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Evaluation of ocular permeation enhancers: in vitro effects on corneal transport of four β-blockers, and in vitro/in vivo toxic activity

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Abstract

The efficacy and toxicity of a series of prospective ocular penetration enhancers (benzalkonium chloride, EDTA, non-ionic surfactants, surface-active heteroglycosides and bile salts) was investigated in vitro, using isolated rabbit corneas. As test drugs four β-blocking agents were used, chosen in order of increasing lipophilicity: atenolol (AT), timolol (TM), levobunolol (LB) and betaxolol (BX). The increased corneal hydration induced by the enhancers was taken as an index of cellular and tissue damage; the ocular irritancy of the agents was also tested in rabbits in vivo. In the absence of enhancers, the apparent corneal permeability coefficients of the four drugs were in the order AT ≅ TM < LB ≥ BX; in general, the enhancers increased the permeation rates of the more hydrophilic drugs, AT and TM, more than those of the other two, more lipophilic ones, LB and BX. The study pointed to some agents (in particular, polyoxyethylene alkyl ethers and bile salts) as effective and safe penetration promoters for AT and TM. Their apparent safety at the tested concentrations was confirmed by their failure to increase the corneal hydration level beyond the 'normal' value, and by their lack of irritant effect in vivo, as evidenced by a Draize test.

Keywords: Ocular penetration; Enhancers; Non-ionic surfactants; Benzalkonium chloride; EDTA; Surface-active heteroglycosides; Bile salts; Atenolol; Timolol; Levobunolol; Betaxolol; Corneal toxicity; Corneal hydration; Draize test

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1. Introduction

The last three decades have witnessed continued efforts aimed at improving the topical bioavailability of ophthalmic drugs. Investigations are being pursued along the following main lines: (i) prolongation of the ocular time of residence of the medication (vehicle approach, mucoadhesives); (ii) increase of the drug penetration characteristics (prodrug approach); and (iii) enhancement of the corneal permeability (enhancer approach) (Patton and Robinson, 1976; Lee, 1993a,b; Liaw and Robinson, 1993).

The last approach, which consists of increasing transitorily the permeability characteristics of the cornea with appropriate substances, known as penetration enhancers or absorption promoters, bears a strict analogy with techniques aimed at facilitating drug penetration through the skin and different epithelia (buccal, nasal, intestinal, rectal, etc.). However, the unique characteristics and great sensitivity of the corneal/conjunctival tissues impose great caution in the selection of enhancers with regard to consideration of their capacity to affect the integrity of epithelial surfaces. In recent years, ocular penetration enhancers have been the object of attentive investigation (Rojanasakul et al., 1990; Ashton et al., 1991a,b; Hayakawa et al., 1992; Liaw and Robinson, 1992; Ismail et al., 1992, Saettone et al., 1994; Sasaki et al., 1995a,b). The present authors felt, however, that additional knowledge on the activity, toxicity, scope and limitations of these substances might be beneficial.

In the present study, the permeation-enhancing effect of benzalkonium chloride, of EDTA and of different non-ionic surfactants, surface-active heteroglycosides and bile salts was tested on rabbit corneas in vitro, using four β-blocking agents as test drugs. These were, in order of increasing lipophilicity, atenolol, timolol, levobunolol and betaxolol. Possible harmful effects of the enhancers on ocular tissues were assessed by determining their influence on corneal hydration in vitro, and the ocular irritancy elicited in rabbits in vivo.

2. Materials and methods

2.1. Materials

The following materials were used as received: benzalkonium chloride (BZ) (Carlo Erba, Milano, Italy), polyoxyethylene 9 lauryl ether (BL-9), polyoxyethylene 23 lauryl ether (Brij® 35), polyoxyethylene 20 stearyl ether (Brij® 78), polyoxyethylene 20 oleyl ether (Brij® 98), ethylenediaminetetraacetic acid, sodium salt (EDTA) (Fluka Chemie AG, Buchs, Switzerland), sodium deoxycholate (DC), sodium taurodeoxycholate (TDC), sodium ursodeoxycholate (UDC), sodium tauroursodeoxycholate (TUDC), digitonin (DIG), escin (ESC), saponin from *Saponaria* species (SAP), reduced glutathione, hydroxypropylmethylcellulose (HPMC), atenolol (AT), betaxolol hydrochloride (BX), levobunolol hydrochloride (LB) and timolol maleate (TM) (Sigma, St. Louis, MO).

All other chemicals were reagent or analytical grade.

2.2. Animals

Male, New Zealand White rabbits were used throughout. The animals used for the ex vivo study (Pampaloni rabbitry, Fauglia, Italy) weighed 2.8–3.5 kg; those used for the studies in vivo (Harlan-Nossan, Milan, Italy) weighed 2.0–2.5 kg. The animals, housed in standard cages in a light-controlled room at 19 ± 1°C and 50 ± 5% R.H., were given a standard pellet diet and water ad libitum.. The animals were treated and used as indicated in the publication 'Guide for the care and use of laboratory animals' (NIH Publication No. 92–93, revised 1985).

2.3. In vitro corneal penetration studies

The rabbits were euthanized with intravenous pentobarbital (Pentothal sodium, Farmaceutici Gellini, Aprilia, Italy). The eyes were proptosed, and the corneas, with a 2 mm ring of sclera, were immediately excised and mounted in a perfusion apparatus (Camber, 1985) maintained at 35 ± 1°C, where the corneal area available for diffusion was 0.78 cm^2. Preheated (35°C) pH = 6.85 glutathione

bicarbonate Ringer (GBR) buffer (Camber, 1985) was added to the epithelial (1.0 ml) and the endothelial (5.0 ml) compartment. To ensure oxygenation and agitation, an O_2-CO_2 (95:5) mixture was bubbled through each compartment at a rate of 3–4 bubbles/s. After 10 min the solution on the epithelial side was withdrawn and substituted with 1.0 ml of a 3.0 mM solution of drug in GBR buffer, with or without enhancer (the concentrations are indicated in Tables 1 and 3). The drug concentration in the donor chamber was low to avoid possible drug effects on corneal integrity over prolonged incubation.

At appropriate intervals, 1.0 ml samples were withdrawn from the endothelial (receiving) compartment, and were immediately replaced with an equal volume of preheated buffer to ensure sink conditions. Each experiment was continued for about 4.5 h, and was performed at least in triplicate.

All analyses were carried out by isocratic reverse phase HPLC (Shimadzu apparatus, Kyoto, Japan); the sensitivity of the assay was 6.0, 4.0, 4.0 and 5.0 μM for AT, TM, LB and BX, respectively.

2.4. Evaluation of corneal hydration levels (HL)

Wet corneal weights, W_w, were obtained after careful removal of the scleral ring; each corneal sample was then desiccated at 100°C for 6 h to give the corresponding dry corneal weight, W_d.

The percent corneal hydration level (HL%), defined as $[1 - (W_d/W_w)] \cdot 100$ was determined both on untreated corneas (removed no later than 30 min after the animals' death) and on corneas recovered from permeation tests performed in the absence and in the presence of enhancers.

Table 1
Physicochemical properties of the four β-blocking agents

Drug	M.W.	pKa	DC[a]	Log PC[b]
AT	266.3	9.32	5.05	−1.77
TM	316.4	9.21	8.91	−1.41
LB	291.4	9.32	15.74	−1.25
BX	307.4	9.38	32.36	−1.02

[a]Experimental distribution coefficient partition coefficient between octanol and pH 6.85 buffer.
[b]Calculated partition coefficient between octanol and pH 6.85 buffer.

2.5. Determination of distribution coefficients

Apparent distribution coefficients (DC), defined as the ratio of drug concentrations in *n*-octanol and in an aqueous phase, were obtained by distributing the drugs for 24 h at 30 ± 1°C between *n*-octanol and pH 7.4 Sörensen phosphate buffer (mutually pre-saturated with each other). The initial drug concentrations in buffer were 50 mM. The volume ratios of *n*-octanol and buffer (1:1 or 1:10) varied depending on drug lipophilicity, and were chosen so that the drug concentration in the buffer phase could be determined by HPLC after equilibration. The partition coefficient (PC) values at the pH of the permeation experiments in vitro (6.85) were calculated from the corresponding distribution coefficients (DC) using the equation reported by Schoenwald and Huang (1983). The DC and Log PC values are reported in Table 1.

2.6. In vivo tests for ocular irritation

The potential ocular irritancy and/or damaging effects of the substances under test were evaluated using a slightly modified version of the Draize test (Draize et al., 1944). All compounds were dissolved in pH 7.4 phosphate buffer solution (PBS), with the exception of digitonin which was suspended in 0.5% p/p HPMC. Each enhancer was tested on three rabbits: the treatment was performed by a single instillation (0.1 ml) of the solution (or suspension) under test into the lower conjunctival sac of one eye; the contra lateral eye was treated with the same volume of pure solvent (PBS or HPMC). Both eyes of rabbits were examined for signs of irritation before treatment, and 30 min, 1, 2, 4, 6 and 24 h after instillation. At 6 and 24 h all eyes were stained with 2% sodium fluorescein and examined under UV light to verify possible corneal lesions. Grading of ocular irritation was performed at each observation time according to Draize et al. (1944).

2.7. Data analysis

2.7.1. Calculation and statistical evaluation of the apparent permeability coefficients (P_{app})

The apparent permeability coefficients (P_{app}) in units of centimeters per second, defined by the expression:

$$P_{app} = \Delta Q/(\Delta t \cdot C_o \cdot A \cdot 3600)$$

where A, the exposed corneal surface area (0.78 cm^2) and C_o, the initial permeant concentration, were calculated from the steady-state slopes of linear plots of the amount of drug in the receiving chamber (Q) vs time (t).

Linear regression analyses (correlation coefficients and slopes) were performed using Statwiew Software (Abacus Concepts, Berkeley, CA).

Statistical differences between P_{app} values were evaluated, using the same software, by one-way analysis of variance, followed by multiple comparisons using the Fisher PLSD (Protected Least Significant Difference) test (Zar, 1984). In the relevant Tables, an asterisk (*) indicates a significant difference at the $P < 0.05$ level.

2.7.2. Statistical analysis of the in vivo data

The in vivo data were statistically evaluated by the Kruskal-Wallis and the Dunn tests, as indicated by Glantz (1994). The areas under curves (AUCs, scores of irritant action vs time after treatment) were calculated according to the Simpson's rule (Tallarida and Murray, 1981).

3. Results and discussion

3.1. Effect of the enhancers on the corneal permeability of the drugs

The order of lipophilicity of the drugs, atenolol (AT) < timolol (TM) < levobunolol (LB) < betaxolol (BX), as defined by their DC and PC values (Table 1), was in agreement with that indicated in different reports (Schoenwald and Huang, 1983; Wang et al., 1991; Ashton et al., 1991a; Sasaki et al., 1995a,b) even if the absolute values differed somewhat from those reported in the literature.

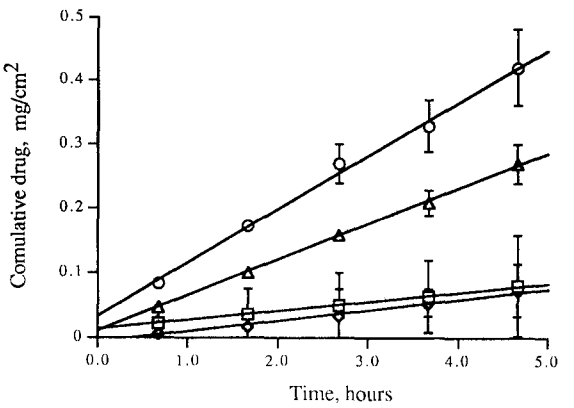

Fig. 1. In vitro transcorneal permeation profiles of the four beta-blocking agents. Key: (◇), TM; (□), AT; (△), BX; (○), LB. Error bars represent S.E. for $n = 4$.

Linear permeation plots with correlation coefficients (r) in the range 0.997–0.999 were obtained in all cases, both in the absence and in the presence of enhancers. A typical graph illustrating the permeation profiles of the drugs in the absence of enhancers is reported in Fig. 1. Table 2 lists the apparent permeability coefficients (P_{app}) and the corneal hydration levels (HL) determined for the permeants in GBR buffer alone, and in the presence of ten prospective enhancers at the stated concentrations. The enhancers were benzalkonium chloride (BZ), digitonin (DIG), saponin (SAP), escin (ESC), four polyoxyethylene ethers (BL9, Brij® 35, 78 and 98), sodium ethylenediaminotetraacetate (EDTA), sodium deoxycholate (DC), sodium taurodeoxycholate (TDC), sodium ursodeoxycholate (UDC) and sodium tauroursodeoxycholate (TUDC).

As shown in Table 2, in the absence of enhancers (GBR buffer) the permeability coefficients of the four drugs were in the order AT ≅ TM < LB ≥ BX, in agreement with the well-known correlations between corneal permeability and drug lipophilicity (Schoenwald, 1993; Ashton et al., 1991a). A significantly lower transcorneal flux was measured for AT and TM with respect to LB and BX. In spite of the lower Papp value shown by the more lipophilic BX (log PC = −1.02) with respect to LB (log PC = −1.25), the two Papp values ($14.71 \cdot 10^{-6}$ and $22.96 \cdot 10^{-6}$, respectively) were not significantly

Table 2
Effect of various enhancers on transcorneal permeation of the drugs under study

Enhancer (conc. % w/w)	HL[a] % (±S.E.)	Apparent permeability coefficients (P_{app}) cm/s·10^6 (±S.E.)			
		AT	TM	LB	BX
GBR[b]	80.4 (0.6)	3.46 (1.23)	3.63 (1.88)	22.96 (3.43)**	14.71 (3.61)**
BZ (0.02)	85.6 (1.7)*	18.04 (3.04)*	9.61 (1.49)	22.76 (6.62)	19.23 (2.70)*
SAP (0.05)	90.0 (1.1)*	56.92 (14.01)*	40.06 (6.30)*	30.27 (3.55)	30.06 (0.73)*
DIG (0.0025)	85.7 (0.6)*	7.72 (3.70)	15.27 (2.67)*	20.40 (2.46)	19.85 (1.20)*
ESC (0.05)	89.0 (0.9)*	23.50 (4.10)*	28.96 (7.36)*	34.65 (3.45)*	16.02 (2.69)
BL-9 (0.1)	87.7 (0.6)*	11.74 (1.86)	26.47 (11.97)*	17.45 (2.70)	16.52 (0.48)
Brij® 35 (0.05)	82.1 (1.5)	36.44 (3.46)*	5.52 (1.33)	16.39 (4.28)	17.77 (17.70)
Brij® 78 (0.05)	82.5 (1.7)	13.39 (3.47)	14.16 (3.31)*	27.63 (2.19)	14.17 (2.50)
Brij® 98 (0.05)	82.7 (1.3)	2.97 (1.73)	3.20 (0.42)	12.90 (3.18)	29.29 (2.35)*
EDTA (0.5)	80.9 (0.8)	4.95 (4.46)	1.72 (0.37)	1.39 (0.67)*	1.86 (0.08)*
DC (0.05)	84.0 (1.1)*	6.69 (2.23)	19.03 (4.29)*	31.83 (3.00)	31.95 (4.73)*
UDC (0.05)	82.4 (0.9)	2.97 (2.48)	7.69 (2.35)*	20.52 (3.77)	23.64 (3.34)*
TDC (0.05)	81.9 (1.5)	20.21 (3.10)*	5.77 (2.73)	12.88 (3.16)	16.94 (2.50)
TUDC (0.05)	80.9 (1.2)	10.42 (3.48)	3.29 (1.61)	34.37 (8.54)	11.16 (2.28)

*Significantly different ($P<0.05$) from the control (GBR buffer, no enhancer).
**Significantly different ($P<0.05$) from AT and TM in GBR buffer (no enhancer).
[a]Corneal Hydration Level.
[b]Glutathione Bicarbonate Ringer (GBR) buffer (no enhancer).

different from one another. The graph in Fig. 2 illustrates the relationship between P_{app} and PC for the drugs, alone and in the presence of some enhancers (SAP, Brij 78, DC and BZ). The dependency of corneal permeation on the lipophilic character of the drugs, and the greater influence of the enhancers on transcorneal permeation of the more hydrophilic ones is clearly apparent.

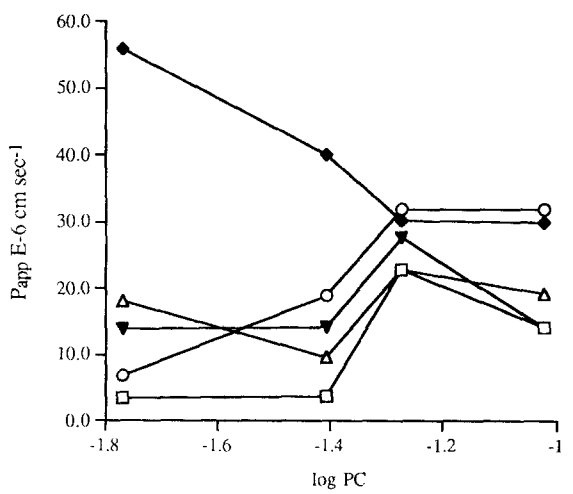

Fig. 2. Graph illustrating the influence of partition coefficient (log PC) on the permeability coefficient (P_{app}) of the four drugs, in the absence (□, GBR buffer) and in the presence of some permeation enhancers: (△) BZ; (▼) Brij 78; (○) DC; (◆) SAP.

In general, the tested enhancers increased the permeation rates of the more hydrophilic drugs, AT and TM, more than those of the other two, more lipophilic ones, LB and BX. To mention only the statistically significant results, the P_{app} value of AT was enhanced 16.4-fold by 0.05% SAP, 10.5-fold by 0.05% Brij® 35, 5.8-fold by 0.05% TDC, and 5.2-fold by 0.02% BZ. In the case of TM the significant P_{app} enhancements were 11.1-fold by SAP, 7.9-fold by ESC, 5.2-fold by DC, 4.2-fold by BL-9, 3.9-fold by Brij® 78, and 2.1-fold by UDC (all at the 0.05% concentration).

The enhancers had little effect on transcorneal penetration of LB: the only significant P_{app} increase was produced by ESC (1.51-fold). In the case of BX, small but significant P_{app} increases were produced by BZ and DIG (1.3-fold), SAP (2.0-fold), Brij® 98 (2-fold), DC (2.3-fold) and UDC (1.6 fold).

Surprisingly, in the present study EDTA was devoid of enhancing activity for AT and TM, and produced a significant reduction (16.5- and 7.9-fold,

Table 3
Effect of bile salts at different concentrations on transcorneal permeation of timolol

Enhancers	HL% ± S.E.	Apparent permeability coefficients (P_{app}) (cm/s · 10^6 ± S.E.)	Relative permeability
GBR	80.4 ± 0.6	3.63 ± 1.88	1
DC (0.025%)	83.0 ± 1.2	8.97 ± 2.13	2.47
DC (0.05%)	84.0 ± 1.9	19.03 ± 4.29*	5.32
DC (0.075%)	88.7 ± 3.1*	29.69 ± 1.51*	8.18
DC (0.1%)	91.0 ± 4.5*	30.06 ± 5.92*	8.28
TDC (0.05%)	81.8 ± 1.4	5.77 ± 2.73	1.59
TDC (0.075%)	82.8 ± 2.2	20.08 ± 4.27*	5.53
TDC (0.1%)	83.9 ± 3.2	18.99 ± 3.39*	5.23
UDC (0.05%)	82.4 ± 1.0	7.69 ± 2.35	2.12
UDC (0.075%)	88.0 ± 4.0*	30.02 ± 5.02 *	8.27
UDC (0.1%)	89.0 ± 4.3*	40.08 ± 0.76 *	11.04
TUDC (0.05%)	80.9 ± 1.2	3.29 ± 1.61	0.91
TUDC (0.075%)	82.2 ± 1.6	3.48 ± 1.64	0.96
TUDC (0.1%)	83.0 ± 3.0	12.07 ± 1.60	3.32

*Significantly ($P < 0.05$) different from the control (GBR buffer).

respectively) of the permeability coefficients of LB and BX. These results are in partial agreement with those of Ashton et al. (1991a,b), who found that 0.5% EDTA showed some enhancing effect for TM and AT, but reduced 1.6-fold the transcorneal flux of LB. Sasaki et al. (1995b), however, in similar experiments reported small but significant P_{app} increases for timolol and for the more lipophilic befunolol (2.3- and 1.6-fold, respectively) caused by 0.5% EDTA. As pointed out by Ashton et al. (1991b), EDTA, a calcium chelator mainly active on the tight junctions, may produce ultra-structural changes in the corneal epithelium, resulting in a water influx and decrease of the overall lipophilic characteristics. This effect might account for the observed permeability reduction observed in the case of the more lipophilic drugs. The contradictory literature reports on the effect of EDTA might possibly be rationalised in terms of different times of contact between the promoter and the corneal tissue, and/or different experimental pH. Further studies will be necessary to clarify this controversial point.

Analysis of Tables 3 and 4, reporting the effect on corneal permeation of TM of different concentration of bile salts and heteroglycosides, shows that only 0.075–0.1% TDC, 0.015% ESC and 0.0025% DIG produced significant P_{app} increases over the control solution, without significantly increasing the HL value.

3.2. Effect of the enhancers on the corneal hydration level (HL)

The percent corneal hydration is a parameter frequently used to evaluate the damage of this tissue. According to Maurice and Riley (1970) the normal cornea has an hydration level of 76–80%. As indicated by Schoenwald and Huang (1983), an 83–92% hydration level, i.e., 3–7 percent units or more over the 'normal' value, denotes damage of the epithelium and/or endothelium. However, there are exceptions to this rule: according to Grass and Robinson (1988) high concentrations (> 0.5%) of EDTA may produce substantial damage and expansion of the intercellular spaces of the corneal epithelium without influencing the normal hydration level.

In the present study, the average hydration level ± S.E. of freshly excised corneas was 80.1% ± 0.9: as shown in Table 2 the same value (80.4% ± 1.1) was obtained from the corneas retrieved at the end of permeation runs in the absence of added enhancers. Higher hydration levels were observed in

Table 4
Effect of the heteroglycosides at different concentrations on transcorneal permeation of timolol

Enhancers	HL% ± S.E.	Apparent permeability coefficients, (P_{app}) (cm/s · 10^6 ± S.E.)	Relative P_{app} values
GBR	80.4 ± 0.6	3.63 ± 1.88	1
SAP (0.010%)	84.0 ± 1.8	7.68 ± 2.20	2.11
SAP (0.015%)	87.0 ± 3.2*	12.07 ± 3.31	3.32
SAP (0.025%)	90.0 ± 4.7*	30.05 ± 1.60*	8.28
SAP (0.050%)	90.0 ± 1.1*	40.06 ± 6.30*	11.03
ESC (0.005%)	81.5 ± 1.5	9.61 ± 3.63	2.65
ESC (0.010%)	82.0 ± 1.9	11.96 ± 3.20	3.29
ESC (0.015%)	83.4 ± 2.1	16.08 ± 5.50*	4.43
ESC (0.025%)	87.4 ± 3.6*	24.99 ± 2.79*	6.88
ESC (0.050%)	89.0 ± 1.0*	28.96 ± 7.36*	7.98
DIG (0.0010%)	81.0 ± 1.4	12.13 ± 5.59	3.34
DIG (0.0025%)	85.7 ± 0.8	15.27 ± 2.67*	4.21
DIG (0.005%)	86.9 ± 1.9*	20.09 ± 4.28*	5.53
DIG (0.010%)	88.3 ± 3.3*	20.09 ± 4.28*	5.53
DIG (0.015%)	88.8 ± 3.4*	22.43 ± 3.63*	6.18

*Significantly ($P<0.05$) different from the control (GBR buffer).

the presence of all enhancers. However, the percent HL increase produced by the three surfactants of the Brij® series and by UDC, TDC and TUDC (concentration, 0.05%) was less than 3 units, and not statistically different from the control. EDTA (0.5%) did not increase significantly the HL value, but at the same time, as said before, it showed no influence on permeation of AT and TM, and exerted a significantly negative effect on permeation of LB and BX. BZ (0.02% w/w, the maximal concentration used as preservative in eyedrops) brought the HL value to 85.6: this agent showed a statistically significant permeation enhancing effect only in the case of AT and BX: the effect was more marked in the case of AT, the most hydrophilic drug of the series. The heteroglycosides SAP and ESC at the 0.05% concentration raised the HL value to 89–90%: a (statistically significant) increase in the 10 units range relative to the physiological value. DIG showed a significant ($p < 0.05$) damaging effect already at the 0.0025% concentration (HL = 85.7%).

A more detailed study on the effect of different concentrations of bile salts on transcorneal permeation of TM, illustrated in Table 3, shows that all agents augmented the HL values with increasing concentration, but statistically significant differences with respect to the controls were observed only for DC and UDC at concentrations above 0.075%. The bile salts which enhanced the permeability coefficient (Papp) of TM to a significant extent (5.3–11.0 folds) without significantly increasing the HL value with respect to the controls were DC 0.05% and TDC 0.075 and 0.1%.

A similar study on the effect of different concentrations of the three heteroglycosides on permeation of TM is illustrated in Table 4. A lower concentration range was used in these studies for DIG with respect to SAP and ESC, since DIG concentrations over 0.015% w/w caused significant swelling and opacification the cornea at the end of the experiments. The results of these tests indicate that SAP produced a significant damaging effect already at the 0.015% concentration (HL, 87.0; $p < 0.05$), while 0.015% ESC and 0.0025% DIG, while not significantly raising the corneal hydration level (83.4 and 85.7%, respectively), increased slightly (4.4- and 4.2-fold), but significantly, the P_{app} of TM. All other statistically significant P_{app} increases of TM observed with the heteroglycosides (relative permeability 6.2–11.0) were associated with high (87–90%) HL values.

An overall view of the relationship between corneal hydration and permeation-enhancing effect of the tested agents is presented in the graph in Fig. 3, based on data from Table 2. The

horizontal axis reports the P_{app} increases for the different drugs + enhancers, relative to the corresponding values in the absence of enhancers, considered equal to unity. The dotted horizontal line in the graph coincides with the 83% corneal hydration level, i.e. the maximal level attainable without producing an irreversible damage (Schoenwald and Huang, 1983). Thus, all points situated below the line can be assumed to correspond to a 'safe' combination of drug + enhancer, even if, as said before, in some cases higher HL values, e.g. 85.7% produced by 0.0025% DIG, were not statistically different from the 'normal' (80.4%) HL value. Inspection of the graph shows that some bile salts (TDC, UDC, TUDC) and two non-ionic surfactants (Brij® 35 and 78), while being active as promoters, did not increase the corneal HL level beyond the safety level. Of particular interest is the permeation-promoting activity of Brij® 35 for AT. The inclined dotted line in the graph indicates the rough correlation existing between corneal hydration and transcorneal drug permeation: with some exceptions (AT + 0.05% Brij-35 or + 0.05% TDC) the increased permeation produced by the enhancers corresponds to an increased hydration of the corneal tissues.

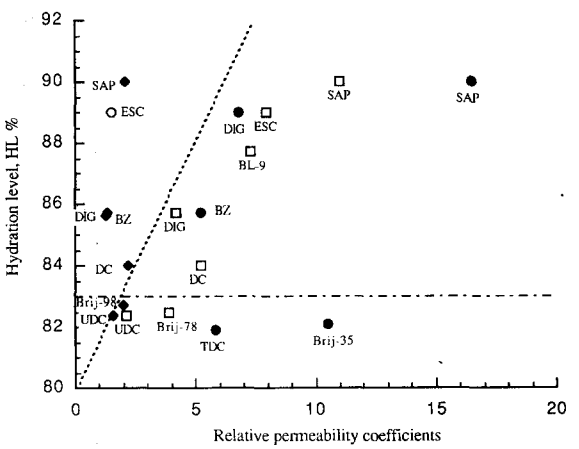

Fig. 3. Relationship between the relative permeability coefficients of the four drugs in the presence of penetration enhancers and the corresponding corneal hydration levels (data from Table 2). Key: (●) AT, (□) TM, (○) LB and (◆) BX.

Table 5
In vivo ocular irritancy of the enhancers at different concentrations (sums of Draize test scores at different observation times)

Enhancer	Concentration (% w/v)					
	0.062	0.125	0.25	0.5	1.0	2.0
DIG	36	68	146	—	—	—
ESC	58	80	148	—	—	—
SAP	—	—	8	30	56	—
DC	—	—	36	68	148*	—
TDC	—	—	—	24	24	—
UDC	—	—	—	4	16	—
TUDC	—	—	—	0	2	—
Brij 78	—	—	—	—	2	8
BL-9	—	—	—	32	74	88[a]
EDTA	—	—	—	0	2	

[a]Corneal lesion.

3.3. *In vivo ocular irritancy*

The results of the in vivo ocular irritancy studies are summarised in Table 5, where the total Draize scores (sum of the test scores calculated for each time point: 0.5–24 h) resulting from treatment with different concentrations of each permeation promoter are reported. The data, plotted as irritation scores at different times vs. the corresponding observation times (30, 60, 120, 240 and 360 min) were also used to calculate the relevant AUC values: these are reported as bar

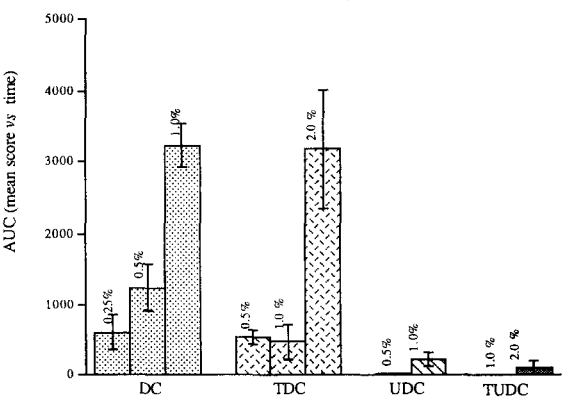

Fig. 4. AUC values (Draize test scores vs. time) for in vivo ocular tolerance of bile salts at different concentrations. Error bars represent S.E. ($n = 3$).

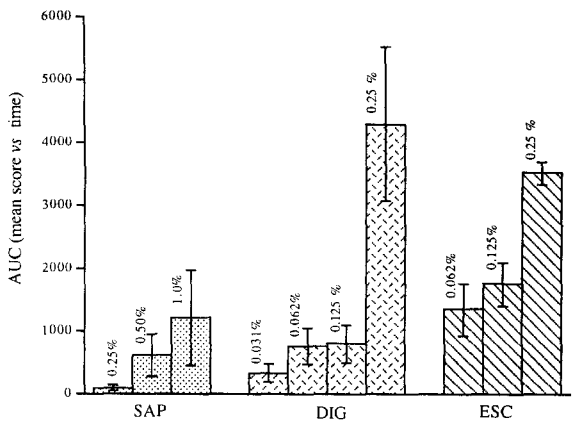

Fig. 5. AUC values (Draize test scores vs. time) for in vivo ocular tolerance of heteroglycosides at different concentrations. Error bars represent S.E. ($n = 3$).

graphs in Fig. 4 (bile salts), Fig. 5 (heteroglycosides) and Fig. 6 (non-ionic surfactants). The studies were performed on all enhancers with the exception of BZ, Brij® 35 and Brij® 98.

Among the bile salts (Fig. 4), DC was the most irritant: it produced a dose-related effect consisting of conjunctival (concentrations, 0.25 and 0.5%) and corneal damage (concentration, 1.0%). TDC appeared well tolerated up to the 1% concentration, but its irritant effect rose sharply at the 2% concentration. The irritant action of this compound was not dose-related between 0.5 and

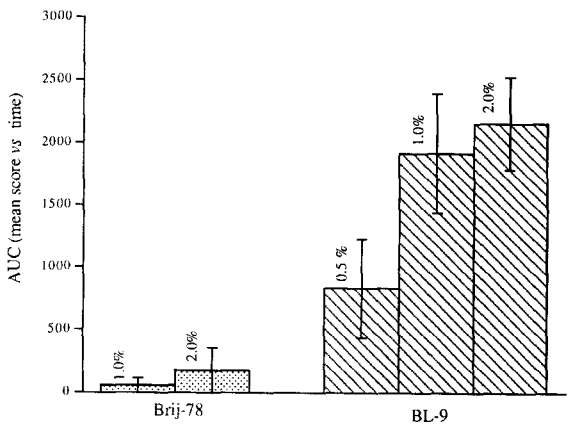

Fig. 6. AUC values (Draize test scores vs. time) for in vivo ocular tolerance of Brij 78 and BL-9 at different concentrations. Error bars represent S.E. ($n = 3$).

1%. UDC (0.5 and 1.0%) and TUDC (1.0 and 2.0%) produced moderate or no irritant effect. At the 0.5% concentration, DC, TDC, UDC and TUDC were significantly different from one another, their irritant activity decreasing in the same order.

Among the saponins (Fig. 5), SAP appeared in general better tolerated: it showed a slight irritant activity at 0.25%, and its irritant AUC value at the 1.0% concentration was not statistically different from those of 0.062 and 0.125% DIG, and those of 0.062 and 1.125% ESC. DIG and ESC showed a very similar ocular irritating effect, which at the highest tested dose (0.25%) was comparable to that produced by 1.0% DC. However, contrary to 1.0% DC, 0.25% DIG and ESC did not produce any corneal damage. Furthermore, 0.25% DIG and ESC, while not differing significantly from one another, were significantly more irritant than 1.0% SAP.

Brij® 78 (Fig. 6) was practically devoid of ocular toxic effects at the maximal tested dose (2.0%), while BL-9 was considerably more irritant; however its effect (not statistically different at the 1.0 and 2.0% concentrations) was significantly lower when compared with similar concentrations of DC and TDC.

The irritant effect in vivo of 0.02% BZ, 0.5% Brij® 35, 0.5% Brij® 98 and EDTA, tested in separate experiments and not reported in the present paper, was practically negligible.

3.4. Conclusions

As a brief premise, some facts and data in the literature relevant to the present study are worth mentioning. The epithelium constitutes the rate-limiting layer in corneal penetration of the more hydrophilic β-blockers: according to Klyce and Crosson (1985) the barrier resides on the very surface of the epithelium, due to the presence of annular tight-junctions or zonulae occludentes. Shih and Lee (1990) in particular, reported an increased penetration in vitro of AT after corneal de-epithelization, while penetration of BX was not affected. It is generally agreed that permeation promoters act by modifying the permeability characteristics of the superficial epithelial cells,

i.e. by facilitating paracellular transport through 'loosening' of the tight junctions (Hochman and Artursson, 1994). These agents, with the exception of EDTA, a Ca^{++} chelator, belong to the general class of surfactants: because of their hydrophilic/lipophilic character they are absorbed on the epithelium, where they may change the physical properties of the cell membranes by removal of phospholipids and also by membrane solubilization.

Benzalkonium chloride (0.005–0.02%) while effective in increasing the corneal permeability in vitro and in vivo of many drugs, is known to cause morphological changes in the epithelium (Green and Tonjum, 1975; Burstein and Klyce, 1977). Saponin (0.5%) was found effective as enhancer for corneal penetration of β-blockers (Sasaki et al., 1995b), and (1.0%) as promoter of systemic absorption of insulin administered via eyedrops (Chiou and Chuang, 1989; Pillion et al., 1994). Digitonin greatly increased the corneal absorption in vitro of a series of different molecular weight polyethylene glycols, but caused significant alteration or complete removal of the epithelial layer (Liaw and Robinson, 1992). Rojanasakul et al. (1990), using laser scanning confocal microscopy and electrophysiological techniques confirmed that all enhancers (in particular, EDTA, digitonin and sodium deoxycholate) significantly increase corneal permeability but may also cause severe cellular membrane damage.

The present investigation confirmed the low corneal permeability of the more hydrophilic β-blockers (atenolol, timolol), and the apparent cellular toxicity of many effective enhancers, as evidenced by their capacity of increasing the corneal hydration level beyond the 'normal' value. However, some of the tested agents proved effective and safe, at least within the limits of the testing methods used in this study. The P_{app} of AT was increased significantly by 0.05% Brij® 35 and TDC (over 10- and 5-fold, respectively) without significantly affecting the corneal hydration level. In the case of TM, Brij® 78 and UDC, at the same concentration (0.05%) produced more modest P_{app} increases (about 4- and 2-fold, respectively), equally without affecting the corneal hydration. BX penetration was safely but scarcely promoted (P_{app} increase, 2- and 1.6-fold, respectively) by Brij® 98 and UDC (both 0.05%). The potential interest of the above-mentioned enhancers is stressed by the fact that, at the 0.05% concentration, none of them induced irritant activity in vivo. Penetration tests in vivo and cellular toxicity tests in vitro are currently carried out in the attempt to further define the limits of practical applicability of these promoters.

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