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Increased corneal hydration induced by potential ocular penetration enhancers: assessment by differential scanning calorimetry (DSC) and by desiccation

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Abstract

The corneal toxicity of some surfactants of possible use as ocular penetration enhancers was investigated by measuring their effect on hydration of rabbit corneas 'in vitro'. The tested substances were benzalkonium chloride (BAC), cetylpyridinium chloride (CPC), ethylenediaminetetraacetic acid disodium salt (EDTA), polyoxyethylene-20-stearyl ether (Brij® 78, PSE), polyethoxylated castor oil (Cremophor® EL, PCO) and sodium deoxycholate (DC). Freshly excised corneas, mounted in perfusion cells, were kept in contact for 1 h with solutions of these agents; corneal hydration was then evaluated by measuring: (a) their total (free + bound) water content by desiccation (gravimetric analysis); and (b) their free water content by differential scanning calorimetry (DSC). The DSC measurements also provided a rough quantitative estimate of corneal solutes. All tested agents significantly influenced corneal hydration, evidently as a consequence of alteration of the corneal epithelium. Although a brief contact with the precorneal tissues 'in vivo' may not prove harmful, the use of these compounds as potential ocular permeation enhancers or otherwise as ingredients of topical ocular formulations for long-term use should be considered with caution.

Keywords: Ocular penetration enhancers; Surfactants; Corneal hydration; Corneal toxicity; Rabbit corneas; DSC; Free water; Bound water

1. Introduction

A sensitive indicator of corneal damage is an increased hydration of this tissue. The main factors controlling corneal hydration, which depends on the water-holding capacity of proteoglycans filling the spaces between collagen fibrils, are the barrier functions of the epithelium and endothelium, the swelling pressure of the stroma, and the endothelium water-pumping mechanism. Intraocular pressure and fluid evaporation from the corneal surface appear to be of lesser importance. When excess fluid penetrates into the stroma as a result of epithelial damage, the ability of proteoglycans to bind water and expand results in increased hydration and corneal edema (Dohlman, 1987).

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The water content of corneal tissue has been assessed by different techniques. These include desiccation (Midelfart, 1987; DeMali and Williams, 1994; Saettone et al., 1996; Doughty, 1999), DSC (differential scanning calorimetry, Castoro et al., 1988; DeMali and Williams, 1994), refractive index measurements (Maurice and Riley, 1970), optical sectioning (Wilson et al., 1984), measurement of thickness changes by ultrasound pachimetry (Chan et al., 1983), Raman spectroscopy (Bauer et al., 1998), NMR and P-NMR spectroscopy (Master and Subramanian, 1982; Kiener et al., 1984; Ceckler et al., 1991; Cheng et al., 1992).

In a previous paper (Saettone et al., 1996) the increased corneal hydration induced by a series of prospective ocular penetration enhancers (benzalkonium chloride, EDTA, non-ionic surfactants, surface-active heteroglycosides and bile salts) was investigated 'ex vivo' on rabbit corneas, using a gravimetric method involving desiccation of the tissue. In continuation of the previous study, this paper reports an investigation where DSC, in conjunction with desiccation, was used to assess corneal hydration. DSC, still very little used in corneal studies, can provide detailed information on the amount and state of water present in animal tissues, by distinguishing free (freezable) from bound (non-freezable) water contents (Aktas et al., 1997). In addition, it can provide a rough quantitative estimate of solutes present in a tissue through assessment of the melting range (ΔT), which increases with increasing solute levels (Carelli et al., 1986).

By this study it was hoped to obtain additional information on the validity of DSC for detection of corneal damages, and on toxic effects induced in corneal tissues by potential ocular penetration enhancers, some of which are also common ingredients of topical ophthalmic formulations.

2. Materials and methods

2.1. Materials

The following chemicals were used as received: benzalkonium chloride (BAC, FeF Chemicals A/S, Køke, Denmark); ethylenediaminetetraacetic acid, disodium salt (EDTA, Merck KGaA, Darmstadt, Germany); polyoxyethylene (20) stearyl ether (PSE, Brij® 78, Fluka, Buchs, Switzerland); polyethoxylated castor oil (PCO, Cremophor® EL, BASF, Ludwigshafen, Germany); cetylpyridinium chloride (CPC) and deoxycholic acid sodium salt (DC, both from Sigma Chemical Co., St. Louis, MO). All solvents, chemicals, etc. were of analytical grade.

2.2. Animals

Male, New Zealand white rabbits weighing 2.5–3.0 kg (Pampaloni rabbitry, Fauglia, Italy) were treated as indicated in the publication 'Guide for care and use of laboratory animals' (NIH Publication No. 93-23, revised 1985). All treatments were carried out under veterinary supervision, and the protocols were approved by the ethical-scientific committee of the University of Pisa. After i.m. administration of 30 mg/kg ketamine (Inoketam 1000 solution, Virbac s.r.l., France) and 5 mg/kg xylazine (Rompum 2% solution, Bayer AG, Leverkusen, Germany) the rabbits were euthanised with an intravenous lethal dose of sodium pentobarbital (Pentothal sodium, Farmaceutici Gellini, Aprilia, Italy), the eyes were then proptosed and the corneas, with a 2 mm ring of sclera, were immediately excised. Great care was taken to avoid folding the corneas, and to prevent contact of their surfaces with a solid body (Maurice, 1997).

2.3. Treatment of corneas with the substances under study

The excised corneas were mounted in perfusion cells (Camber, 1985) maintained at 32 ± 0.5 °C. The cells, made of acrylic plastic, consisted of a donor compartment (epithelial side, volume 1.0 ml) and a receiving compartment (endothelial side, volume 5.0 ml). The area occupied by the cornea between the two compartments was 0.78 cm^2. After positioning the cornea in the apparatus, 5.0 and 1.0 ml of preheated pH = 6.85 glu-

tathione bicarbonate Ringer buffer (GBR) were added to the receiving and donor compartments, respectively; to ensure oxygenation and agitation, a mixture of 95% O_2–5% CO_2 was bubbled through each compartment at a rate of 3–4 bubbles/s (Camber, 1985). After allowing the corneal conditions to equilibrate for 10 min, the solution on the epithelial side was withdrawn and substituted with 1.0 ml of a solution of an agent under study in GBR (Table 1). Each perfusion experiment was continued for 1.0 h and was repeated at least six times.

2.4. Evaluation of corneal hydration levels (HL)

The effect of the agents under study on corneal hydration was evaluated by measuring: (a) the total (free + bound) water content of corneas by desiccation (gravimetric analysis); and (b) the free water content by DSC.

2.4.1. Gravimetric analysis

The corneas were removed from the perfusion apparatus; then, after carefully removing any surface water by gently blotting with filter paper, a 9 mm button was trephined from the central portion. The button was divided into two halves: of these one was weighed, desiccated at 100 °C to constant weight (6 h) and weighed again. All tested samples were from different animals.

The percent corneal hydration level (HL%) was obtained as follows:

$$[1 - W_d/W_w] \times 100,$$

where W_d and W_w are the dry and wet corneal weights, respectively.

2.4.2. DSC analysis

DSC measurements were performed using a Mettler TA 3000 Thermal Analysis System, consisting of TC-10 TA processor, DSC20 measuring cell and printer/plotter. At end of each perfusion study, one half of each corneal button, obtained as described in the previous section, was further divided into two halves: a corner of one of these (corresponding to the central cornea) was cut with surgical scissors and immediately weighed to 0.1 mg accuracy (weight range, 4.0–6.0 mg). All tested portions were from different animals. The corneal samples were then hermetically sealed into an aluminium crucible; after refrigerating at − 30 °C for 15 min, DSC curves were obtained by heating the samples at a programmed rate of 2.0 °C/min. The DSC curves recorded the differential heat flow (ΔQ) as a function of time.

The area under the curve gives the heat energy (joules) used to melt the 'bulk' water in the sample. The latent heat of melting (ΔH) in J/g and the interval of fusion (ΔT, difference between temperatures corresponding to complete melting and 10% melting) were determined by the purity analysis method automatically executed by the instrument. The latent heat of melting was converted into the amount of freezable water per gram of sample by calculating the ratio between the heat of fusion of the sample and that of the unit mass of doubly-distilled water ($\Delta H = 333.88$ J/g) determined during the calibration runs.

In both cases (gravimetric and DSC analysis) the smallest amount of detectable water was 0.01 mmoles.

Three typical DSC curves (pure water, treated and untreated cornea) are shown in Fig. 1.

Table 1
Concentrations of the agents tested in the study

Agent	Concentration (% w/v)		
BAC	0.01	0.002	0.001
CPC	0.001		
EDTA	0.5		
PSE	0.005	0.001	1E-5
PCO	0.5		
DC	0.05		

2.5. Statistical analysis

The statistical significance of the differences between means was evaluated using an ANOVA test. The evaluation included group comparisons using the Fisher PLSD test (Zar, 1984). Differences were considered statistically significant at $P < 0.05$.

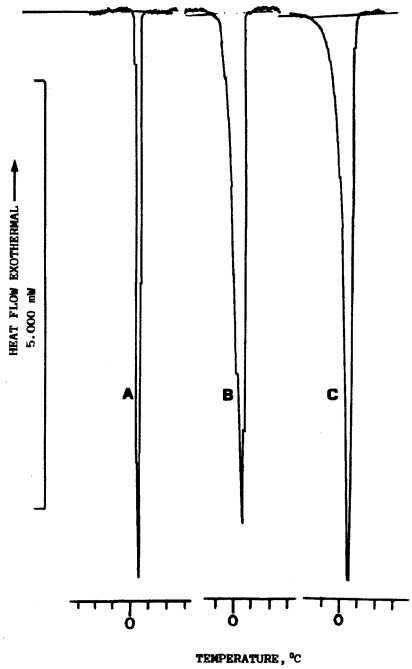

Fig. 1. Typical DSC curves reported as example: A, pure water; B, cornea treated with PSE 0.001%; C, untreated cornea.

3. Results

The percent corneal hydration values determined gravimetrically (after desiccation) and by DSC after 1 h treatment with the test agents are listed in Table 2. The hydration level of corneas kept in contact with GBR alone for 1 h was $81.6 \pm 0.28\%$ by gravimetric analysis, and $71.6 \pm 0.08\%$ by DSC. These values were taken as controls for the data obtained by each technique. The lower DSC hydration value depends on the fact that this technique measures only the 'free' (freezable) water, thawing at 0 °C. Table 2 also lists in Column C the values corresponding to the 'bound' water present in the samples: these values were obtained by subtracting the 'free' water (Column B) from the 'total' water contents (Column A). The gravimetric hydration changes ('total' water) with respect to the controls for all treated corneas are illustrated graphically in Fig. 2.

As indicated in Column A of Table 2 and in Fig. 2, all tested agents, with the exception of CPC, which significantly *decreased* the total water content of corneas, induced a significant increase of this parameter. Inspection of Columns B and C of Table 2, and of Fig. 3 (which illustrates graphically the 'free' and 'bound' water changes with respect to the control) reveals, however, a particular picture. Only 0.001 and 0.005% PSE (Brij® 78) produced a significant increase ($+4.3\%$) of the percent free water corneal content (while, at the same time, decreasing the bound water) while 0.00001% PSE, PCO and DC produced non-significant increases. The two higher BAC concentrations, CPC and EDTA produced non-significant *decreases* of corneal free water. The bound water (Column C, Table 2 and Fig. 3C) was increased significantly ($+3.1\%$) by EDTA, and significantly *decreased* by PSE at the two higher concentrations (-2.9 and 3.2%, respectively). All other agents produced non-significant increases of bound water.

The intervals of fusion, or melting ranges (ΔT, °C) of control and of treated corneas are reported, with the relevant standard errors, in Column D of Table 2. The ΔT variations with respect to the control value are illustrated graphically in Fig. 4. The observed melting range of control corneas was 2.73 ± 0.03 °C: statistically significant increases of this parameter were produced by BAC at the two higher concentrations, by CPC and by EDTA; PSE at the two lower concentrations produced non-significant ΔT increases, and a significant ΔT *decrease* at the highest (0.005%) concentration. PCO also induced a significant ΔT decrease, while DC had no significant effect on ΔT.

4. Discussion

As indicated by Schoenwald and Huang (1983) who cite Maurice and Riley (1970) in their support, in most species the water content of the normal cornea ranges from 3.2 to 4 g/g dry weight, corresponding to a 76–80% hydration level; hydration levels increased up to 83–92% would indicate damage of the epithelium and/or

Table 2
Summary of data obtained by desiccation (A) and by DSC (B, C, D) (means ± SEM; $n = 6$)

Treatment of cornea	A	B	C	D
	Total H_2O, %	Free H_2O, %	Bound H_2O, % (A − B)	Melting range, (ΔT, °C)
GBR (control)	81.6 ± 0.28	71.6 ± 0.08	9.9 ± 0.20	2.7 ± 0.03
BAC 0.001%	82.7 ± 0.05[a]	71.5 ± 1.85	11.2 ± 1.90	2.7 ± 0.03
BAC 0.002%	82.2 ± 0.32[a]	70.8 ± 0.22	11.4 ± 0.54	2.9 ± 0.09[a]
BAC 0.01%	82.7 ± 0.60[a]	70.6 ± 0.76	11.6 ± 0.65	2.9 ± 0.05[a]
CPC 0.001%	80.2 ± 0.28[a]	69.9 ± 1.48	10.3 ± 1.19	3.0 ± 0.05[a]
EDTA 0.5%	83.0 ± 0.20[a]	70.0 ± 0.35	13.0 ± 0.55[a]	2.9 ± 0.04[a]
PSE 0.00001%	83.7 ± 0.57[a]	72.8 ± 0.79	10.8 ± 0.08	2.9 ± 0.08
PSE 0.001%	83.0 ± 0.36[a]	76.0 ± 1.45[a]	7.0 ± 1.09[a]	2.8 ± 0.22
PSE 0.005%	82.7 ± 0.09[a]	75.9 ± 1.56[a]	6.7 ± 0.65[a]	2.6 ± 0.10
PCO 0.5%	82.5 ± 0.36[a]	71.8 ± 0.85	10.7 ± 1.20	2.5 ± 0.12[a]
DC 0.05%	84.2 ± 0.33[a]	74.0 ± 0.01	10.1 ± 0.33	2.7 ± 0.04

[a] Significantly different ($P < 0.05$, Fisher PLSD test) from control A: gravimetric analysis; B: DSC analysis; C = A − B; data are expressed as % w/w.

of endothelium. Maurice (1984) however, also on the basis of previous literature reports, indicates for rabbit corneas average water contents ranging between 2.95 and 3.5 g/g dry weight, corresponding approximately to 75–78% hydration.

In the present investigation the total water content of freshly excised rabbit corneas was 80.1 ± 0.69% ($n = 6$); this value was lower that of control corneas, kept in contact for 1 h with GBR buffer in the perfusion cells (81.6 ± 0.28%), but not significantly different from it. On account of the slightly higher 'normal' value observed in this study with respect to previous literature data (Maurice, 1997) and of the uncertainty about a 'toxic' hydration range, an assumption of damage was made when the observed hydrations were statistically different from the control values.

Potential citotoxic effects of the GBR buffer equilibrated with 95% O_2–5% CO_2 have been indicated by Doughty (1995). However, this incubation solution is commonly used in studies dealing with corneal permeability (Camber, 1985; Rojanasakul and Robinson, 1989; Rojanasakul et al., 1990; Saettone et al., 1996).

A full discussion on the actual, complex mechanisms by which the tested agents exert their action on corneal hydration and ion content is outside the scope of the present paper. However, a few considerations follow. A comparison of the results observed with BAC and CPC is of particular interest. These compounds belong to the class of cationic surfactants and possess close chemical characteristics. However, CPC reduced significantly the *total* water content of corneas, while BAC had the opposite effect. As shown in Fig. 3,

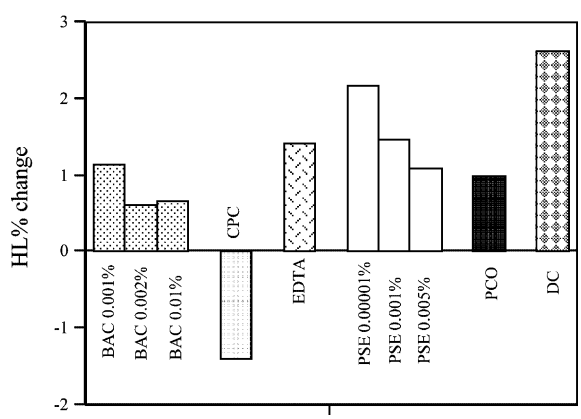

Fig. 2. Percent variation, with respect to control, of total water content of corneas treated with the test agents (gravimetric data). All values are statistically different ($P < 0.05$) from the control.

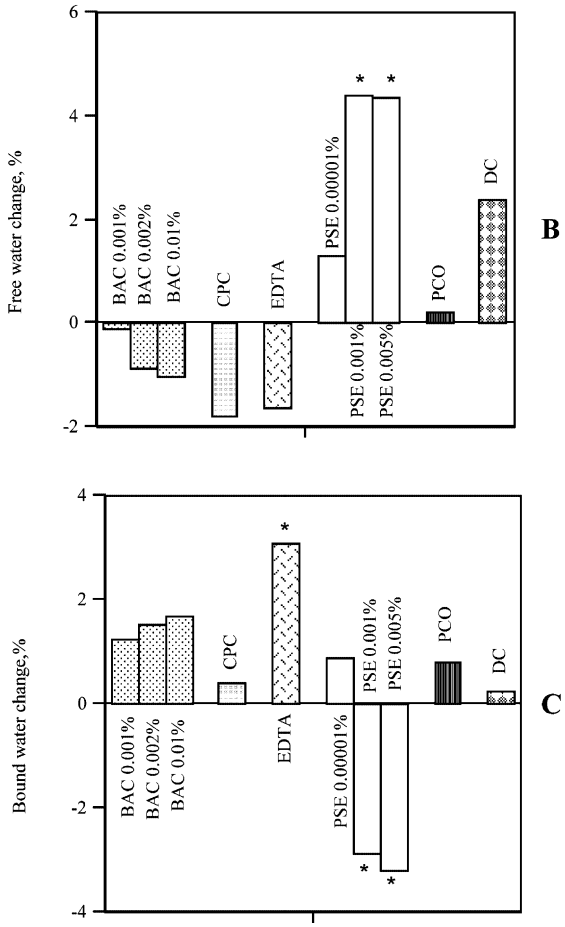

Fig. 3. Percent variation, with respect to control, of 'free' (B) and 'bound' (C) water content of corneas treated with the test agents. An asterisk (*) indicates a statistically significant ($P < 0.05$) difference from the control.

the decreased hydration produced by CPC occurred mainly at the expense of free water, while the bound water remained essentially unchanged. Increasing concentrations of BAC also slightly decreased the free corneal water, but had a greater influence on bound water. Interestingly, a recent study (Doughty, 1999) indicated an identical inhibitory effect of BAC and CPC on the water absorption properties of sheep corneal stroma in vitro.

BAC, the most commonly used preservative in ophthalmic solutions, has been shown to cause, in common with many surfactants, a dose-dependent disruption of the plasma membranes of epithelial cells of corneal epithelium as well as loosening of cells junctions (Green and Tonjum, 1971). It seems to disrupt the integrity of the outermost epithelial layers by causing desquamation of the top layers of corneal epithelium (Pfister and Burstein, 1976). Its promoting effect on corneal permeability of different drugs such as e.g. prednisolone phosphate (Green and Downs, 1974), pilocarpine (Green and Downs, 1975), F2a prostaglandin (Camber and Edman, 1987) etc. has been reported. The observed increase of bound water produced by BAC (Fig. 3 C), as well as the effect on corneal permeability, can be ascribed to the disrupting action exerted by the surfactant on corneal epithelium.

CPC has been reported to induce effects similar to BAC on corneal epithelial cells (Green, 1976) such as disorganisation and breakdown of the physiological barrier, resulting in increased permeability. Its positive influence on corneal permeability in vitro to sodium fluorescein (Green and Tonjum, 1971) and to penicillin (Godbey et al., 1979) has been reported. However, unpublished data from our laboratory indicated that, when tested at the same concentration, its activity on transcorneal permeation of timolol was one-half as that of BAC. Its remarkable corneal toxicity

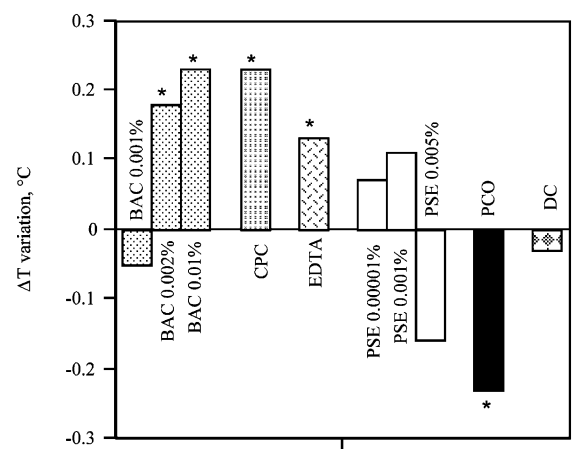

Fig. 4. Melting range (ΔT) variation, with respect to control, of corneas treated with the test agents. An asterisk (*) indicates a statistically significant ($P < 0.05$) difference from the control.

has been emphasised in recent papers (Lin and Hemming, 1996; Furrer et al., 1999). One particularity of CPC, not shared, or shared to a much lesser extent with BAC, is a strong interaction with stromal proteoglycans, which represent the main components of the ground substance of the cornea (Landemore et al., 1991; Doughty, 1999). Since the ground substance plays a significant role in the corneal hydration, the precipitation of proteoglycans or the activity of specific proteolytic enzymes can reduce swelling of the corneal stroma. The observed reduction induced by CPC on total and free water content of corneas might be explained on this basis.

EDTA increased significantly the content of corneal bound water ($+3.1\%$), while decreasing to a non-significant extent the free water. EDTA is a calcium chelator, mainly active on the tight junctions between epithelial cells, whose integrity seems to be dependent on Ca (Grass and Robinson, 1988a,b; Grass et al., 1988). It is known to produce ultra-structural changes in the corneal epithelium, resulting in decrease of the overall lipophilic characteristics of this tissue and in expansion of intercellular spaces (Ashton et al., 1991). These physiological alterations of corneal epithelium or endothelium are correlated with changes in corneal hydration (Adams et al., 1992) and with drug permeability through whole corneas (Grass and Robinson, 1988a,b). The increase in ΔT value produced by EDTA can be correlated to the ability of this agent to modify the salt concentration of tissue fluids by chelating calcium ions, and to physiological variations due to the loss of tight junctions integrity.

PSE [Brij® 78, polyoxyethylene (20) stearyl ether] and PCO (Cremophor® EL, polyethoxylated castor oil) are non-ionic surfactants, of occasional use in eyedrops as drug solubilisers. Sodium deoxycholate (DC), a bile salt, is an anionic surfactant. Different bile salts have been tested as permeation enhancers for buccal transmucosal drug delivery (Kurosaki et al., 1988; Zhang et al., 1994; Steward et al., 1994; Senel et al., 1994). In general the surfactants, at low concentrations, are incorporated into the lipid bilayer, with consequent changes of the physical properties of the cell membranes. When the lipid bilayer is saturated, mixed micelles begin to form, resulting in removal of phospholipids from the cell membranes and in membrane solubilisation (Hochman and Artursson, 1994). In our previous study (Saettone et al., 1996) 0.05% PSE as well as 0.05% DC, was found to increase significantly the transcorneal permeation of timolol. Increasing concentrations of PSE (from 0.00001 to 0.005%) increased significantly the 'total' and the 'free' corneal hydration; the highest concentration also decreased significantly the concentration of corneal solutes, possibly as the result of dilution of corneal solutes.

In conclusion, in the present tests 'in vitro' on isolated rabbit corneas, all tested agents appeared to influence significantly corneal hydration, presumably as a consequence of alterations of the corneal epithelium. Their influence on total, free and bound water, as well as their effect on corneal solutes was specified. Although a brief contact with the precorneal tissues 'in vivo' may not prove harmful, the use of these compounds as potential ocular permeation enhancers or otherwise as ingredients of topical ocular formulations for long-term use should be considered with some caution.

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