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Research paper

Thiolated quaternary ammonium–chitosan conjugates for enhanced precorneal retention, transcorneal permeation and intraocular absorption of dexamethasone

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ABSTRACT

Previously, a quaternary ammonium (N^+)–chitosan (Ch) conjugate ($N^+(60)$–Ch) characterized by short pendant chains, made of 1.7 ± 0.1 adjacent diethyl-dimethylene-ammonium groups, substituted onto the primary amino group of the chitosan repeating units (degree of substitution, 59.2 ± 4.5%) was used to synthesize a multifunctional non-cytotoxic thiomer ($N^+(60)$–Ch–SH(5)), carrying 4.5 ± 0.7% thiol-bearing 3-mercaptopropionamide besides quaternary ammonium groups. The present work was aimed at evaluating the potential of $N^+(60)$–Ch–SH(5) and $N^+(60)$–Ch as bioactive excipients for dexamethasone (DMS) eyedrops. The DMS permeability across excised rabbit cornea was enhanced over the control value by the thiomer and the parent polymer to about the same extent (3.8 vs. 4.1 times). The mean precorneal retention time and AUC in the aqueous of DMS instilled in rabbit eyes via eyedrops were enhanced by the thiomer (MRT = 77.96 ± 3.57 min, AUC = 33.19 ± 6.96 μg ml^{-1} min) more than the parent polymer (MRT = 65.74 ± 4.91 min, AUC = 21.48 ± 3.81 μg ml^{-1} min) over the control (MRT = 5.07 ± 0.25 min, AUC = 6.25 ± 0.65 μg ml^{-1} min). The quaternary ammonium ions were responsible for both permeabilization of corneal epithelium and polymer adhesion to precorneal mucus, while the thiols increased the latter. This synergistic action is the basis of the higher thiomer bioactivity *in vivo*. A good ocular tolerability of the chitosan derivatives resulted from *in vivo* experiments.

1. Introduction

The topical treatment of extra- or intraocular diseases with eyedrops is the best accepted by patients. Treatment with eyedrops, however, usually entails coping with a poor bioavailability because the precorneal area, i.e., the site of drug action/absorption, is rapidly cleared of drugs by protective mechanisms of the eye, such as blinking, basal and reflex tearing, and nasolacrimal drainage. From here derives the need of frequent instillations, and hence the risk of side effects. An approach to increasing the bioavailability of drugs administered by eyedrops has been the reduction of drainage rate by increasing the viscosity of the preparation [1–6] or resorting to mucoadhesive polymers [7]. In those cases where a well-tolerated topical treatment is desired to implement an intraocular therapy, eyedrops can be made to contain an effective, biocompatible, non-irritant polymeric corneal permeability enhancer. Chitosan, a well-known polysaccharide obtained by deacetylation of chitin, can help in this respect. The tremendous potential of chitosan in the pharmaceutical area has been illustrated by several review articles [8–17]. These point out that chitosan is biodegradable, has low toxicity and good ocular tolerability, exhibits bioadhesion and permeability-enhancing properties and also physicochemical characteristics that make it suitable for the design of ocular drug delivery vehicles. Chitosan has been found to enhance drug penetration across not only cell monolayers, such as the intestinal [18–21] and nasal [22] epithelia, but also stratified cell layers, such as the buccal [23,24], vaginal [23] and corneal [25,26] epithelia. However, using unmodified chitosan in eyedrops with the purpose of permeabilizing the cornea is not quite rational, because this polymer is insoluble in tear fluid, and hence inactive at the physiological pH of 7.4. *N*-trimethylchitosan (TMC), a polycationic derivative of chitosan, soluble in tear fluid at physiological pH, has been obtained by partially quaternizing the primary amino group of chitosan. TMC significantly enhanced the *in vivo* transcorneal absorption rate of ofloxacin in rabbits [27] and the apparent permeability of the very lipophilic dexamethasone and that of the very polar fluorescein sodium salt, across the excised rabbit cornea [28]. The absorption-enhancing efficacy of TMC was thought to depend on its charge density which, in neutral or alkaline environments, is determined by its quaternization degree [29]. In the light of this consideration, water-soluble chitosan derivatives, bearing substituted short pendant chains containing a number, n, of adjacent quaternary ammonium groups (N^+–Ch), were prepared [30]. These

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derivatives enhanced the apparent permeability of dexamethasone across the excised rabbit cornea to about the same extent as TMC, whereas the enhancing effect of the most substituted N^+–Ch derivative on the permeability of fluorescein sodium across the same substrate was neatly stronger. Zambito et al. [28] tested this chitosan–quaternary ammonium conjugate for its ability to promote the intraocular penetration of dexamethasone or fluorescein *in vivo* in rabbit eyes. The polymer enhanced peak concentration and AUC of fluorescein in the aqueous humour remarkably more than those of dexamethasone; however, the enhanced parameters of the former remained lower than the control parameters of the latter. A comparatively high fraction of free, unsubstituted primary amino groups were still available on the backbone of N^+–Ch for covalent attachment of thiol-bearing compounds, via formation of 3-mercaptopropionamide moieties. This has in fact led to water-soluble thiolated chitosan–quaternary ammonium conjugates (N^+–Ch–SH) the epithelial permeability-enhancing potential of which was tested using the Caco-2 cell monolayer and the excised rat jejunum as substrates [31]. On the basis of the obtained results, the quaternary ammonium groups of these derivatives were ascribed the ability to reversibly open the epithelial tight junctions and also perturb the plasma membrane of the epithelial cells. On their part, the thiol groups were believed to keep the polymer adherent to the epithelium by reacting with the thiols of the epithelial mucus to form disulphide bonds, thus favouring the permeability-enhancing action of the positive ions.

In the light of the previous literature information, the purpose of the present work has been to ascertain whether the above synergism of quaternary ammonium and thiol groups also characterizes the interaction of the N^+–Ch–SH derivatives with the corneal epithelium and could be taken advantage of to increase the transcorneal permeability and intraocular availability of topically applied dexamethasone. Indeed this drug, fairly permeable across the cornea, is currently formulated as a suspension in commercial eyedrops. It was felt that a significant and safe enhancement of corneal permeability by the above thiomer could allow improvement of the therapeutic protocol. Excised rabbit corneas mounted in perfusion cells have been used to evaluate polymer effects on dexamethasone corneal permeability. Polymer effects on drug retention in tear fluid and intraocular bioavailability have been studied by *in vivo* experiments with rabbits where the tear fluid and aqueous humour were analyzed periodically to obtain relevant concentration *vs.* time curves.

2. Materials and methods

2.1. Materials

Dexamethasone (DMS) (Sigma); chitosan minimum 90% deacetylated from shrimp shell (Ch) (Chitoclear FG90, Primex, Drammen, Norway); Novesina collirio (oxybuprocaine), (Mipharm, Milano, Italy); pentothal sodium (Farmaceutici Gellini, Aprilia, Italy) were used. The commercial Ch had an average viscometric molecular weight of 590 kDa. Its deacetylation degree, determined by IR or NMR, was 90% or 82%. A chitosan–quaternary ammonium conjugate with a degree of substitution by pendant chains of 59.2 ± 4.5% and a number, n, of adjacent quaternary ammonium groups in pendant chains of 1.7 ± 0.1 (N^+(60)–Ch) were synthesized by Zambito et al. [32]. This derivative was thiolated to obtain a degree of substitution by thiol-bearing groups of 4.5 ± 0.7% (N^+(60)–Ch–SH(5)), by Zambito et al. [31]. These authors demonstrated that this thiomer is stable in the lyophilized state. Considering that the synthesis of the Ch derivatives caused no chain fragmentation [31,32], the MWs of the end products could be calculated from the MW of the starting Ch, knowing the degrees of substitution by pendant chains and thiol-bearing groups, and the n value. The calculated values are 1162 kDa for N^+(60)–Ch and 1166 kDa for N^+(60)–Ch–SH(5).

2.2. Permeation measurements across excised rabbit cornea

Male, New Zealand albino rabbits of 4.5–5.0 kg were used. They were treated as prescribed in the publication 'Guide for the care and use of laboratory animals' (NIH Publication No. 92–93, revised 1985). All experiments were carried out under veterinary supervision, and the protocols were approved by the Ethical-Scientific Committee of the University of Pisa. The rabbits were euthanized with intravenous pentobarbital. The eyes were proptosed, and the corneas, with a 2-mm ring of sclera, were immediately excised and mounted in perfusion cells fabricated according to Camber [33]. The corneal area available for diffusion was 0.78 cm^2. Each cell was maintained at 35 ± 1 °C. Glutathione bicarbonate Ringer buffer pH 6.8 (GBR) was added to the donor (1.0 ml) and receptor (3.0 ml) compartments. To ensure oxygenation and agitation, an O_2–CO_2 (95:5) mixture was bubbled through each compartment (3–4 bubble/s). After equilibration for 10 min, the solution in the donor side was replaced with 1.0 ml of a DMS suspension 0.3% w/v in GBR (control), or in GBR containing 1% w/v of the polymer under test. At intervals, 100 μl of the receptor solution was withdrawn for analysis by HPLC [34] and replaced with an equal volume of fresh preheated buffer. Each run had a 4.0-h duration and was repeated six times. Three rabbits (six corneas) were used for each of the DMS suspensions tested.

2.3. Permeation data treatment

For each permeation run, a value of the apparent permeability coefficient, P^*_{app}, of DMS across the cornea was calculated from the following equation, assuming passive diffusion under steady-state conditions [28,30]:

$$P^*_{app} = \frac{dM}{dt}\frac{1}{AC_0 f_F} \quad (1)$$

where $\frac{dM}{dt}\frac{1}{A}$, the permeation flux, is the slope of the linear portion of the cumulative amount permeated per unit surface area vs. time plot, C_0 is the initial concentration of permeant dissolved in the donor solution and f_F is the fraction of permeant free from binding to polymer. In the case of DMS, which was suspended in the donor, the product $C_0 f_F$ is theoretically equal to the drug solubility in the polymer-free donor phase (0.12 mg/ml [28]), whatever the DMS–polymer binding. For each plot, the linear regression analysis was extended to the set of data points that gave the best fit, as judged from the r^2 value. Also the lag time, L^*, that is the time axis intercept of the regression line, was calculated for each plot. The single P^*_{app} and L^* values were averaged to calculate the mean apparent permeability, P_{app}, and the mean lag time, L ($n = 6$). The mean cumulative amount permeated per unit area in any given time was calculated to plot each permeation profile and to determine T_{4h}, i.e., the cumulative transport over the whole time of experiment. The significance of the difference between two P_{app}, or L, or T_{4h} values was assessed by the Student's t-test ($P < 0.05$). For the chitosan derivatives that produced a significant P_{app} increase, this was measured by the enhancement ratio (ER), defined as the ratio between the P_{app} values obtained in the presence and in the absence of the enhancer.

2.4. Gravimetric evaluation of corneal hydration levels

At the end of each permeation run, the cornea was removed from the perfusion apparatus, and the percent corneal hydration level was evaluated by measuring the total water content of the

cornea by desiccation. After carefully removing the remaining sclera, the trimmed cornea was gently blotted dry, and the wet corneal weight (W_w) was determined (10^{-5} g). The sample was then desiccated in an oven at 100 °C for 6 h after which it attained a constant weight (dry corneal weight, W_d). The corneal hydration level (HL) was calculated as $[1 - (W_d/W_w)]100$. Each of the HL values listed in Table 1 is the mean of six corneas (three rabbits).

2.5. Preparation of ophthalmic drops

For *in vivo* tests, three types of medicated ophthalmic drops were prepared having the following compositions:

- 0.3% w/v DMS suspended in isotonic phosphate-buffered (pH 7.4, 0.0375 M) saline (PBS) (control).
- 0.3% w/v DMS suspended in PBS, containing 1% w/v N^+(60)–Ch.
- 0.3% w/v DMS suspended in PBS, containing 1% w/v N^+(60)–Ch–SH(5).

The drops contained a dispersion of DMS particles (<1.5 μm) obtained by spray-drying a 0.1 mg/ml drug solution (Mini Spray Dryer BÜCHI B-191, inlet and outlet air temperatures, 150 °C and 60 °C, respectively; spray nozzle, 0.7 mm; feed flow, 8 ml/min). The micronized drug particles were suspended by vigorous stirring in PBS (control) or PBS containing previously dissolved polymer. They persisted in the suspended state until the ophthalmic drops were instilled. Iodometric titration of the –SH groups of N^+(60)–Ch–SH(5), carried out as described previously [31], showed that these groups remained stable in the ophthalmic drops for at least 24 h at ambient temperature. Such a stability was considered sufficient for ophthalmic drops being prepared from the lyophilized thiomer and instilled in rabbit eyes.

2.6. Rheological analysis

Rheograms of the eyedrops were recorded at 35 °C in the 0–200 s^{-1} shear-rate interval over 300 s, with a Haake RS1 rheometer equipped with the coaxial cylinders Z40 (rotor) and Z41 (stator). Data were acquired and analyzed by Rheo Win Pro software (Haake).

2.7. In vivo tests

Male, New Zealand albino rabbits of 4.0–4.5 kg, maintained under standard stabulation conditions, were used. The animals were treated as specified in Section 2.2. A total of 30 rabbits were used for all *in vivo* tests. Nine of these were used, at least 2 weeks after the last test, for the *ex vivo* experiments described in Section 2.2.

2.7.1. Irritation tests

To evaluate the irritation of rabbit eyes caused by the ophthalmic drops under study, a modified Draize test was carried out following the procedure described by Lallemand et al. [35].

2.7.2. Determination of DMS elimination kinetics from tear fluid

For determining the kinetics of DMS disappearance from tear fluid, one drop (50 μl), corresponding to 0.15 mg of DMS, was instilled, by a Gilson pipette, into the lower conjunctival sac of each rabbit, with care to avoid spillage. At intervals, tear fluid samples were collected from the lower marginal tear strip using 1.0-μl disposable glass capillaries (Microcaps, Drummond Scientific Co., USA), which were flushed with 1.0-μl water. After further dilution with 100 μl of water, the samples were directly injected for analysis by HPLC [34]. For each of the three eyedrop types studied, described in Section 2.5, eight elimination curves were obtained, each determined in a single eye of different animals by withdrawing tear fluid samples at 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 60, 90, 120, 150, 180 min from instillation. Twenty-four animals were used for this study.

2.7.3. Measurement of DMS intraocular penetration

For measuring DMS intraocular penetration, one eyedrop was instilled in the rabbit eye as described in the preceding section. After a pre-established time from instillation, namely, 30, 60, 90, 120, 150 or 180 min, with each polymer-containing eyedrop type, and 30, 45, 60, 75 or 90 min, for the control, each rabbit was anaesthetized by instilling one drop of Novesina®, then 60–80 μl of aqueous humour was aspirated from the anterior chamber, using a 1.0-ml insulin syringe fitted with a 29-gauge needle. Single eyes of at least six animals were used for each data point. Both eyes of each animal were used for different data points. At least 102 aqueous humour samples were needed for this study. In order to limit the number of animals, each eye was re-used, after a 2-week interval to let it recover from the piercing. For analysis, each sample was mixed with an equal volume of acetonitrile, then it was centrifuged, and 20 μl of the supernatant were analyzed by HPLC [34]. Thirty animals were used for this study. Twenty-four of these had previously been used for the study described in the preceding section.

2.8. In vivo data treatment

The concentration in tear fluid (C_{TF}) vs. time data, obtained as described in the previous section, was used to calculate the mean residence time of DMS in tear fluid (MRT), according to the relevant non-compartmental technique [36]. This parameter resulted from the ratio, AUMC/AUC, between the area under the $C_{TF} \cdot t$ vs. t curve, *i.e.*, area under momentum curve (AUMC) and the area under the C_{TF} vs. t curve, *i.e.*, area under curve (AUC). AUMC and AUC were calculated by the linear trapezoidal rule, between time 0 and the time when C_{TF} dropped below the minimum quantifiable value. For each elimination curve, the corresponding MRT was calculated; thus, for each case studied, eight MRT values were obtained, of which mean and SE were calculated. Difference significance between two means was evaluated by the Student's t-test ($P < 0.05$). Also the maximum residence time of the drug at quantifiable concentrations in tear fluid (RT_{max}) was reported. This time corresponded to the last point of the C_{TF} vs. time plot. In this plot

Table 1
Data on DMS permeation across excised rabbit cornea from Ringer buffer pH 6.8 containing 0.3% (w/v) DMS and 1% (w/v) of different Ch derivatives. Means ± SD of at least six runs.

Polymer	HL, %	L, h	Flux, μg cm^{-2} h^{-1}	P_{app} 10^6, cm s^{-1}	ER	T_{4h}, μg cm^{-2}
Control	76.79 ± 1.81	1.35 ± 0.11	0.63 ± 0.07	1.75 ± 0.19	–	1.83 ± 0.19
N^+(60)–Ch	79.69 ± 2.67	1.51 ± 0.20**	2.56 ± 0.22*	7.11 ± 0.61*	4.06	6.44 ± 0.02*,**
N^+(60)–Ch–SH(5)	77.76 ± 1.82	1.09 ± 0.08*,**	2.38 ± 0.14*	6.61 ± 0.39*	3.78	6.93 ± 0.34*,**

HL, corneal hydration level; L, lag time; P_{app}, apparent permeability; ER, enhancement ratio; T_{4h}, cumulative transport over the whole time of experiment (4 h).
* Significantly different from control.
** Significantly different from each other ($P < 0.05$).

for each time interval, the mean of eight C_{TF} values obtained with different animals was reported. The minimum quantifiable C_{TF} value was 2.5 μg/ml, considering the necessity to dilute the withdrawn samples at least 1:50 v/v.

The AUC for the aqueous humour was calculated by the linear trapezoidal rule between 0 and 150 min. The statistical methods described by Schoenwald et al. [37] were used in comparing AUC values. The significance of differences between pharmacokinetic parameters was evaluated by the Student's *t*-test ($P < 0.05$).

3. Results and discussion

3.1. Permeation studies

The experimental design for determining corneal permeability has also been used in previous works [28,33]. The hydration level (HL%) of the corneal tissue has been considered a sensitive indicator of tissue integrity. According to the literature, the normal water weight contained in the rabbit cornea ranges between 75% and 78% (see, e.g., Ref. [38]), a corneal damage being indicated by hydration levels increased to 83% or more [39]. In the present work, the corneal hydration was determined, at the end of each permeation run, by the usual gravimetric method [28,39–42]. The resulting HL% values are listed in Table 1. All are below 80.1 ± 0.69% ($n = 6$), a value reported by Monti et al. [39] for freshly excised rabbit corneas, which indicates that neither the permeant nor the chitosan derivatives produced any substantial damage to the tissue in the perfusion apparatus.

The data on DMS permeation in the absence (control) or in the presence of each of the chitosan derivatives under study (1% w/v) are presented in Fig. 1, while the relevant parameters are listed in Table 1. All regressions, applied to the linear portion of each plot, are significant ($r^2 \geqslant 0.96$). For the control, a P_{app} value was obtained in the present work significantly lower than the corresponding value obtained in the previous work [28] in the same conditions ($1.75 \pm 0.19\ 10^{-6}$ vs. $4.38 \pm 1.28\ 10^{-6}$ cm s^{-1}). This difference reflects the difference in weight between the rabbits used in the two works (4.5–5.0 vs. 2.5–3.0 kg), implying a difference in age. Indeed, a significant decrease in rabbit corneal permeability with age was reported [43]. The P_{app} values determined in the presence of the chitosan derivatives indicate that both polymers significantly enhanced the apparent permeability of DMS across the cornea and that the respective effects were not significantly different. DMS is believed to cross epithelia *via* the transcellular route [31], hence the enhancing effect is thought to be due to the quaternary ammonium cations, present on both polymers, perturbing the lipid bilayer of the corneal cell membrane. In fact, an enhanced release of extra-cellular lactate dehydrogenase, indicative of a similar perturbation [44], was observed by Rassu et al., following contact of human umbilical vein endothelial cells with a chitosan derivative equal to N$^+$(60)–Ch [45]. On the other hand, the thiol groups of N$^+$(60)–Ch–SH(5) are thought to exert no parallel enhancing effect because, otherwise, this polymer would have resulted more effective in enhancing the P_{app} than the non-thiolated derivative. Anyway with N$^+$(60)–Ch–SH(5), the cumulative DMS transport over the whole time of experiment is significantly higher, due to a shorter lag time. As a plausible hypothesis, this effect might be ascribed to thiol groups causing a stronger adhesion of the thiomer to the mucous corneal surface. Altogether a higher DMS intraocular absorption-promoting potential of the thiomer results from the ex vivo permeation tests, which indicate that the permeabilizing effect of this polymer on the cornea is faster. This consideration is of particular relevance because the contact time of drugs instilled by eyedrops is usually short.

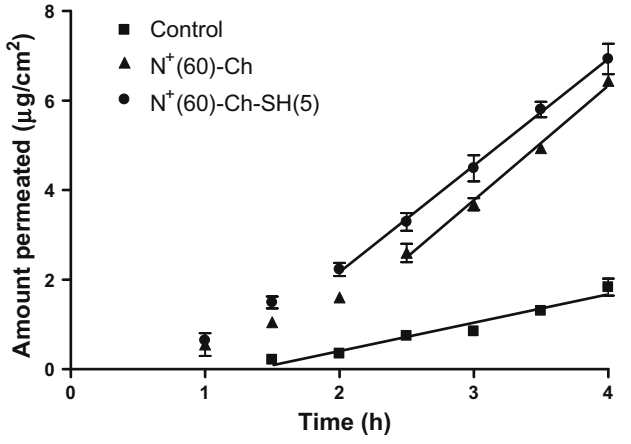

Fig. 1. Effects of chitosan derivatives on DMS permeation across excised rabbit cornea. Means ± SD of six runs. Regression lines are reported ($r^2 > 0.96$).

3.2. Rheological analysis

The ophthalmic drops containing N$^+$(60)–Ch or N$^+$(60)–Ch–SH(5) have shown a Newtonian rheological behaviour. The viscosity of the drops containing the thiolated derivative is lower than that for the non-thiolated one (2.5 vs. 5 mPa s). Bernkop-Schnürch et al. [46] reported increased viscosity and elasticity properties of thiolated compared to unmodified chitosan and ascribed this effect to the oxidation of thiol groups at physiological pH values, resulting in the formation of inter-chain disulphide bonds. This is apparently not the case with the present N$^+$(60)–Ch–SH(5) thiomer, which maintained a Newtonian rheological behaviour with a viscosity lower than that of the parent polymer. In fact, as stated in Section 2.5, the number of thiol groups of N$^+$(60)–Ch–SH(5) remained stable for at least 24 h at pH 7.4, whereas that of the chitosan–TBA conjugate 100 described by Bernkop-Schnürch et al. [46] decreased significantly after just 1 h at pH 6, less favourable than 7.4 to thiol oxidation. No substantiated, specific explanation of the above differences in behaviour is currently available. We can just refer to the considerable structural differences between N$^+$(60)–Ch–SH(5) and the chitosan–TBA conjugates (not oxidized), responsible for quite a different rheological behaviour at physiological pH (Newtonian vs. viscoelastic). Also, lacking is a substantiated explanation of the reduction of N$^+$(60)–Ch viscosity following thiolation. This effect may tentatively be ascribed to the replacement of the hydrophilic primary amino groups on about 5% of the N$^+$(60)–Ch repeating units by comparatively hydrophobic 3-mercaptopropionamide moieties, with consequent reduction of the amount of water molecules bound to such units. This might lead to a chain conformation change involving a reduced interchain friction.

3.3. In vivo tests

3.3.1. Eye irritation

The modified Draize test [35] revealed a slight conjunctival discharge and a very slight reddening of the conjunctiva, but no chemosis, during the first 30 min after application of the ophthalmic drops. In none of the six rabbits did the I_{irr} score exceed 3. Then, on the whole, the polymers studied exhibited a fair ocular tolerability.

3.3.2. Elimination kinetics from tear fluid

Data in Table 2 show that both polymers increased either MRT or RT_{max} exceedingly, the effect of the thiomer being stronger. These results can be explained by the polymers being mucoadhe-

Table 2
Polymer effects on DMS residence time in tear fluid of rabbits after instillation of one ophthalmic drop (50 μl) containing 0.3% w/v DMS and 1% w/v of different Ch derivatives. MRT, mean residence time; RT_{max}, maximum residence time at measurable concentrations (⩾2.5 μg/ml).

Polymer	MRT, min	RT_{max}, min
Control	5.07 ± 0.25	15
N^+(60)–Ch	65.74 ± 4.91*,**	150
N^+(60)–Ch–SH(5)	77.96 ± 3.57*,**	180

Means ± SD of eight values obtained with different animals.
* Significantly different from control.
** Significantly different from each other ($P < 0.05$).

sive. Such a mucoadhesivity is ascribed to cooperative interactions of polymers with the glycoproteins of the mucus covering the ocular surface, triggered by the electrostatic attraction between the positively charged quaternary ammonium groups of the chitosan derivatives and the negative charges of the glycoproteins. The thiomer is still more mucoadhesive because its thiol groups can give exchange reactions with disulphide bonds within the mucus or oxidation reactions with cysteine-rich subdomains of mucus glycoproteins [47,48], both resulting in the formation of disulphide bonds between polymer and mucus.

3.3.3. Pharmacokinetics in the aqueous

Previous work [28] has shown that the paracellular route across the cornea, which is the more likely for very polar molecules, remains difficult to penetrate despite the permeabilizing action of chitosan derivatives. In fact, the water-soluble tobramycin sulphate was unable to reach detectable levels in the aqueous humour even when its retention on the ocular surface was maximized, by applying an erodible ocular insert, and the cornea was treated with

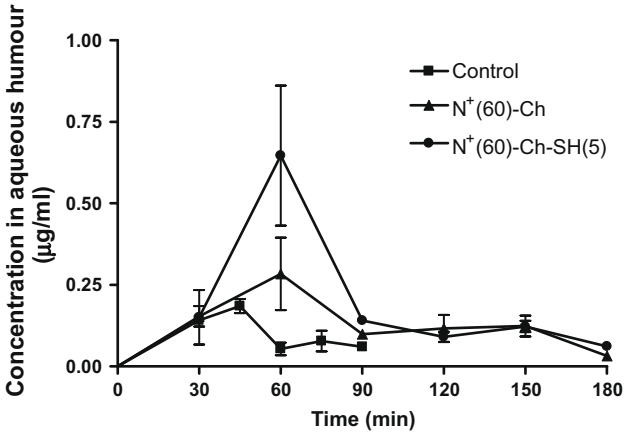

Fig. 2. Pharmacokinetics in the aqueous following instillation of ophthalmic drops (see text for composition). Means ± SD of at least six values obtained with different animals.

the permeability enhancer TMC [34]. On the other hand, the use of a polymeric enhancer may turn profitable for improving the effectiveness of existing commercial eyedrop formulations of fairly permeable drugs, such as DMS. The profiles of DMS concentration in the aqueous *vs.* time seen in Fig. 2 and the relative pharmacokinetic data, listed in Table 3, in particular, C_{max} and AUC_{0-150}, show that in the presence of the chitosan derivatives, the intraocular absorption of DMS is significantly increased with respect to the control and that the effect of the thiomer is significantly stronger than that of the non-thiolated derivative. Such a strengthened effect is believed to be the result of a synergistic action of the quaternary ammonium and thiol groups present on the former. The ex vivo permeation tests discussed in Section 3.1 have shown that the quaternary ammonium cations are responsible for the permeabilization of the cornea, while the *in vivo* measurements of the precorneal elimination kinetics have suggested that the above cations and thiol groups act synergistically to prolong DMS contact with the ocular surface, thus allowing the thiomer more time to exert its permeabilizing action. It is known that the thiols of thiomers formulated in neutral aqueous solution are prone to oxidation, so the present N^+(60)–Ch–SH(5) is expected to pose chemical stability issues. These have been overcome by lyophilizing the polymer for storage [31].

4. Conclusions

The questions that have prompted the present research have been answered. In fact, the synergism of quaternary ammonium and thiol groups of the N^+(60)–Ch–SH(5) derivative, which was found to be at the basis of the interaction of this polymer with the intestinal epithelium, has been shown to also characterize its interaction with the corneal epithelium. Consequently, the thiomer has resulted more effective than the parent non-thiolated derivative in promoting the transcorneal absorption of DMS and shows promise of enhancing the intraocular bioavailability of the relevant ophthalmic drops and allowing improvement of the therapeutic protocol. The quaternary ammonium ions of the thiomer are responsible for both the permeabilization of corneal epithelium and the polymer mucoadhesion, while the thiols increase the latter. This synergistic action is at the basis of the polymer bioactivity. The determination of the corneal hydration level at the end of the ex vivo experiments and the observation of ocular irritation signs in the course of the *in vivo* experiments have indicated a good ocular tolerability of the chitosan derivatives investigated. Their use in ophthalmic drops is made comparatively simple by their water solubility and absence of viscosity-increasing effect, especially in the case of the thiomer. This leaves room for further increasing the intraocular bioavailability by adding an inert, biocompatible viscosity enhancer. To get the better of thiol stability problems, N^+(60)–Ch–SH(5) could be stored in the lyophilized state and dissolved extempore in the DMS suspension. In fact it has been shown that the –SH groups remain stable in the ophthalmic drops at least 24 h at ambient temperature.

Table 3
Pharmacokinetic data in the aqueous following instillation of one ophthalmic drop (50 μl) containing 0.3% w/v DMS and 1% w/v of different Ch derivatives.

Polymer	C_{max}, μg ml^{-1}	T_{max}, min	AUC_{0-150}, μg ml^{-1} min	AUC_{rel}
Control	0.185 ± 0.022	45	6.25 ± 0.65	1
N^+(60)–Ch	0.284 ± 0.111**	60	21.48 ± 3.81*,**	3.44
N^+(60)–Ch–SH(5)	0.646 ± 0.215*,**	60	33.19 ± 6.96*,**	5.31

Means ± SD of at least six values obtained with different animals.
* Significantly different from control.
** Significantly different from each other ($P < 0.05$).

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