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Permeability of pilocarpic acid diesters across albino rabbit cornea in vitro

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Summary

Corneal uptake and permeability of various alkyl and aryl diesters of pilocarpic acid in isolated albino rabbit cornea were investigated in vitro in diffusion cells. The permeability coefficient for pilocarpine was 2.77×10^{-6} cm/s. The fractional distribution of pilocarpine in the epithelial side, cornea and endothelial side at 4 h was 85.0, 11.0 and 4.0%, respectively. The corneal permeability coefficient of some pilocarpic acid diesters was several times higher (maximum 3.4-fold). No intact prodrug was observed in the endothelial side. A parabolic relationship between the logarithm of the apparent partition coefficient (1-octanol-pH 7.4 phosphate buffer) (log PC) and the corneal permeability was noticed and the permeability of the most lipophilic derivate was less than that of pilocarpine. In contrast, corneal uptake was increased with increasing lipophilicity being almost complete with a log PC value of 7.70. Corneal permeability and the rate of enzymatic hydrolysis of the compounds correlated well. The corneal permeability of lipophilic pilocarpine diesters (log PC $\geq$ 2.87) given as prodrug seems to be controlled by the formation of pilocarpine in the corneal epithelium rather than by the absorption of prodrugs into the epithelium or their epithelium-stroma transport rate. The optimal lipophilicity for improving corneal permeability (i.e., rate of ocular pilocarpine delivery), was observed at log PC values of 3–4, but more extensive corneal uptake by the most lipophilic compounds suggests that the largest ocular bioavailability may be obtained with larger values of log PC.

Introduction

Glaucoma is one of the most serious ocular diseases. Pilocarpine, a direct-acting cholinergic muscarinic agonist, is widely used in the treatment of this disease. Although pilocarpine has a good pharmacodynamic effect on the intraocular pressure, it has some disadvantages. Only 1–2% or less of an instilled pilocarpine dose gains access to the inner eye (Chrai and Robinson, 1974; Lazare and Horlington, 1975). The duration of the intraocular pressure lowering by pilocarpine is short, and consequently the frequency of administration is 3–6 times a day which leads to decreased patient compliance (Norell, 1980). Frequent drug administration also increases the fre-

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quency of ocular side effects like miosis and accommodation problems.

One of the most difficult problems to overcome when working with ophthalmic drugs, is the poor ocular absorption, which for the most part prevents the drug from reaching its intraocular site of action. Also pilocarpine shows low ocular bioavailability (1–2%) due to the rapid solution drainage from the application site (Chrai et al., 1973; 1974), induced lacrimation (Lee and Robinson, 1979), drug protein binding (Mikkelson et al., 1973a,b; Chrai and Robinson, 1976), tear turnover (Patton and Robinson, 1975) and conjunctival absorption (Patton and Robinson, 1976). These factors compete with productive corneal ocular absorption of the drug. Another disadvantage of topically ocularly administered pilocarpine is its extensive systemic absorption (Salminen et al., 1984).

During the past two decades considerable research effort has been devoted to enhancing ocular drug bioavailability (for references see Shell, 1984). For this purpose new ophthalmic vehicles and dosage forms have been proposed. In order to lengthen the contact time of pilocarpine with the ocular surface, experiments with vehicles containing viscosity-increasing agents have been performed (Green and Downs, 1975); these include gel forming systems (Gurny, 1981). Thus far, such methods have only resulted in moderate success: small improvements in ocular pilocarpine bioavailability and ocular side effects are not avoided (Grass and Robinson, 1984). A membrane-controlled drug delivery system, Ocusert, is another method that has been used to minimize the frequency of pilocarpine applications (Quigley et al., 1975). Controlled release inserts provide long duration of action and decreased intensity of ocular side effects of pilocarpine, but patient acceptance has been a problem with solid inserts (Lee and Robinson, 1986).

Another possibility to increase ocular absorption is to improve the ability of the drug to penetrate through the cornea, the main route of drug absorption into the eye. One promising way to do this is formulation of prodrugs. The prodrug dipivefrin, a pivalic acid diester of epinephrine, is an example of a drug with improved corneal penetration when compared with the parent drug (Kass et al., 1979). The esterification has made the molecule more lipophilic which increases its corneal permeability and ocular bioavailability. It has recently been shown that compared to pilocarpine some pilocarpic acid diesters have improved miotic activity in rabbits (Mosher et al., 1987).

We synthesized diesters of pilocarpic acid with different lipophilicities. Bundgaard et al. (1985, 1986) demonstrated that in the presence of human plasma or rabbit corneal tissue homogenates pilocarpine is formed from pilocarpic acid diesters in quantitative amounts through a sequential process involving enzymatic hydrolysis of the *O*-acyl bond followed by the spontaneous lactonization of the intermediate pilocarpic acid monoester (Scheme 1). The main purpose of this study was to compare the permeability of a series of pilocarpic acid diesters across excised albino rabbit corneas and to evaluate the structure-corneal permeability relationship of these prodrugs.

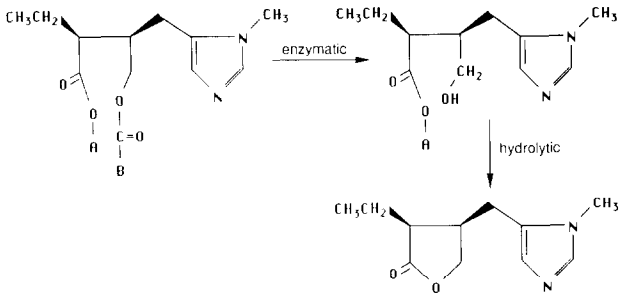
Materials and Methods

Materials

Adult male and female albino rabbits (New Zealand strain), weighing between 2.0 and 4.8 kg, were used as the animal model. Lighting was maintained on a 10 h dark/14 h light cycle, and the animals were fed a regular diet with no restrictions on the amount of food or water consumed. Pilocarpine hydrochloride was a gift from Huhtamäki OY Leiras (Finland) and isopilocarpine nitrate was purchased from Aldrich Chemie (Germany). Pilocarpic acid diester fumarates were synthesized and identified as described elsewhere (Järvinen et al., 1991a). Structures of each prodrug are shown in Scheme 1. HPLC-grade methanol was from J.T. Baker (Deventer, The Netherlands). All other chemicals used were of analytical grade.

Dissection of the rabbit cornea

The rabbits were killed by a marginal ear-vein injection of a lethal dose of T-61 vet. (Hoechst,



1 A = -CH2- B = -CH3

2 A = -CH2- B = -CH2CH3

3 A = -CH2- B = -(CH2)3CH3

4 A = -CH2- B = -(CH2)6CH3

5 A = -CH2- B =

6 A = -CH2- B = Cl

7 A = -CH2- B = -CH2CH3

Scheme 1.

Munich, Germany). The eye was then proptosed and a small transverse incision was made about 5 mm posteriorly from the limbus and, subsequently, the cornea with the scleral ring was carefully cut out with small scissors. The conjuctival and scleral tissue served as a gasket and permitted the cornea to be suspended within the corneal ring during the experiment. The exposed cornea of the proptosed eye was carefully placed on a corneal holder, which maintained the cornea curvature and held the eye in place. Care was taken to avoid contamination of the epithelial surface with blood or physical trauma to the tissue. Slight suction was applied to the tissue to tightly secure it on the mounting device as described earlier (Schoenwald and Huang, 1983). Using forceps, first the lens and then the iris were delicately removed leaving the cornea as a transparent film. Thread was used to bind this tissue to the corneal ring. At this point, suction was removed and the corneal ring was mounted with the tissue in the center of the perfusion chambers. Five to eight corneas were used for each permeability determination. The mounting device and corneal holder have been described in detail previously (Schoenwald and Huang, 1983).

Permeability experiment

Glutathione bicarbonated Ringer's (GBR) solution (O'Brien and Edelhauser, 1977) was used throughout the perfusion studies. This solution is reported to preserve tissue integrity of an excised cornea over 6 h. 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). Solutions were stored in a refrigerator and they were used within 3 weeks to prevent mold growth. Equal volumes of both solutions were mixed just prior to use.

Within 20 min from the killing of the animal the cornea was mounted and clamped between two cylindrical compartments of the perfusion chamber. GBR solution, pre-adjusted to pH 7.65 and temperature 37 °C, was added first to the endothelial side (3.2 ml) to prevent the cornea from buckling. Immediately thereafter, 3.0 ml of this solution containing 150–572 μM of pilocarpine or 7–282 μM of pilocarpic acid diester in GBR buffer was added to the epithelial side. A mixture of O_2 and CO_2 (95%:5%) was bubbled in both compartments at a rate of three to five bubbles/s to provide mixing and steady solution pH. Samples of 200 μl were withdrawn from the receiving side for the period of 4 h. Each sample was immediately replaced with an equal volume of GBR solution. The first sample was withdrawn within 2 min after starting the experiment and served as a control to detect any leakage. Subsequent samples were taken approximately every 20 min for 1 h and then every 30 min through the 4

h period. At the end of the experiment a sample was taken also from the epithelial side to detect the remaining prodrug.

Drug assay

High-performance liquid chromatography (HPLC) was performed with a system consisting of a Beckman pump Model 116, a variable-wavelength U.V.-detector Beckman Type 166 ($\lambda = 215$ nm), System Gold data module (Beckman Instruments Inc., San Ramon, U.S.A.), Marathon autosampler (Spark Holland, Emmen, The Netherlands) with a column thermostat, and a Rheodyne 7080-080 injection valve equipped with a 20-μl loop. Pilocarpine, isopilocarpine, pilocarpic acid, and isopilocarpic acid were determined with a deactivated LC18-DB Supelcosil column (Supelco, Bellefonte, U.S.A.) (250 × 4.6 mm) with 5 μm particles at 40 °C. The solvent system was 5% KH_2PO_4 (pH 2.5):methanol (97% : 3%) with a flow rate of 1.5 ml/min. In some experiments pilocarpine was assayed with a μBondapak C18 column (Waters, Milford, U.S.A.) (300 × 3.9 mm) packed with 10 μm particles at ambient temperature with a solvent system consisting of 5% KH_2PO_4, (pH 2.5):methanol (90% : 10%) and the flow rate was 1.5 ml/min. A deactivated Supelcosil LC8-DB column (Supelco, Bellefonte, U.S.A) (150 × 4.6 mm) with 5 μm particles were used for determination of pilocarpic acid diesters. The solvent system was 0.02 M KH_2PO_4 (pH 4.5):methanol (29% : 71%) with an effluent flow rate of 1.0 ml/min. pH was measured with Orion SA 520 pH meter (Boston, MA, U.S.A.) at the temperature of study.

Hydration levels

The hydration level of the cornea gives an indication of its condition. After each permeability experiment, the cornea was removed from the mounting rings, and the remaining scleral tissue and conjunctiva were cut away. The cornea was weighed and then dried in an oven at 50 °C overnight. After reweighing, the water content of the cornea was calculated. The normal cornea has a hydration level of 76–80% (Maurice and Riley, 1970). If manipulation of the cornea led to damage of the epithelium and/or endothelium, then the hydration level would rise. Corneas with a hydration level of 83% or greater were considered damaged as suggested by Schoenwald and Huang (1983).

Calculation of permeability coefficients and lag times

The apparent corneal permeability coefficient (P_{app}, cm/s) was determined according to:

$$P_{app} = \frac{\Delta Q}{\Delta t \cdot 60 \cdot A \cdot C_0} \quad (1)$$

where Q is the total amount permeated at time t, $\Delta Q/\Delta t$ (the slope of the linear portion of the graph) is the steady-state flux of pilocarpine to the receiver side (μmol/min), 60 is the conversion of minutes to seconds, A is the corneal surface area (in this study 1.17 cm^2) and C_0 is the initial donor side drug amount (μmol). The mean surface area of cornea ($n = 42$) was determined making an incision to the cornea and by flattening it on a plastic sheet and drawing a continuous line along the limbus. Thereafter the area was determined from the plastic by cut-and-weigh method.

When the steady-state portion of the drug permeated vs time graph is extrapolated, the point of intersection at time axis gives the lag time of permeation (t_L). This is the time required for a penetrant to establish a steady concentration gradient within the membrane separating the donor from the receptor compartment.

Results

The hydration levels of corneas which had been in the perfusion apparatus for 4 h was 79.8 ± 2.5% (mean ± S.D., $n = 50$). Corneal hydration levels for each group are presented in Table 1.

The lag times before the steady-state permeation are shown in Table 2. The lag times of pilocarpic acid diesters are inversely related to the permeability coefficients (Tables 1 and 2). In other words, the more rapidly penetrating diesters have shorter lag times of permeation.

TABLE 1

Remaining pilocarpine or intact prodrug on the epithelial side, penetrated pilocarpine in the endothelial side, corneal membrane permeability (P_{app}) and corneal hydration levels for each compound (numbers in parentheses are S.D. with n = 3–8)

Compound	Remaining pilocarpine or intact prodrug on the epithelial side (%)		Penetrated pilocarpine in the endothelial side (%)	P_{app} ($\times 10^{-6}$) [a] (cm/s)	Corneal hydration (%)
	Pilocarpine	Prodrug			
Pilocarpine	84.98 (2.48)	–	4.00 (0.90)	2.77 (0.63)	79.45 (0.86)
1	38.64 (11.91)	34.11 (10.24)	12.55 (1.88)	8.43 (1.40)	80.20 (2.75)
2	38.05 (6.25)	19.26 (5.65)	14.26 (4.13)	9.30 (2.63)	77.71 (3.84)
3	68.65 (13.69)	7.68 (4.68)	12.50 (3.72)	8.00 (1.81)	80.84 (2.56)
4	32.62 (5.26)	0.20-3.50 [b]	2.41 (0.71)	1.76 (0.38)	78.18 (1.61)
5	24.84 (6.88)	17.05 (5.33)	7.70 (2.35)	5.69 (1.44)	80.88 (1.70)
6	37.43 (5.12)	19.04 (6.61)	8.15 (2.83)	5.10 (1.27)	79.87 (2.91)
7	6.83 (1.70)	16.48 (6.74)	1.26 (0.51)	0.93 (0.25)	81.18 (1.57)

[a] Corneal membrane permeability (mean ± S.D.).
[b] Range of variation. $n = 6$. Four values were below the detection limit (corresponds to 0.2% remaining). Mean of the determined values gives 3.5%.

A typical permeability experiment (Fig. 1) shows permeation of pilocarpine and compound 2 across excised rabbit corneas. The penetrated fraction of pilocarpine to the endothelial side in 4 h of perfusion varied between 1.26 and 14.26% in the case of pilocarpic acid diesters and for pilocarpine it was 4.00% (Table 1). The permeability coefficient of pilocarpine was 2.77×10^{-6} cm/s. Pilocarpine permeability when given as pilocarpic acid diesters was $0.93–9.30 \times 10^{-6}$ cm/s (Table 1). These results indicate that the best prodrug permeability (compound 2) is more than 3-fold compared to pilocarpine.

HPLC analysis showed that examined pilocarpic acid diesters had been completely hydrolyzed during the passage through the cornea; no intact prodrug was detected in the receiver side. Furthermore, no other metabolites could be seen except pilocarpine and its degradation products: pilocarpic acid, isopilocarpine and isopilocarpic acid. Fraction of degradation products of pilocarpine was less than 10% of pilocarpine concentration in the endothelial side.

TABLE 2

Lag time (t_L), lipophilicity (log PC), and enzymatic lability ($t_{1/2}$) of pilocarpine and pilocarpic acid diesters

Compound	t_L (min) [a]	Log PC [b,c] (RSD,%)	$t_{1/2}$ (min) [c] 80% human serum
Pilocarpine	18 ± 7	0.01 (34.3)	–
1	12 ± 4	2.87 (0.4)	16
2	11 ± 3	3.30 (1.2)	6
3	22 ± 4	4.43 (0.3)	9
4	62 ± 9	7.70 (1.0)	38
5	29 ± 12	4.57 (0.2)	18
6	29 ± 9	5.64 (1.9)	23
7	68 ± 12	4.40 (0.3)	33

[a] Diffusional lag time, (mean ± S.D.).
[b] Apparent partition coefficient between 1-octanol and phosphate buffer of pH 7.40.
[c] Data from Järvinen et al. (1991b).

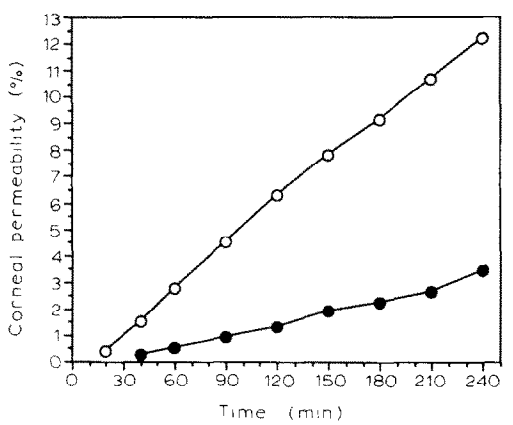


Fig. 1. Permeability rate of pilocarpine (●) and compound 2 (○) across single excised rabbit corneas as a function of time. The initial concentrations on the epithelial side for pilocarpine and compound 2 were 254 and 212 μM, respectively.

Analysis of the epithelial side showed both intact prodrug and free pilocarpine (Table 1). At 4 h the fraction of intact prodrug on the epithelial side varied between 0.2 and 34.1% of the initial amount, and the fraction of pilocarpine in the epithelial side was between 6.8 and 68.7% of the total pilocarpine added as pilocarpic acid diesters. Control experiments revealed that all the pilocarpic acid diesters were chemically stable in the perfusion medium without tissue. At 4 h the amounts of pilocarpine absorbed by the cornea and remaining in the epithelial side were 11.0 ± 2.9 and $85.0 \pm 2.5\%$, respectively. The corneal uptake determined as the loss of prodrug in the epithelial side at 4 h is dependent on the lipophilicity of the prodrug used (Tables 1 and 2). The largest uptake was with the most lipophilic derivative (compound 4) and the smallest with the most hydrophilic ones (compounds 1 and 2) (Tables 1 and 2).

The corneal permeabilities vary parabolically with the lipophilicity (Fig. 2). The regression curve is represented by: $\log P_{app} = -0.038384(\log PC)^2 + 0.26671(\log PC) - 5.5553$, where $r = 0.9793$. The optimal lipophilicity for improving corneal penetration, in terms of log PC, was 3–4. The

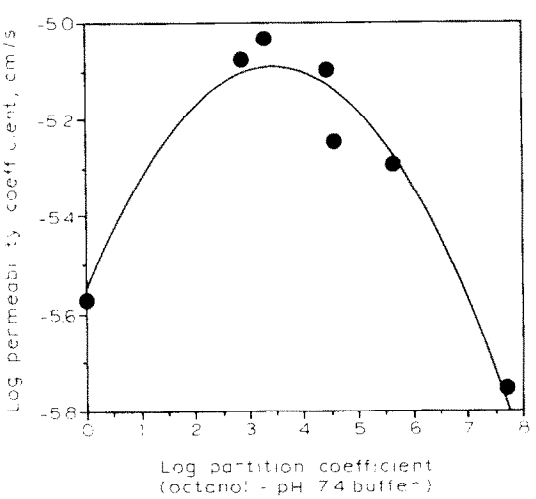


Fig. 2. Log–log plot of permeability coefficient (pH 7.65) through epithelium vs apparent partition coefficient (1-octanol–pH 7.4 phosphate buffer). The regression curve is represented by: $\log P_{app} = -0.038384(\log PC)^2 + 0.26671(\log PC) - 5.5553$, where $r = 0.9793$.

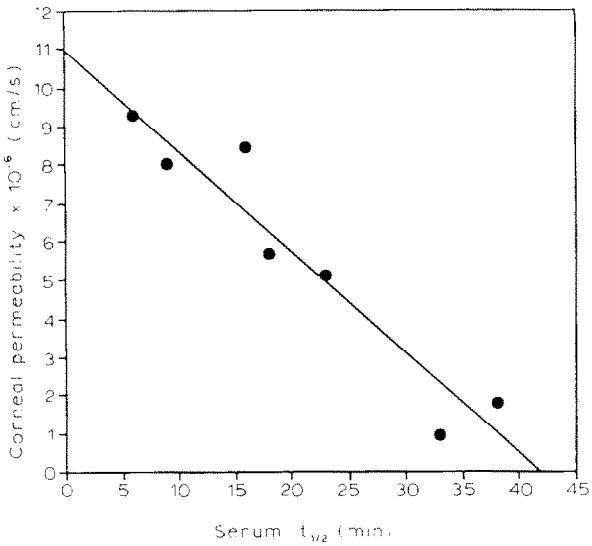


Fig. 3. Corneal permeability coefficient of pilocarpic acid diesters vs 80% human serum $t_{1/2}$. The linear regression line is represented by: $P_{app} = -0.26379(\text{serum } t_{1/2}) + 10.988$, where $r = 0.9524$.

half-lives of the pilocarpic acid diester prodrugs in 80% human serum varied between 6 and 38 min (Table 2). Our results also indicate a good correlation between corneal permeability and serum $t_{1/2}$ (Fig. 3). The linear regression line is represented by: $P_{app} = -0.26379(\text{serum } t_{1/2}) + 10.988$, where $r = 0.9524$.

Discussion

In terms of drug penetration, the cornea can be considered as consisting of three primary layers: epithelium, stroma and endothelium (Huang et al., 1983). Epithelium, being lipoidal in nature, represents a diffusional barrier to relatively hydrophilic pilocarpine (Sieg and Robinson, 1976).

The present study shows that in vitro all lipophilic pilocarpic acid diesters are more extensively taken up by the rabbit cornea than the parent pilocarpine (Table 1). The uptake is increased by the increased lipophilicity of the examined substance suggesting uptake primarily by the lipoidal corneal epithelium (Table 1). However, similar dependence was not observed between lipophilicity and corneal permeability (Fig. 2).

The relationship was parabolic and the optimal lipophilicity for corneal penetration, in terms of log PC value, was about 3–4. Our results, showing a parabolic relationship between the corneal permeability and the apparent partition coefficient, are in accordance with earlier experiments. With steroids (Schoenwald and Ward, 1978), *n*-alkyl-*p*-aminobenzoate esters (Mosher and Mikkelson, 1979), beta-blockers (Schoenwald and Huang, 1983), and timolol prodrugs (Chien et al., 1988) optimal log PC values for corneal permeability have been determined to be 2.9, 2.5–2.6, 2.9 and 2.5, respectively. Interestingly, Mosher et al. (1987) showed a parabolic correlation between apparent relative bioavailability of the pilocarpine diester prodrugs and their log PC values. The optimal log PC was 4.2–4.4. For lipophilic diesters (log PC over 4) (Fig. 2) the correlation between corneal permeability and lipophilicity was inverse and consequently absorption to corneal epithelium does not control drug penetration.

The esterase activity in the corneal epithelium is approx. 2-times higher than in the stroma-endothelium (Petersen et al., 1965; Lee et al., 1982) and therefore the residence time of a prodrug in the epithelium has a significant impact on ocular bioavailability. All pilocarpic acid diesters studied are completely hydrolyzed during the penetration through rabbit cornea and only pilocarpine reached the endothelial side of the perfusion apparatus. The remaining amount of diester on the epithelial side after 4 h of perfusion correlates inversely with lipophilicity of the examined compounds (Tables 1 and 2).

Interestingly, a good correlation existed between the corneal permeability and the rate of enzymatic hydrolysis (Fig. 3). Although the rank order of log PC and $t_{1/2}$ values (Table 2) was about the same the correlation between the rate of enzymatic hydrolysis and corneal permeability is not due to decreased epithelium-stroma transfer rate of pilocarpic acid diesters, because no intact prodrug penetrated through the cornea. Consequently, the corneal permeability of pilocarpine given as a prodrug is rather controlled by the formation of pilocarpine in the corneal epithelium and not by the absorption of prodrugs into the epithelium. This is clearly seen in the case of compounds 3 and 7 (Tables 1 and 2). Despite their similar log PC values the corneal permeability of compound 3 was about 9-fold compared to compound 7 possibly due to faster cleavage of compound 3 by esterases (Table 1). Formation of pilocarpine from the prodrugs may be indirectly affected by prodrug lipophilicity, since very lipophilic diesters are expected to be primarily localized in cell membranes instead of cytoplasm. This may reduce the rate of enzymatic cleavage and permeability coefficient.

Our study shows that pilocarpic acid diesters are effectively taken up by the cornea due to their lipophilicity and subsequently hydrolyzed by corneal esterases releasing pharmacologically active pilocarpine. In conclusion, pilocarpic acid diesters are potentially useful prodrugs of pilocarpine in improving ocular drug delivery.

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