Penetration of Timolol Ester Prodrugs in the Pigmented Rabbit

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The main objective of this study was to investigate how enzymatic lability would affect the extent of corneal and conjunctival penetration of a series of alkyl, cycloalkyl, and aryl ester prodrugs of timolol in the pigmented rabbit. Enzymatic lability of the prodrugs was studied in corneal epithelial and conjunctival homogenates, while their corneal and conjunctival penetration was determined using the isolated tissues in the modified Ussing chamber. The straight-chain alkyl and the unsubstituted cycloalkyl esters were hydrolyzed more rapidly than their corresponding branched-chain and substituted analogues as well as the aryl esters. The corneal and conjunctival penetration of all prodrugs, regardless of enzymatic lability, varied parabolically with lipophilicity. Moreover, the enzymatically more labile straight-chain alkyl esters penetrated the cornea and the conjunctiva more readily than the more stable branched-chain esters of comparable lipophilicity. Enzymatic lability is, therefore, an additional factor that should be considered in designing alkyl ester prodrugs with improved ocular drug delivery characteristics. Enzymatic lability does not, however, play as important a role as lipophilicity in the corneal and conjunctival penetration of cycloalkyl and aryl ester prodrugs.

KEY WORDS: prodrugs; timolol; enzymatic lability; lipophilicity; corneal penetration; conjunctival penetration.

INTRODUCTION

Timolol is a nonselective β-adrenergic antagonist widely used in the treatment of open-angle glaucoma. About 25% of the patients cannot, however, tolerate treatment because of cardiovascular and respiratory complications (1). Since 1987, we have been investigating the use of prodrugs to improve the corneal penetration of timolol, thereby reducing the required dosage and incidence of systemic side effects (2–5). *O*-Butyryl timolol, which is 50 times more lipophilic than timolol, was absorbed into the aqueous humor four to five times better than timolol, while yielding similar plasma timolol concentrations (2). Further, *O*-valeryl timolol and *O*-pivaloyl timolol, which possess similar lipophilic characteristics but different degrees of susceptibility to esterase-mediated hydrolysis, were absorbed into the aqueous humor and plasma to different extents. This finding suggests that, in addition to lipophilicity, susceptibility to hydrolysis may influence the extent of prodrug absorption, a possible factor which has not been considered previously. Thus, the main objective of this study was to investigate how enzymatic lability could affect the extent of corneal and conjunctival penetration of a series of alkyl, cycloalkyl, and aryl ester prodrugs of timolol in the pigmented rabbit. These prodrugs are listed in Table I, and their structures are shown in Scheme I. Another objective of this study was to determine how prodrug derivatization could affect the ratio of corneal-to-conjunctival penetration of timolol and, by virtue of their respective involvement in ocular and systemic absorption (6,7), affect the ratio of ocular-to-systemic timolol absorption.

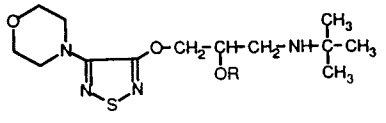
MATERIALS AND METHODS

Materials

Male pigmented rabbits, weighing about 2 kg, were purchased from Irish Farm Rabbitry (Los Angeles, CA). Timolol maleate was obtained from Sigma Chemicals (St. Louis, MO) or kindly donated by LEO Pharmaceuticals (Copenhagen, Denmark). Timolol ester prodrugs as hydrochloride or fumarate salts were synthesized as described by Bundgaard *et al.* (4,8). All other chemicals were either HPLC or reagent grade and were used as received.

Assay

Timolol and its prodrugs were quantitated using reversed-phase HPLC on a Beckman Ultrasphere ODS column (25 cm × 4.6 mm; particle size, 5 μm) fitted with a Brownlee Labs Newguard precolumn (1.5 cm), as previously described (3). The HPLC system consisted of a SCL-6A system controller, two LC-6A pumps, a SIL-6A autoinjector, a SPD-6A spectrophotometric detector, and a CR-3A integrator (Shimadzu Instruments, Baltimore, MD). The mobile phase was a mixture of acetonitrile and water containing 1% triethylamine HCl at pH 3. The proportion of acetonitrile in the mobile phase was increased linearly from 23 to 60% for the first 10 min and kept at 60% for the next 9 min. The flow rate was 1 ml min^{-1}. Propranolol hydrochloride (25 μg ml^{-1}) served as the internal standard. Timolol and its prodrugs were monitored at 294 nm. The retention time of ti-



I: R = H

II: R = $-\overset{O}{\overset{\|}{C}}-R'$

Scheme I

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Table I. Pseudo First-Order Rate Constants (k) for the Hydrolysis of Various Timolol Prodrugs in Buffer Solution (pH 7.4) and Homogenates of the Corneal Epithelium and the Conjunctiva of the Pigmented Rabbit at 37°C

No.	Prodrug	k (10^4 min^{-1})[a]		
		pH 7.4 buffer	Corneal epithelial	Conjunctival
		Alkyl esters		
2	*O*-Acetyl timolol	177.5 ± 2.0	199.4 ± 63.7	186.0 ± 63.5
3	*O*-Propionyl timolol	199.5 ± 47.1	210.4 ± 97.4	257.6 ± 59.0
4	*O*-Butyryl timolol	73.8 ± 0.2	254.9 ± 15.9	296.3 ± 6.7
5	*O*-Isobutyryl timolol	115.5 ± 6.5	159.9 ± 4.2	208.0 ± 9.0
6	*O*-Valeryl timolol	88.8 ± 1.2	221.0 ± 4.0	173.9 ± 4.1
7	*O*-Pivaloyl timolol	13.7 ± 0.1	53.8 ± 2.8	35.2 ± 1.0
8	*O*-Neopentanoyl timolol	14.1 ± 1.6	19.8 ± 1.2	20.1 ± 1.1
9	*O*-Hexanoyl timolol	65.7 ± 1.9	207.8 ± 5.0	131.8 ± 1.9
10	*O*-2-Ethylbutyryl timolol	22.9 ± 1.6	27.1 ± 1.8	31.6 ± 2.4
11	*O*-Octanoyl timolol	64.3 ± 6.3	128.8 ± 36.0	126.1 ± 13.6
		Cycloalkyl esters		
12	*O*-Cyclopropanoyl timolol	24.9 ± 1.2	22.3 ± 1.4	20.9 ± 3.3
13	*O*-1′-Methylcyclopropanoyl timolol	12.1 ± 1.8	22.3 ± 1.4	29.3 ± 1.9
14	*O*-2′-Methylcyclopropanoyl timolol	13.5 ± 1.0	13.4 ± 1.5	24.2 ± 2.4
15	*O*-Cyclobutanoyl timolol	296.5 ± 8.7	251.8 ± 18.7	599.4 ± 63.3
16	*O*-Cyclopentanoyl timolol	125.9 ± 5.7	386.4 ± 17.9	950.7 ± 4.6
17	*O*-Cyclohexanoyl timolol	87.9 ± 5.6	496.2 ± 38.6	355.3 ± 15.1
		Aryl esters		
18	*O*-Benzoyl timolol	41.6 ± 2.1	37.2 ± 1.5	156.0 ± 27.0
19	*O*-*o*-Methylbenzoyl timolol	23.9 ± 1.2	16.7 ± 1.8	13.9 ± 1.5
20	*O*-*p*-Methylbenzoyl timolol	28.9 ± 2.6	71.9 ± 2.2	246.8 ± 44.6
21	*O*-*p*-Methoxybenzoyl timolol	14.2 ± 1.4	14.9 ± 0.7	27.3 ± 1.2
22	*O*-2-(Benzoyloxymethyl)benzoyl timolol	344.4 ± 37.1	134.5 ± 7.2	260.2 ± 22.1
23	*O*-2-Aminobenzoyl timolol	0.9 ± 0.6	3.7 ± 1.2	3.3 ± 1.5
24	*O*-2-Methylaminobenzoyl timolol	13.5 ± 1.1	11.6 ± 1.6	7.0 ± 0.7
25	*O*-Benzoyl carbamate timolol ester	52.9 ± 3.5	24.8 ± 0.5	37.0 ± 1.4
26	*O*-3-Thienyl timolol	25.6 ± 0.9	28.7 ± 2.4	66.3 ± 1.7

[a] Mean ± SD (n = 3).

molol and its prodrugs ranged from 7 to 20 min. The sensitivity of the assay was 0.5 nmol with respect to timolol and its prodrugs. The intra- and interrun variations were 5 and 7.5%, respectively.

Prodrug Hydrolysis in Corneal Epithelial and Conjunctival Homogenates

Preparation of Corneal Epithelial and Conjunctival Homogenates. Corneal epithelial and conjunctival homogenates were prepared as previously described (9). Following euthanasia by an injection of sodium pentobarbital solution (Eutha-6, Western Medical Supply Co., Arcadia, CA) into a marginal ear vein of the rabbit, its palpebral conjunctiva was excised and its corneal epithelium was scraped off the cornea using a No. 11 surgical blade. The tissue specimens were homogenized in 1–2 ml of ice-cold 10 m*M* phosphate buffer (pH 7.4) using an Elvejehm–Potter homogenizer and centrifuged at 1500*g* at 4°C for 10 min to remove cellular and nuclear debris. The resulting supernatant was diluted with isotonic phosphate buffer to a protein concentration of 0.25 mg/ml, which was determined using a dye-binding assay with bovine serum albumin as the standard (10).

Prodrug Hydrolysis. Hydrolysis of timolol prodrugs was studied by incubating, in triplicate, 500 μl each of prodrug solution (0.2 m*M*) and conjunctival or corneal epithelial supernatant at 37°C. At predetermined times up until 240 min, 50 μl was withdrawn from the incubation mixture and mixed with 150 μl of acetonitrile to precipitate the tissue proteins, thereby terminating the reaction. Fifty microliters of a 25 μg/ml propranolol solution, the internal standard, was then added. Following centrifugation at 1500*g* for 10 min, 20 μl of the supernatant was injected into the HPLC. The pseudo first-order rate constants of prodrug hydrolysis were obtained by following prodrug disappearance.

Corneal and Conjunctival Penetration of Timolol and Its Prodrugs

A modified procedure reported by Chang *et al.* (2) was used. Rabbits were euthanized by a rapid injection of sodium pentobarbital solution (Eutha-6, Western Medical Supplies,

Table II. Corneal and Conjunctival Permeability Coefficients (P_{app}) of Timolol and Its Prodrugs and Their Ratios (C/J) as a Function of Lipophilicity (log PC)

No.	Compound	log PC[a]	P_{app} (10^5 cm sec^{-1})[b]		C/J
			Corneal	Conjunctival	
1	Timolol	−0.04	1.13 ± 0.19	3.32 ± 1.28	0.34
		Alkyl esters			
2	*O*-Acetyl timolol	1.12	2.31 ± 0.48	5.25 ± 0.66	0.44
3	*O*-Propionyl timolol	1.62	2.93 ± 0.14	6.06 ± 1.51	0.48
4	*O*-Butyryl timolol	2.08	3.23 ± 0.45	6.28 ± 0.24	0.51
5	*O*-Isobutyryl timolol	2.19	1.81 ± 0.24	3.41 ± 0.24	0.53
6	*O*-Valeryl timolol	2.67	3.13 ± 0.28	5.97 ± 0.24	0.52
7	*O*-Pivaloyl timolol	2.68	1.36 ± 0.61	2.38 ± 0.15	0.57
8	*O*-Neopentanoyl timolol	3.09	1.55 ± 0.27	1.90 ± 0.06	0.82
9	*O*-Hexanoyl timolol	3.35	2.08 ± 0.11	3.83 ± 0.66	0.54
10	*O*-2-Ethylbutyryl timolol	3.26	0.71 ± 0.07	1.04 ± 0.02	0.68
11	*O*-Octanoyl timolol	4.66	0.89 ± 0.19	0.42 ± 0.04	2.13
		Cycloalkyl esters			
12	*O*-Cyclopropanoyl timolol	1.74	2.66 ± 0.21	4.15 ± 0.31	0.64
13	*O*-1′-Methylcyclopropanoyl timolol	2.22	3.30 ± 0.12	4.31 ± 0.54	0.77
14	*O*-2′-Methylcyclopropanoyl timolol	2.26	2.59 ± 0.08	5.85 ± 0.17	0.44
15	*O*-Cyclobutanoyl timolol	2.36	1.53 ± 0.03	2.77 ± 0.36	0.55
16	*O*-Cyclopentanoyl timolol	2.75	1.74 ± 0.03	3.47 ± 0.30	0.50
17	*O*-Cyclohexanoyl timolol	3.30	0.68 ± 0.10	1.03 ± 0.10	0.66
		Aryl esters			
18	*O*-Benzoyl timolol	2.55	1.68 ± 0.25	2.04 ± 0.07	0.82
19	*O*-*o*-Methylbenzoyl timolol	3.02	0.42 ± 0.02	0.69 ± 0.03	0.61
20	*O*-*p*-Methylbenzoyl timolol	3.11	1.18 ± 0.18	0.93 ± 0.06	1.27
21	*O*-*p*-Methoxybenzoyl timolol	2.65	1.48 ± 0.22	1.41 ± 0.28	1.05
22	*O*-2-(Benzoyloxymethyl)benzoyl timolol	4.37	0.33 ± 0.02	0.28 ± 0.02	1.18
23	*O*-2-Aminobenzoyl timolol	2.51	1.72 ± 0.13	2.55 ± 0.10	0.68
24	*O*-2-Methylaminobenzoyl timolol	3.04	0.82 ± 0.05	1.62 ± 0.27	0.72
25	*O*-Benzoyl carbamate timolol ester	1.75	2.65 ± 0.21	3.68 ± 0.50	0.72
26	*O*-3-Thienyl timolol	2.27	2.17 ± 0.33	2.79 ± 0.42	0.78

[a] PC is the partition coefficient between *n*-octanol and 0.05 *M* phosphate buffer at pH 7.4. The data are from Ref. 4.
[b] Mean ± SD; n = 4–6.

Arcadia, CA) into the marginal ear vein. The eyes were immediately enucleated and the corneas or the orbital portion of the palpebral conjunctivas were excised and mounted in a lucite-block modified Ussing chamber (9). Two and one-half milliliters of glutathione bicarbonate Ringer's (GBR) solution (11) was added to the endothelial (serosal) side. An equal volume of the same solution containing 3 m*M* timolol or its prodrug was immediately added to the epithelial (mucosal) side. Mixing in each chamber was achieved by bubbling a mixture of 95% O_2 and 5% CO_2. The temperature of the chamber was kept at 37°C by a circulating water bath. At predetermined times up until 240 min, 50-μl aliquots were sampled from the endothelial side and immediately replaced by an equal volume of GBR solution. The aliquots were assayed for drug and prodrug by reversed phase HPLC.

After correcting for the corneal (1.075-cm^2) and conjunctival (0.95-cm^2) surface area and initial drug or prodrug concentration in the donor reservoir, the apparent corneal and conjunctival permeability coefficients were calculated from the linear portion of a plot of micromoles of total prodrug in the receiver compartment vs time, where total prodrug refers to the sum of micromoles of intact prodrug and timolol formed. There are two reasons for this simplistic approach. First, the prodrug concentration never reached steady state due to concurrent hydrolysis and rapid penetration, making it difficult to calculate the permeability coefficient for intact prodrug under nonsink conditions. Second, it is the total prodrug that penetrates the cornea and conjunctiva which determines the ocular and systemic bioavailability (6,7), respectively, since the prodrug will eventually be hydrolyzed completely within the eye and the bloodstream.

No attempt was made to correct for chemical and enzymatic hydrolysis of any prodrug in the donor and receiver solutions. As pointed out in a previous study (12), the corneal epithelium is saturated with prodrug within minutes of contact. Consequently, it is the prodrug in the corneal epithelium rather than in the bathing solution which establishes the concentration gradient for prodrug diffusion across the cornea. This is also assumed to be the case in conjunctival prodrug penetration.

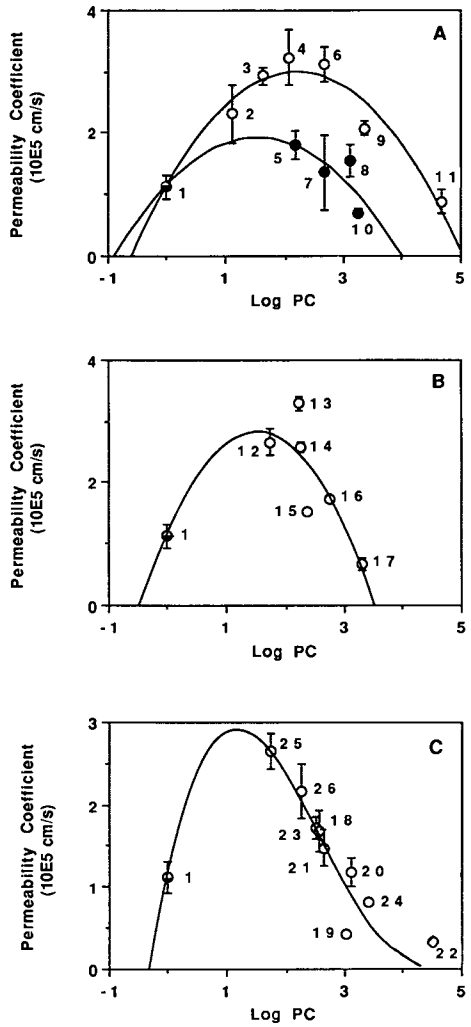

Fig. 1. Influence of prodrug lipophilicity (log PC) on the corneal penetration of alkyl (A), cycloalkyl (B), and aryl (C) esters of timolol in the pigmented rabbit. In A, ○ refers to straight-chain alkyl esters, ● refers to branched-chain alkyl esters, and ◒ refers to timolol. Error bars represent standard deviation (n = 4–6). Where not shown, the error bars are smaller than the size of the symbols. See Table I for key to the numbering of compounds.

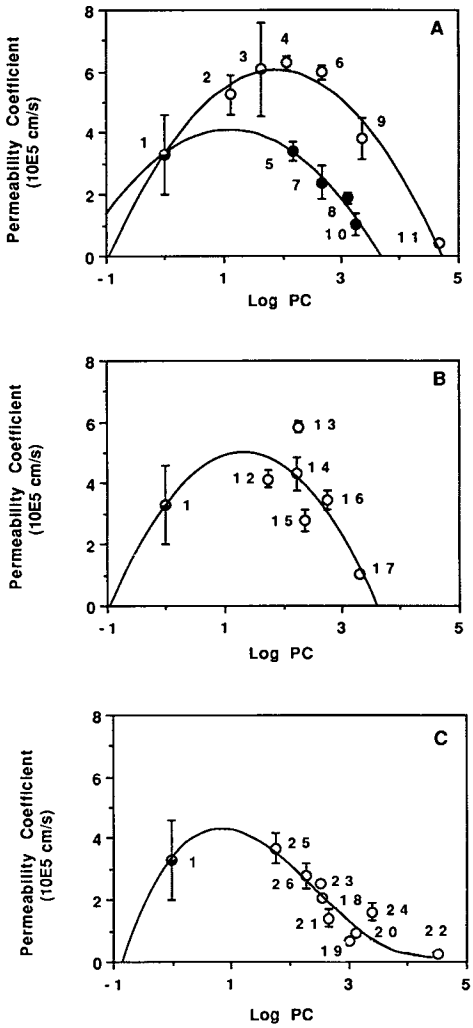

Fig. 2. Influence of prodrug lipophilicity (log PC) on the conjunctival penetration of alkyl (A), cycloalkyl (B), and aryl (C) timolol esters in the pigmented rabbit. In A, ○ refers to straight-chain alkyl esters, ● refers to branched-chain alkyl esters, and ◒ refers to timolol. Error bars represent standard deviation (n = 4–6). Where not shown, the error bars are smaller than the size of the symbols. See Table I for key to the numbering of compounds.

RESULTS

The majority of the prodrugs were more stable in the corneal epithelial than in the conjunctival homogenate (Table I). Moreover, the straight-chain alkyl and the unsubstituted cycloalkyl esters were hydrolyzed more rapidly than their corresponding branched chain and substituted analogues as well as the aryl esters. For example, *O*-butyryl timolol was hydrolyzed more rapidly than the *O*-isobutyryl ester, *O*-valeryl timolol was hydrolyzed more rapidly than the *O*-pivaloyl and *O*-neopentanoyl esters, *O*-hexanoyl timolol was hydrolyzed more rapidly than the *O*-benzoyl ester, and *O*-cyclobutanoyl timolol was hydrolyzed more rapidly than the *O*-1′-methyl- and *O*-2-methylcyclopropranoyl esters.

The apparent permeability coefficients of timolol prodrugs across the cornea and conjunctiva as a function of prodrug lipophilicity (4) are listed in Table II and shown in Figs. 1 and 2, respectively. The corneal and conjunctival permeability coefficients of the alkyl esters varied parabolically with the logarithm of the n-octanol/pH 7.4 buffer partition coefficient, with the straight-chain alkyl esters in one group and the branched ones in the other (Figs. 1A and 2A). Susceptibility to esterase-mediated hydrolysis appeared to be the differentiating characteristic between them. Generally speaking, as the hydrolytic rate constant increased, so did the permeability coefficient (Fig. 3). In the absence of additional hydrophilic esters for evaluation, a parabolic relationship was assumed for the cycloalkyl (Figs. 1B and 2B) and aryl esters (Figs. 1C and 2C).

Combining all the timolol ester prodrugs, two groups also emerged (Fig. 4). Members of the group that penetrated the cornea and conjunctiva more readily included all the straight-chain alkyl esters, *O*-cyclopropanoyl timolol (No. 12) and *O*-1′-methylcyclopropanoyl timolol (No. 13) in the case of corneal penetration and *O*-2′-methylcyclopropanoyl

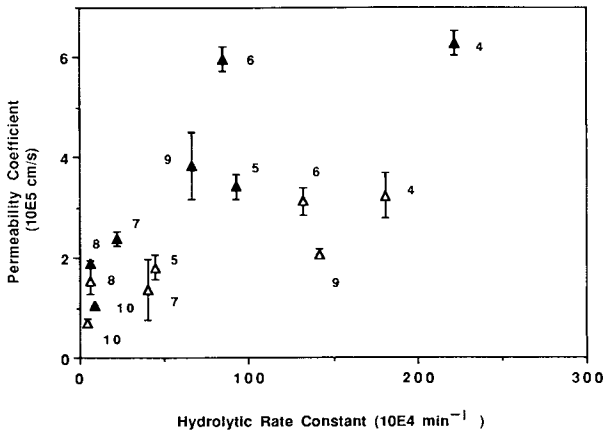

Fig. 3. Influence of susceptibility to esterase-mediated hydrolysis, expressed as hydrolytic rate constant, on the permeability coefficient of straight-chain ester prodrugs of timolol across the isolated cornea (△) and conjunctiva (▲) of the pigmented rabbit, The hydrolytic rate constant has been corrected for chemical hydrolysis by substracting the rate constant in buffer control in Table I from that in the corneal epithelial or conjunctival homogenates. Error bars represent standard deviation (n = 4–6). Where not shown, the error bars are smaller than the size of the symbols. See Table I for key to the numbering of compounds.

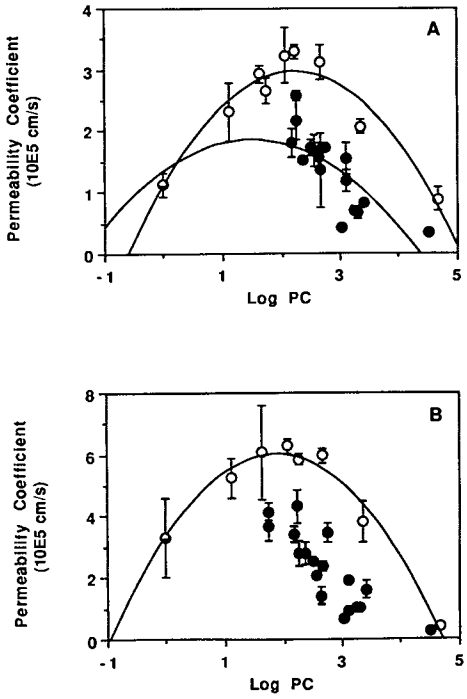

Fig. 4. Influence of prodrug lipophilicity (log PC) on the corneal (A) and conjunctival penetration (B) of timolol ester prodrugs in the pigmented rabbit. Error bars represent standard deviation (n = 4–6). Where not shown, the error bars are smaller than the size of the symbols. (◒) Timolol; (○) straight-chain alkyl esters as well as *O*-cyclopropanoyl timolol (No. 12) and *O*-1′-methylcyclopropanoyl timolol (No. 13) in the case of corneal penetration and *O*-2′-methylcyclopropanoyl timolol (No. 14) in the case of conjunctival penetration; (●) all branched alkyl esters, all the cycloalkyl esters except those named above, and all the aryl esters.

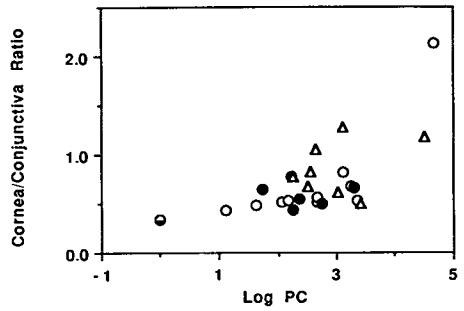

Fig. 5. Influence of prodrug lipophilicity (log PC) on the ratio of corneal-to-conjunctival penetration of timolol prodrugs. (◒) Timolol; (○) alkyl esters; (●) cycloalkyl esters; (△) aryl esters.

timolol (No. 14) in the case of conjunctival penetration. Members in the other group that penetrated the cornea and conjunctiva less readily included all the branched alkyl esters, all the cycloalkyl esters except those named above, and all the aryl esters.

Overall, the prodrugs improved the ratio of corneal-to-conjunctival penetration of timolol (the C/J ratio) about two times (Fig. 5). Compared with 0.34 for timolol, the C/J ratio was 0.72 ± 0.51 (n = 10) for the alkyl esters, 0.59 ± 0.12 (n = 6) for the cycloalkyl esters, 0.85 ± 0.26 (n = 9) for the aryl esters, and 0.74 ± 0.36 (n = 25) overall. A notable exception was the *O*-octanoyl ester, which showed the highest C/J ratio.

DISCUSSION

The parabolic dependence of corneal drug penetration on drug lipophilicity is well documented (13–16). This relationship has been interpreted as a shift in the rate-limiting mass transfer step across the trilaminate cornea—the highly lipophilic epithelium, the hydrophilic stroma, and the moderately lipophilic endothelium—with changes in drug lipophilicity (14). Deviation from the parabolic relationship has been reported by Grass and Robinson (17), when compounds of diverse chemical structure and molecular size are considered. Nevertheless, it is reasonable to expect that within a homologous series the parabolic relationship will hold, which in fact guided our earlier attempts to design timolol prodrugs (2–5). The present study demonstrates that the conjunctival penetration of timolol prodrugs also obeys a parabolic relationship and that enzymatic lability is another factor that influences the extent of corneal and conjunctival penetration of alkyl ester prodrugs of timolol (Figs. 1A and 2A). The effect of hydrolysis on prodrug penetration is to be expected from its effect on the concentration gradient governing permeation. By analogy to the situation in the transdermal penetration and metabolism of a diester of salicyclic acid [methyl 2-(ethoxycarbonyloxy)benzoate] (18), the concentration gradient between the corneal epithelial cells and beyond is steeper for prodrugs that are enzymatically labile, allowing them to penetrate the cornea and conjunctiva more readily than the more stable ones.

Our study has also provided an important hint on the potential use of prodrugs to reduce the systemic absorption of timolol while still improving ocular absorption or at least not compromising it. The main characteristic of these prodrugs is a corneal permeability coefficient which is at least as

large as that of timolol and a conjunctival permeability coefficient which can only be as large as that of timolol (Table II). Such prodrugs include the branched alkyl esters *O*-pivaloyl (No. 7) and *O*-neopentanoyl timolol (No. 8); the cycloalkyl ester *O*-cyclobutanoyl timolol (No. 15); and the aryl esters *O*-benzoyl (No. 18), *O*-*p*-methylbenzoyl (No. 20), *O*-*p*-methoxybenzoyl (No. 21), *O*-2-aminobenzoyl (No. 23), and *O*-3-thienyl timolol (No. 26) (Table II). The overall C/J ratio of these prodrugs, 0.81 ± 0.23 (n = 8), is more favorable than that of timolol, 0.34. Interestingly, these esters are also relatively resistant to enzymatic hydrolysis in the corneal epithelial and conjunctival homogenates (Table I).

In summary, enzymatic lability is an additional factor besides lipophilicity that can be exploited to improve ocular drug absorption by a homologous series of alkyl ester prodrugs. There is preliminary evidence that certain cycloalkyl and aryl ester prodrugs, which are not prone to enzymatic hydrolysis, are poorly absorbed across the conjunctiva. They are, therefore, good candidates to consider for the purpose of reducing systemic drug absorption. Such a possibility is currently being investigated.

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