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Effect of iontophoresis on transcorneal permeation 'in vitro' of two β-blocking agents, and on corneal hydration

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Abstract

Purpose of the present investigation was to examine the effect of iontophoresis on permeation of two β-blocking agents, timolol maleate (TM) and betaxolol hydrochloride (BX) across rabbit corneas in vitro. Continuous or pulsed current of variable intensity and duration was applied, and possible corneal damage due to the electric treatment was assessed by measuring the corneal hydration level. The effect of iontophoresis on corneal permeation of the relatively more hydrophilic TM was much greater than the effect on the more lipophilic BX. It was found that for both drugs the iontophoretically driven transcorneal penetration is governed only by current density and overall time of treatment, irrespective of the type of treatment (single or repeated) and of current (constant or pulsed). For both drugs all significant permeation increases due to iontophoresis were invariably accompanied by a significant increased corneal hydration, indicative of damage to the corneal epithelium. Even if the present in vitro data cannot be extrapolated to an in vivo treatment, they confirm the potential risk associated with ocular iontophoresis.

Keywords: Rabbit cornea; Iontophoresis in vitro; Corneal hydration; Corneal toxicity; Timolol; Betaxolol

1. Introduction

Drug delivery to the inner eye still constitutes a critical problem in ocular therapeutics. Deep ocular fluids and tissues cannot be efficiently reached by topical administration, while ophthalmic drugs administered systemically have poor access to the inner eye because of the blood–aqueous and blood–retinal barriers. Subconjunctival and retrobulbar injections do not produce adequate drug levels, while direct intracameral or intravitreal delivery is an invasive procedure potentially involving serious intraocular complications.

Iontophoresis, which consists of applying a weak electrical current to drive charged drug molecules across tissue barriers, is one of the proposed alternative techniques for delivery of therapeutic drug doses to the inner eye. Several applications in experimental and clinical ophthalmology of iontophoresis, particularly for the treatment of bacterial/viral infections, inflammations, glaucoma, etc., have been reported (Hughes

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and Maurice, 1984; Rootman et al., 1988a; Grossman et al., 1990; Hill et al., 1993; Sarraf and Lee, 1994; Behar-Cohen et al., 2002). Ocular iontophoresis, although effective, noninvasive and associated with minimal discomfort for patients, is not entirely harmless for ocular tissues (Rootman et al., 1988b; Grossman and Lee, 1989). This justifies further studies aimed at evaluating its scope and limitations.

Purpose of the present investigation was to examine the effect of continuous or pulsed current, of variable intensity and duration, on permeation of two β-blocking agents across rabbit corneas in vitro. A concomitant purpose was the assessment of possible tissue damages due to the treatment. These were evaluated by measuring the corneal hydration level, which is a sensitive indicator of damage of this tissue (Dohlman, 1987). Timolol maleate (TM) and betaxolol hydrochloride (BX) were chosen as model drugs on account of their different *n*-octanol/water apparent distribution coefficients.

2. Materials and methods

2.1. Materials

TM and BX were from Sigma-Aldrich Srl (Milano, Italy). All other chemicals, reagents, solvents etc. were of analytical grade.

2.2. Animals

New Zealand albino rabbits (Harlan-Nossan, Milan, Italy), weighing 2.5–3.0 kg, were employed. 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 rabbits 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). All experiments conformed with the ARVO 'Statement for the use of animals in ophthalmic and visual research', and were carried out under veterinary supervision after approval of the protocols by the ethical-scientific committee of the University of Pisa.

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 cell (Camber and Edman, 1987) modified to contain two ports for introduction of electrodes on each corneal side. The cell was maintained at 35±1 °C, and the corneal area available for diffusion was 0.78 cm^2. Preheated (35 °C) glutathione bicarbonate Ringer buffer (GBR) was added to both 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 per s. After 10 min equilibration the solution on the epithelial side was withdrawn and substituted with 1.0 ml of a 3.0 mM solution of the test drug in GBR. To ensure sink conditions, at appropriate time intervals 1.0 ml of the receptor solution was withdrawn for analysis, and replaced with an equal volume of fresh preheated buffer. Each experiment had a 4.0 h duration, and was repeated at least six times.

The iontophoresis setup consisted of a custom-made current generator, capable of delivering constant or pulsed direct current (DC) of different intensity, a 15 kΩ precison resistor and an auto-ranging microvolt digital multimeter (Model 197A, Keithley Instruments, Inc., Cleveland, OH, USA). The current was applied using AgCl-coated silver wires (diameter 1.0 mm) for the cathode and the same electrodes, electrochemically reduced in sodium chloride solution, for the anode (Laneri et al., 1999). In iontophoretic transport experiments, the anode was introduced in the donor (epithelial) compartment and the cathode in the receptor (endothelial) compartment of the cell.

The current was applied following two different procedures: (a) only in the initial phase of experiments, for 10 or 40 min (single treatment, ST); or (b) during the whole experiment, for 0.5, 5.0, or 10.0 min followed by 30 min intervals (repeated treatment, RT). Two main types of current were applied: (a) constant direct current (CDC), intensity ranging between 0.5 and 5.0 mA; or (b) pulsed

current with square waveform, PDC (intensity, 5.0 mA; period, 500 ms; on/off ratio, 1/2; frequency, $v = 0.5$ Hz) (Liu et al., 1988). The combination of different parameters gave eight iontophoretic treatments, summarized in Table 1.

2.4. Analytical methods

The drug concentration in the samples was determined by HPLC (liquid chromatograph with LC 6A pump and 20 μl Rheodyne injector, SPDM6A detector and computer integrating system, Shimadzu Corp., Kyoto, Japan). The column (30 cm × 3.9 mm) was packed with μ-Bondapack C18 (pore size 10 μm, Waters, USA-Milford, MA). TM analysis was carried out using 60:40 v/v methanol-0.05 M pH 3.5 phosphate buffer as mobile phase (flow rate 1.0 ml/min): the retention time and the detection wavelength were 4.9 min and 294 nm. In the case of BX the mobile phase (flow rate = 1.0 ml/min) was 10:30:60 v/v 0.1 M pH 3.0 phosphate buffer:acetonitrile:water. The retention time and the detection wavelength were 6.0 min and 221 nm.

2.5. Evaluation of corneal hydration levels

At the end of each experiment the corneas were removed from the perfusion apparatus and the percent corneal hydration level was evaluated by gravimetric analysis. Wet corneal weights, W_w, were obtained after careful removal of the scleral ring; each corneal sample was then desiccated at 100 °C for 12 h to give the corresponding dry corneal weight, W_d. The percent corneal hydration level (HL%), defined as $[1-(W_d/W_w)]100$ was determined on corneas recovered from permeation tests performed both in the absence and in the presence of electric current.

2.6. Data analysis

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

$$P_{app} = \frac{\Delta Q}{\Delta t C_o A 3600}$$

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

The enhancement factors (EF), expressing the relative activity of each treatment, were calculated from the ratio $P_{app}(b)/P_{app}(a)$, where $P_{app}(b)$ and $P_{app}(a)$ are the apparent permeability coefficients in presence and in absence of treatment, respectively.

Linear regression analyses (correlation coefficients and slopes) were performed using STATVIEW 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 protected least significant difference (PLSD) test (Zar, 1984). In Table 2 an asterisk (*) indicates a significant difference at the $P < 0.05$ level.

3. Results

The transcorneal permeation data for TM and BX under different iontophoretic conditions are listed in Table 2. The Table reports, for each treatment, the apparent permeability coefficients P_{app} and the EF values; all data are the average of at least six determinations.

The two different types of treatment (repeated, RT, and single, ST) were designed to evaluate the relative influence of current intensity and treatment duration on transcorneal permeation of the two drugs. As shown in the Table, when the corneas were treated for 0.5 min with CDC of 0.5 or 1.0 mA intensity, at 30 min intervals (RTs 1 and 2) TM permeation increased 1.8 and 2.4 times, respectively, when compared with the untreated control (difference not statistically significant).

When the exposure time was increased from 0.5 to 5.0 min, while maintaining a CDC of 1.0 mA intensity (RT 3 vs. 2) the apparent permeation rate of TM was unchanged. However, when the CDC intensity was raised to 2.0 mA (RT 4 vs. 3) a significant increase of the EF (4.3-fold) was

Table 1
Experimental conditions used in the iontophoretic tests

Treatment number	Current type	Current intensity (mA)	Current density (mA/cm^2)	Single treatment (ST)	Repeated treatment (RT)	Duration of application (min)
1	Constant (CDC)	0.5	0.64	–	√	0.5
2	Constant (CDC)	1.0	1.28	–	√	0.5
3	Constant (CDC)	1.0	1.28	–	√	5.0
4	Constant (CDC)	2.0	2.56	–	√	5.0
5	Constant (CDC)	2.0	2.56	√	–	10.0
6	Constant (CDC)	5.0	6.41	√	–	10.0
7	Pulsed (PDC)	5.0	6.41	√	–	40.0
8	Pulsed (PDC)	5.0	6.41	–	√	10.0

Table 2
Transcorneal permeation (Papp) and corneal hydration (HL%) data for TM and BX under different iontophoretic conditions

Treatment number	TM Papp $10^6 \pm$S.E. (cm/s)	TM EF	TM HL%$\pm$S.E.	BX Papp $10^3 \pm$S.E. (cm/s)	BX EF	BX HL%$\pm$S.E.
Control	2.3$\pm$0.9	1.0	81.0$\pm$0.4	15.0$\pm$1.1	1.0	80.4$\pm$0.4
1	4.2$\pm$0.8	1.8$\pm$0.3	81.2$\pm$0.4	–	–	–
2	5.5$\pm$0.5	2.4$\pm$0.2	81.5$\pm$0.3	–	–	–
3	5.3$\pm$0.5	2.3$\pm$0.2	81.7$\pm$0.3	–	–	–
4	10.0$\pm$1.7*	4.3$\pm$0.7	83.3$\pm$0.5*	24.3$\pm$0.3*	1.6$\pm$0.2	83.0$\pm$0.2*
5	3.8$\pm$0.2	1.6$\pm$0.1	81.8$\pm$0.3	18.1$\pm$0.2	1.2$\pm$0.1	82.0$\pm$0.3
6	10.1$\pm$0.1*	4.4$\pm$0.6	83.0$\pm$0.2*	25.6$\pm$0.1*	1.7$\pm$0.2	83.3$\pm$0.4*
7	17.5$\pm$4.2*	7.5$\pm$1.8	83.3$\pm$0.2*	26.1$\pm$0.5*	1.7$\pm$0.3	83.4$\pm$0.3*
8	18.5$\pm$1.7*	7.9$\pm$0.7	83.8$\pm$0.3*	24.6$\pm$0.3*	1.6$\pm$0.2	83.8$\pm$0.3*

*, Significantly different from control ($P < 0.05$, Fisher PLSD test).

observed. A single 10 min treatment (10 min, 2.0 mA) with CDC (treatment 5) had a small, non-significant influence on the permeation rate of TM, while a significant effect with respect to the control (EF = 4.4) was observed in ST when the CDC intensity was raised to 5.0 mA (treatment 6). In treatments 7 and 8 pulsed current (PDC) of 5.0 mA intensity instead of CDC was applied: in the first case the treatment was single (40 min), and on the other it was repeated (10 min, with 30 min intervals). In both cases significant EF increases of TM permeation were observed (7.5- and 7.9-fold, respectively, for treatments 7 and 8).

In the case of BX, the transcorneal permeation rate in the control experiments was faster than that of TM, in agreement with the greater lipophilic character of BX (apparent octanol/buffer distribution coefficients for TM and BX, 8.9 and 32.4, respectively), and with the well-known correlations between corneal permeability and drug lipophilicity (Ashton et al., 1991; Schoenwald, 1993; Saettone et al., 1996). Treatments 1–3, which had proven poorly effective with TM, were omitted for BX. As in the case of TM, iontophoretic treatments 4, 6, 7 and 8 produced significant increases of the permeation rate of BX; the EF, however, were modest and ranged between 1.6 and 1.7. For both drugs, the best overall results in terms of increased permeation rate were observed with treatment 8 (10 min RT with pulsed current).

At the end of each experiment the percent hydration level (HL%) of the corneas was determined in order to assess possible corneal damages due to the applied electric field. This parameter is a sensitive indicator of corneal alteration (Schoenwald and Huang, 1983; Monti et al., 2002). According to Maurice and Riley (1970) the normal corneal hydration level is 76–80%, and, as indicated by Schoenwald and Huang (1983) 83–92% hydration level, i.e. 3–7% units or more over the 'normal' value, denotes damages of the epithelium and/or endothelium. The HL% values of corneas submitted to different iontophoretic treatments in the experiments with TM and BX are also listed in Table 2. The control corneas, maintained in contact with the drug solutions for 4 h in the absence of current, showed an hydration level of 81.0$\pm$0.4% for TM and 80.4$\pm$0.4% for BX. An assumption of damage was made when the observed hydrations were significantly different from the respective control values.

All significant permeation increases due to iontophoretic treatment, both for TM and for BX (treatments 4, 6, 7 and 8) were invariably accompanied by a significant increase of corneal hydration: the most effective treatments raised the HL% to about 83%.

4. Discussion

The present investigation showed that some iontophoretic treatments (in particular treatments 6, 7 and 8 where constant or pulsed current of 5.0 mA intensity was used) significantly increased

transcorneal permeation of TM and BX. The effect of iontophoresis on transcorneal permeation of TM was greater than for BX, and this can be attributed to the relatively greater hydrophilicity of the former drug. A similar effect had been observed in a study on the activity of some corneal penetration enhancers on transcorneal penetration in vitro of four beta-blocking agents (Saettone et al., 1996). The enhancers (different types of surface-active agents, acting on the integrity of the corneal epithelium) increased the permeation rates of the more hydrophilic drugs, atenolol and TM, more than those of the other two, more lipophilic ones, levobunolol and BX. This was attributed to disruption of the lipophilic corneal epithelium, which constitutes the main obstacle to permeation of hydrophilic drugs. Alteration of this barrier increases the passage of hydrophilic drugs (whose penetration occurs mainly through a restricted 'pore', or aqueous diffusional pathway) while affecting to a lesser extent the permeation of lipophilic ones (cf. Grass et al., 1988).

According to Hughes and Maurice (1984) the following equation quantitates the amount of drug, Md, that penetrates the epithelium after transcorneal iontophoresis:

$$\mathrm{Md} = \frac{i\mathrm{PdCd}t}{F(\mathrm{PdCd} + \mathrm{PiCi})}$$

where i and t are the current density and the duration of iontophoresis, respectively; F is the Faraday constant, Pd is the permeability of tissue to the drug, Cd is the drug concentration, and Ci and Pi are the concentration of additional ionized substances competing with the drug to carry the current, and their tissue permeability, respectively. A plot of the EF values for TM and BX versus the product of i and t (the variables governing penetration) is presented in Fig. 1. As the graph suggests, for both drugs the iontophoretically driven transcorneal penetration is governed only by current density and overall time of treatment: there is no apparent influence of type of treatment (single or repeated) and of current (constant or pulsed). The experimental points in the graph corresponding to a statistically significant increase of the corneal hydration level are marked with the

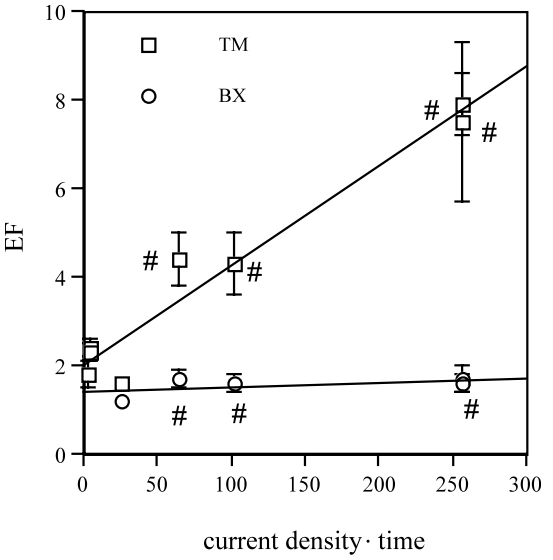

Fig. 1. Influence of the product of current density × time on transcorneal permeation of TM and BX. The points marked with # correspond to a significantly increased corneal hydration.

symbol. It is clearly apparent that only non-significant penetration enhancements could be obtained without concomitant corneal toxicity. The electric field can induce orientation of some constituents of the epithelium, resulting in alteration of corneal texture: this and other factors (locally increased temperature, ions, etc.) may be responsible for the increased corneal hydration associated with increased transcorneal permeation. The contribution to corneal hydration of electroosmotic solvent flow (from anode to cathode) during iontophoretic drug permeation should also be considered. The convective solvent flow across negatively charged membranes (such as the cornea), beside assisting the diffusion of a positively charged permeant, can increase the amount of water in the tissue (Srinivasan et al., 1989; Srinivasan and Higuchi, 1990). Damaging effect of iontophoresis to corneal tissues have been reported by several authors (cf., e.g. Rootman et al., 1988b; Grossman and Lee, 1989; Hughes and Maurice, 1984). As indicated by Behar-Cohen et al. (2002), only using controlled iontophoretic parameters and a low current density clinical or histological lesions can be avoided. In the present in vitro

experiments only a 2.4-fold permeation increase of TM (not statistically significant), and practically no increase of BX permeation could be obtained without a concomitant increase of corneal hydration.

The present data are indicative of the sensitivity of the in vitro cornea to electric treatment. Of course, a profound difference can exist between in vitro and in vivo situations, since the in vivo cornea has some capacity to restore in time its original biological parameters. In any case, even if these results cannot be extrapolated to an in vivo treatment, also in view of the relatively high current densities used, they point to potential toxicity risks associated with iontophoresis.

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