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Enhancer effects on *in vitro* corneal permeation of timolol and acyclovir

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The aim of this study was to evaluate the ability of two non-toxic skin penetration enhancers, *N*-methylpyrrolidone (NMP) and a positively charged phospholipid mixture (PS), to increase *in vitro* corneal permeation of timolol maleate (TM) and acyclovir (AC) in comparison with two corneal absorption promoters, polyethylene glycol octadecyl ether (Brij 78) and sodium taurocholate (TA). *In vitro* experiments were performed on corneas from albino rabbits which were mounted in a perfusion apparatus. The concentrations of the enhancers being tested were: Brij 78 1%, PS 1%, TA 1%, NMP 5%, NMP 10%. The safety of the enhancers being tested was assessed *in vitro* by determining their effects on corneal hydration and *in vivo* by means of a modified Draize test. Calculating the amount of drug permeated at different time points (90 and 180 min) we observed that TA, PS and NMP 5% significantly increased the cumulative amount of AC permeated after 90 min but only PS was effective after 180 min. TA, Brij 78 and PS were able to increase significantly the amount of TM permeated after 90 min but after 180 min only Brij 78 retained its effect. TA, Brij 78 and NMP 10% significantly increased the percent hydration levels (% HL) compared to the control while PS and NMP 5% did not affect % HL. The results of *in vivo* ocular tolerability studies showed that the enhancers which caused an *in vitro* increase of % HL produced *in vivo* conjunctival and/or corneal damages. The results of this study suggest that PS could be regarded as a potential corneal enhancer to increase the intraocular bioavailability of AC and TM.

1. Introduction

Using conventional ocular dosage forms, generally less than 10% of an applied dose is absorbed across the cornea into the eye [1, 2]. This has been attributed to many concurrent factors such as limited rate of corneal absorption and precorneal loss processes, which include rapid tear drainage of the instilled dose, binding to tear protein and noncorneal absorption [3, 4].

In recent years, many attempts have been made to improve topical bioavailability of ophthalmic drugs. The most promising approaches include prolongation of drug retention in the precorneal area [5, 6], use of prodrugs with a better penetration profile [7, 8], and enhancement of corneal permeability by using absorption promoters [9]. This last approach has gained much attention due to the discovery that additives commonly used in ophthalmic formulations can act as penetration enhancers [10, 11].

Recently, studies investigating skin penetration enhancers as ophthalmic promoters have produced some interesting results. Azone, a well-known skin penetration enhancer, was able to effectively increase *in vitro* corneal permeation of different drugs but it caused corneal swelling even at low concentrations [12]. The cornea bears a close analogy with the skin in the respect that they are both multilayered biological membranes and their outermost layers are tightly junctioned and act as a barrier to the penetration of exogenous substances. However, great differences exist between the skin and the cornea since the horny layer consists of keratinized, dehydrated, and dead cells whereas the corneal epithelium contains living and hydrated cells. Due to these differences, the use of skin penetration enhancers to improve corneal drug permeability requires a careful selection with respect to their effect on the integrity of the corneal epithelial surface.

In this study the promoting effect of two non-toxic skin penetration enhancers, *N*-methylpyrrolidone (NMP) and a positively charged phospholipid mixture (PS) was investigated on rabbit cornea *in vitro* and compared to that of other corneal penetration enhancers, using one β-blocking agent (timolol maleate) and an antiviral agent (acyclovir) as test drugs.

Timolol (TM) and acyclovir (AC) were chosen as model drugs since they show low corneal permeability but different water solubility, being well and poor water soluble, respectively. NMP and PS were selected as test promoters on the basis of their enhancement ability and their safety. Many investigations [13] have shown that NMP is effective in increasing skin permeation of both hydrophilic and lipophilic molecules, while showing low toxicity. Phospholipids are regarded as safe promoters to improve drug permeability through the skin [14]. Since the cornea car-

ries a negative charge, we thought it would be interesting to evaluate the enhancement effect of a positively charged phospholipid mixture.
The safety of the enhancers being tested was assessed *in vitro* by determining their effects on corneal hydration and *in vivo* by means of a modified Draize test [15].

2. Investigations, results and discussion

The penetration profiles of TM and AC through the isolated corneas from GBR solutions with and without enhancers are shown in Fig. 1 and 2, respectively. Linear permeation plots with correlation coefficients in the range 0.989–0.999 were obtained in all cases, both in the presence and in the absence of promoters. The apparent permeability coefficient (P_{app}), the lag time and the enhancement factor (E.F.) of TM and AC, determined for the permeants in GBR buffer alone (control) and in the presence of the enhancers being tested, are summarized in Table 1 and 2, respectively. In the absence of enhancers (control) the apparent permeability coefficients of TM and AC were in accordance with those reported elsewhere [17, 23]. Among the enhancers tested, only Brij 78 showed a significant promoting effect on P_{app} values by increasing P_{app} of TM 1.4-fold compared to the corresponding control. As reported [9], penetration enhancers are more effective in increasing corneal permeability of hydrophilic molecules which are poor permeants. Therefore, the enhancement of P_{app} observed for TM could be attributed to its greater hydrophilicity compared to AC (Log P of TM $= -1.41$; Log P of AC $= 0.06$).
Apart from Brij 78, all the promoters tested failed to increase the P_{app} of both TM and AC. Interestingly, NMP 10% decreased the P_{app} of TM whereas NMP 5% did not significantly affect the P_{app} values of this drug. Negative effects of promoters on the P_{app} have already been reported [12]. In our experiments, the concentration-dependent decrease of the P_{app} induced by NMP could be due to NMP well-known solubilizing properties [13] which could increase drug solubility in the tissue thus reducing drug thermodynamic activity and hence the driving force for the permeation process. NMP was used at 5–10% because these concentrations are regarded as the lowest limits for NMP to be effective as a skin permeation enhancer [13]. In order to evaluate the effect of lower NMP concentrations on TM and AC *in vitro* corneal permeation, experiments were performed using NMP in the range

Table 1: Permeability coefficient (P_{app}), lag time, enhancement factor (E.F.) and percentage hydration level (% HL) of timolol in the presence and in the absence of corneal penetration enhancers

Enhancer	$P_{app} \times 10^6$ (±S.D.) (cm/s)	Lag time (min)	E.F.	% HL (±S.D.)
Control	26.13 ± 4.61	7.29	1.00	78.11 ± 2.56
TA 1%	27.58 ± 2.40	−16.60	1.06	83.24 ± 1.04[b]
Brij 78 1%	34.10 ± 3.59[a]	5.55	1.37	85.92 ± 0.21[b]
PS 1%	26.98 ± 4.06	−25.60	1.03	75.14 ± 1.86
NMP 5%	32.80 ± 2.05	3.10	1.26	79.80 ± 0.99
NMP 10%	19.53 ± 1.21	6.14	0.75	82.96 ± 0.63[b]

[a] $p < 0.01$ compared to the control
[b] $p < 0.05$ compared to the control

Table 2: Permeability coefficient (P_{app}), lag time, enhancement factor (E.F.) and percentage hydration level (% HL) of acyclovir in the presence and in the absence of corneal penetration enhancers

Enhancer	$P_{app} \times 10^6$ (±S. D.) (cm/s)	Lag time (min)	E. F.	% HL (±S.D.)
Control	3.91 ± 0.92	53.71	1.00	79.65 ± 0.90
TA 1%	4.42 ± 1.78	15.62	1.13	85.43 ± 3.22[a]
Brij 78 1%	3.83 ± 1.63	24.17	0.99	85.53 ± 1.30[a]
PS 1%	3.72 ± 0.49	9.47	0.95	77.47 ± 1.12
NMP 5%	3.98 ± 0.78	3.30	1.01	71.55 ± 3.61
NMP 10%	3.32 ± 0.04	2.51	0.85	83.00 ± 0.57[a]

[a] $p < 0.01$ compared to the control

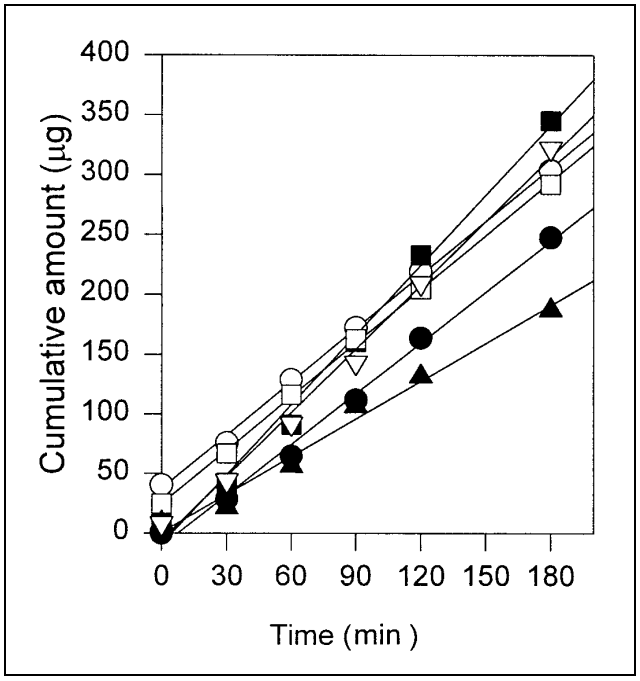

Fig. 1. *In vitro* corneal permeation profile of Timolol in the presence and in the absence of enhancers. (●) Timolol; (○) PS; (■) Brij 78; (□) TA; (▽) NMP 5%; (▲) NMP 10%

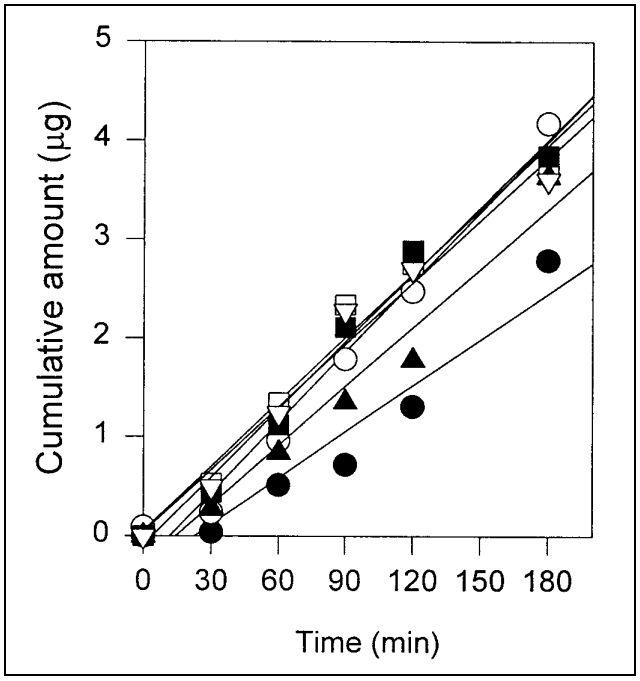

Fig. 2. *In vitro* corneal permeation profile of Acyclovir in the presence and in the absence of enhancers. (●) Acyclovir; (○) TA; (□) PS; (▲) Brij 78; (■) NMP 5%; (▽) NMP 10%

Table 3: Cumulative amount permeated after 90 min (Q_{90}) and 180 min (Q_{180}) and corresponding enhancement factors (E.F.) of timolol and acyclovir in the presence and in the absence of corneal penetration enhancers

Enhancer	Drug							
	Timolol				Acyclovir			
	Q_{90} (±S.D.) (μg)	$E.F._{90}$	Q_{180} (±S.D.) (μg)	$E.F._{180}$	Q_{90} (±S.D.) (μg)	$E.F._{90}$	Q_{180} (±S.D.) (μg)	$E.F._{180}$
Control	111.973 (±16.248)	1.00	247.725 (±38.230)	1.00	0.721 (±0.351)	1.00	2.792 (±0.368)	1.00
TA 1%	162.613[a] (±19.622)	1.45	292.077 (±34.866)	1.18	1.784[a] (±0.303)	2.47	4.179 (±1.119)	1.50
Brij 78 1%	160.514[a] (±22.293)	1.43	345.323[a] (±25.365)	1.39	1.350 (±0.539)	1.72	3.633 (±1.665)	1.30
PS 1%	172.119[a] (±24.325)	1.54	303.730 (±48.255)	1.23	2.335[a] (±0.826)	3.24	3.744[a] (±0.419)	1.34
NMP 5%	143.250 (±16.080)	1.28	322.220[a] (±17.806)	1.30	2.110[a] (±0.439)	2.93	3.851 (±0.794)	1.38
NMP 10%	114.027 (±53.090)	1.02	187.91 (±4.206)	0.76	1.508 (±0.275)	2.09	3.403 (±0.445)	1.22

[a] $p < 0.05$ compared to the control

0.1–1%. No significant effect of NMP 0.1–1% on TM and AC permeability coefficients was observed (data not shown). Concentrations higher than 10% were not tested since an increase of corneal hydration was observed using NMP 10%.

As reported in Table 1 and 2, all the enhancers tested shortened the lag time of TM and AC compared to the control. However, no relationship was observed between the type of enhancer used and the lag time shortening since the promoters most effective in reducing the lag time of AC showed only a slight effect on the lag time of TM. Interestingly, negative lag time values were observed for TM corneal permeation in the presence of TA 1% or PS 1%. This finding would suggest an initial fast permeation of TM followed by a slower permeation through the corneal tissue in the presence of these enhancers. Therefore, we thought it would be interesting to calculate the amount of drug permeated at different time points. The cumulative amount of drug permeated after 90 min (Q_{90}) and 180 min (Q_{180}) and the corresponding E.F. values, both for TM and AC, are reported in Table 3. TA, PS and NMP 5% significantly increased the cumulative amount of AC permeated after 90 min but only PS was able to enhance the Q_{180} value of AC, although the E.F. value after 180 min was markedly lower than that determined after 90 min. As regards timolol, TA, Brij 78 and PS were able to increase significantly its Q_{90} value, although to a low extent (E.F. about 1.5). After 180 min TA and PS were not effective in increasing the cumulative amount of permeated TM whereas Brij 78 retained its effect. The cumulative amount of TM permeated after 90 min in the presence of NMP 5% did not differ from the control but it was significantly higher than that of control after 180 min.

Table 4: Diffusion coefficients (D_m) and partition coefficients (K_m) of timolol and acyclovir in the presence and in the absence of corneal penetration enhancers

Enhancer	Drug			
	Timolol		Acyclovir	
	$D_m \times 10^9$ (cm^2/s)	K_m	$D_m \times 10^9$ (cm^2/s)	K_m
Control	5.64	17.82	0.76	19.62
TA 1%	N.C.[a]	N.C.[a]	2.63	6.42
Brij 78 1%	7.41	18.66	1.70	8.64
PS 1%	N.C.[a]	N.C.[a]	4.34	3.30
NMP 5%	13.26	9.54	12.46	1.20
NMP 10%	6.70	11.16	16.44	0.78

[a] N.C = not calculated because lag time was negative

These results suggest that NMP could require a longer application time than that used in our experiments to exert its promoting effect on TM corneal permeation.

In order to elucidate the mechanism of action of the promoters being tested, apparent diffusion coefficients (D_m) and partition coefficients (K_m) both of TM and AC in the presence and in the absence of enhancers were calculated and reported in Table 4. Regarding control solutions, AC showed a greater K_m value compared to TM but its D_m was lower. The difference observed between AC and TM penetration parameters could be attributed to the greater lipophilicity of AC compared to TM. As shown in Table 4, all the enhancers tested increased D_m values of both TM and AC in comparison to the corresponding controls but they were not able to significantly affect TM and AC K_m values, apart from Brij 78 which enhanced only the K_m of timolol. These results suggest that an increase of D_m modified drug permeation profiles by shortening the lag time and enhancing the cumulative amount permeated in the early period of the permeation process. However, after 180 min only the promoter (Brij 78) which provided an increase of K_m was able to enhance the P_{app} of the drug. Similarly, other authors [22] reported that differences of drug corneal permeability coefficients could be determined by partition parameters rather than diffusion parameters. As shown in Table 3, PS, a positively charged phospholipid, significantly increased AC cumulative amount permeated after 90 and 180 min. The cornea possesses permeselective properties allowing preferential penetration of noncharged or positively charged molecules [24]. However, corneal permeselectivity is a complex phenomenon which combines active and passive contribution deriving from cell membrane activity and electrostatic shunt activity, respectively. The results of our experiments suggest that the effectiveness of charged ocular permeation promoters depends on both their charge and their ability to affect drug partition and diffusion coefficient since PS was effective in increasing permeation of AC but not of TM.

A prerequisite for a molecule to be regarded as a useful corneal absorption promoter is the lack of ocular toxicity. *In vitro* evaluation of corneal damages is commonly performed by determining the percent corneal hydration [20]. As shown in Table 1 and 2, the percent corneal hydration level (% HL) determined in the absence of enhancers, both for TM and AC, was in the range 76–80% which is regarded as the normal hydration level for undamaged corneas [20]. Among the enhancers tested, TA, Brij 78 and NMP 10% significantly increased% HL values compared to the control while PS and NMP 5% did not affect cor-

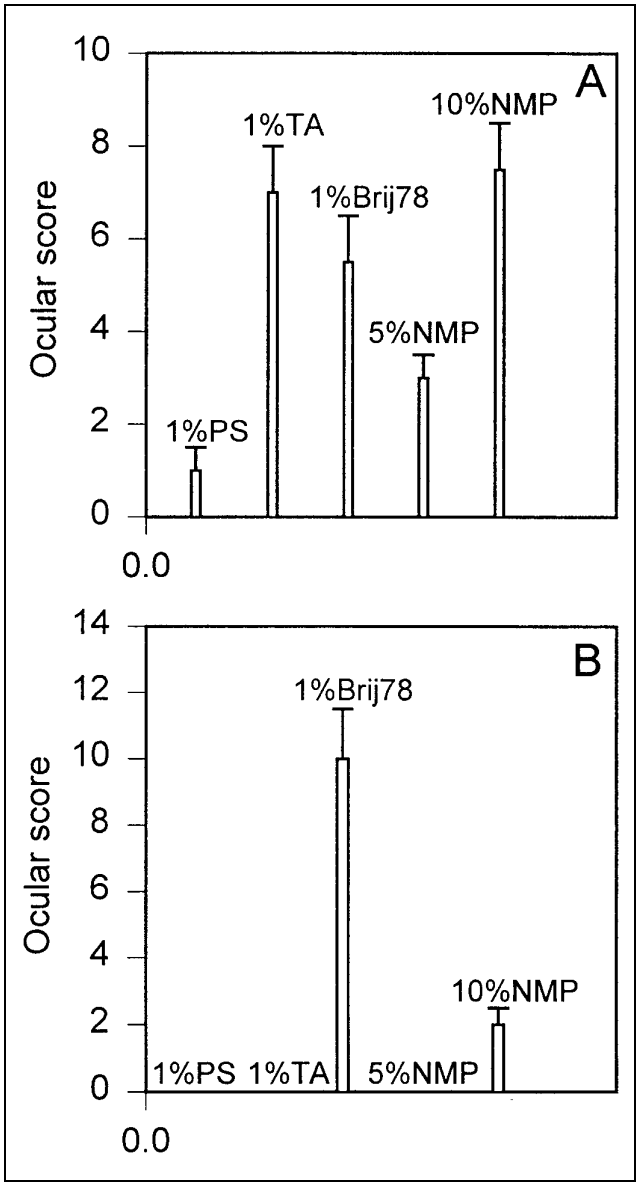

Fig. 3. Effects of enhancers on conjunctival inflammation after 10 min (A) and 16 h (B) from the last instillation. Each bar represents the mean ± S.D. of 4–6 observations

neal hydration levels. The results of *in vivo* ocular tolerability studies of the enhancers tested are shown in Fig. 3. Repetitive topical administration of PS did not show any sign of ocular intolerance *in vivo* since no signs of inflammation were observed both in the conjunctiva and in the cornea. TA produced a mild conjunctival inflammation after 10 min from the last instillation but disappeared at the second observation (16 h). Brij 78 caused a mild conjunctival inflammation with corneal desepithelization that was more evident after 16 h. Recently, other authors [20] showed that a single instillation of Brij 78 or TA on rabbit eye was devoid of any ocular effect even at the maximal tested dose (2%). On the contrary, in our experiments both TA and Brij 78 caused a mild conjunctival inflammation and corneal damage after repeated administration. The damaging effects observed in our experiments could be attributed to the different pattern of treatment. In our studies, a multiple administration pattern was chosen since it more closely resembles the actual use in clinical therapy. NMP 5% produced a moderate conjunctival inflammation that was lower compared to NMP 10%. However, conjunctival inflammation produced by NMP 10% markedly decreased after 16 h. No signs of corneal toxicity were observed after instillation of NMP 5–10%. The results of *in vivo* ocular tolerability studies are in good agreement with the *in vitro* data on corneal hydration since the enhancers which caused *in vivo* conjunctival and/or corneal damages produced also an *in vitro* increase of % HL.

In conclusion, the results of this study suggest that PS can be considered as a potential enhancer for AC and could be used in topical ophthalmic formulations to increase the intraocular bioavailability of this drug. On the contrary, TA, Brij 78 and NMP could not be considered as perspective corneal penetration enhancers for TM and AC due to their lack of effectiveness and/or their damaging effects. In vivo studies are planned to assess the effects of PS on corneal permeation of AC in rabbits so as to evaluate the influence of this promoter on AC ocular pharmacokinetics.

3. Experimental

3.1. Materials

Acyclovir (AC), timolol maleate (TM), 1-methyl-2-pyrrolidinone (NMP), sodium taurocholate (TA) were bought from Sigma Chemicals (St. Louis, USA). The positively charged phospholipid mixture (PS) extracted from pigs was a kind gift of Bausch & Lomb-Fidia Oftal (Italy). Polyethylene glycol octadecyl ether (Brij 78) was obtained from Fluka (Buchs, Switzerland). All other chemicals were either reagent or analytical grade, and were used as received.

3.2. Animals

Male New Zealand albino rabbits (Charles River, Calco, Italy) 1.8–2.0 kg, free of any signs of ocular or gross abnormalities, were used. Animal procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research.

3.3. In vitro experiments

The rabbits were sacrificed by intravenous injection of 0.3 ml/kg euthanasia solution (Tanax, Hoechst AG, Germany). The whole eyes were enucleated from their sockets and the corneas with a 2 mm ring of sclera were immediately excised in accordance with the procedure described elsewhere [16, 17]. Various tissues of the eye were dissected leaving the cornea suspended within a corneal ring which was then placed and clamped between two compartments of the perfusion chamber (Precision Instrument Design, Los Altos, CA, USA).

In vitro experiments were performed according with the methods reported by others [16]. The chamber was thermostated to maintain the cornea and the perfusion solution at 35 °C. Glutathione bicarbonate Ringer's solution (GBR) was used throughout the perfusion studies since it has been reported to preserve the integrity of excised cornea for up to 6 h [18]. Mounting of corneas in the perfusion apparatus was completed within 20–40 min of death of the animal. Chambers were equilibrated at 35 °C at least 1 h prior to the experiment.

A measured volume (4.5 ml) of preheated (35 °C) GBR buffer was added first to the endothelial compartment to prevent the cornea from buckling. An equal volume of drug solution in GBR buffer, with or without enhancer, was then added to the epithelial compartment. Drug concentration (w/v) in the donor compartment was kept low to avoid the effect of the drug on corneal integrity during prolonged incubation (TM 0.09%; AC 0.01%). The concentration of the enhancers being tested (TA, NMP, Brij 78, PS) ranged from 0.1 to 10% for each of the drugs tested (see Table 1).

To ensure mixing and oxygenation an $O_2:CO_2$ (95:5) mixture was bubbled through each compartment at a rate of 3–4 bubble/s.

One milliliter aliquots were withdrawn from the receiving compartment at appropriate intervals over a 180-min period for analysis of drug content. Each sample was immediately replaced with an equal volume of preheated buffer to maintain a constant volume. Each experiment was performed in triplicate.

3.4. Drug assay

Drug content in the samples withdrawn from the receiving compartment was determined spectrophotometrically at 286 nm for AC and 294 nm for TM. A standard working curve was constructed daily from a known concentration of each drug in GBR solution. The sensitivity of the assay was 0.5 μg/ml for AC and 1.0 μg/ml for TM.

3.5. Evaluation of corneal hydration level

Wet corneal weight (W_w) was obtained after careful removal of the scleral tissue; each cornea was then desiccated at 60 °C overnight to give the corresponding dry corneal weight (W_d).
The percent corneal hydration level (% HL) was calculated as one minus the ratio of the dried and the wet corneal weights multiplied by 100 [12].
Corneas with % HL above 83% were considered damaged [17].

3.6. Evaluation of ocular inflammation

Ocular inflammation was quantified using a slit-lamp at various times according to a modified Draize test [15] by two observers who were unaware of the treatment. TA, Brij 78 and PS were tested at 1%, NMP was administered at 5 and 10%. Fifty microliters of solution was topically administered every 30 min for 6 h thereafter (twelve treatments). At the end of the treatment, two observations at 10 min and 16 h were performed to evaluate the ocular tissues. Corneal integrity was evaluated by methylene blue staining. Methylene blue was chosen because it does not rapidly diffuse through the stroma and therefore it provides a more accurate determination of the extent of epithelial damages [19].

3.7. Data analysis

AC and TM steady state fluxes were calculated by linear regression analysis in the linear portion of the curve obtained plotting the cumulative amount of drug permeated against time. The lag time (L_t) was determined as the intersection of the regression line with the x-axis.
The apparent corneal permeability coefficient P_{app} (cm/s) was determined from the steady state flux values as previously reported [20].
Apparent diffusion coefficients (D_m) values were calculated as the ratio of the squared thickness of the cornea (h^2) and the lag time multiplied by 6 [21]. For permeation purposes, other authors [22] assumed that the thickness of the cornea is 0.03708 cm The partition coefficients (K_m) values were calculated from the relationship: $K_m = D_m P_{app}/h$ [21].
Results are expressed as the mean ± S.D and Student's t-test was used to estimate the significance of the differences between mean values. Values of $p < 0.05$ were considered statistically significant.

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