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Research paper

Effects of *N*-trimethylchitosan on transcellular and paracellular transcorneal drug transport

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Abstract

The effects of *N*-trimethylchitosan (TMC) on the transcorneal transport of dexamethasone, taken as a marker of the transcellular penetration route, and of tobramycin, a marker of the paracellular route, were studied by assessing the TMC effect on the intraocular pharmacokinetics of each marker. The drugs were topically applied via erodible inserts (weight, 20 mg; diameter, 6 mm; drug dose, 0.3 mg) based on poly(ethylene oxide), containing 10% w/w medicated TMC microspheres (diameter <2.5 μm). Before application, drug release and insert erosion kinetics, and release mechanism were studied in vitro. With either drug, introduction of 10% TMC into insert did not substantially alter the release and erosion rates, hence this formulation was apt to isolate the transcorneal penetration enhancing effect of TMC. Ocular pharmacokinetics were determined in the rabbit model. TMC produced significant increases of dexamethasone C_{max} (5.69 ± 0.49 vs. 3.07 ± 0.31 μg/ml) and AUC (619.3 ± 32.5 vs. 380.5 ± 32.0 μg min/ml) in the aqueous with respect to the reference TMC-free insert. On the other hand, TMC was unable to yield tobramycin concentrations in the aqueous exceeding the determination limit (0.5 μg/ml). In conclusion, TMC enhances transcorneal transport via the transcellular route, whereas it is unable to effectively open the tight junctions between corneal cells.
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Keywords: Dexamethasone; Tobramycin; *N*-Trimethylchitosan; Transcorneal absorption; Absorption enhancer; Transcellular transport; Paracellular transport; Ocular drug delivery; Mucosal delivery; Polymeric biomaterials

1. Introduction

Extensive investigation of the potential of chitosan, the deacetylated form of chitin, in ocular drug delivery has been stimulated by such properties of this polymer as mucoadhesiveness [1,2] and ability to enhance the permeability of mucous membranes [2,3]. A survey of the literature [4–8] has revealed that chitosan is endowed with excellent ocular tolerance [9], favourable rheological behaviour [10], ability to prolong precorneal drug retention [9] and to enhance transcorneal permeability [5,11].

The effectiveness of chitosan as a transmucosal absorption enhancer, however, is severely limited by its insolubility at the neutral pH of the tear fluid. This inconvenience can be circumvented by synthesizing the partially quaternized derivative *N*-trimethylchitosan chloride (TMC), which is soluble irrespective of pH. Indeed, TMC has proved a potent permeation enhancer of hydrophilic molecules and macromolecules across the intestinal epithelium [12–14]. It was reported that TMC acts on epithelial cell monolayers by opening the tight junctions between adjacent cells through an interaction of the polycationic polymer with the negatively charged sites on the cell membrane and/or in the tight junctions. Such an action would favour paracellular drug transport [3,12,13,15,16]. In a recent paper [17], it was shown that TMC polymers of medium to high quaternization degree also have the ability to significantly enhance the permeability of the fluroquinolone antibiotic

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ofloxacin across a stratified epithelium, such as the cornea. These data concur with other literature results in indicating electrostatic interactions as the basis of the permeability enhancing mechanism for drugs of different physicochemical properties across epithelia of different structure. However, the data could not clarify whether TMC affects the paracellular or the transcellular route of transcorneal transport, since it is still unknown by which route ofloxacin is transported. In fact, although this molecule is rather hydrophilic than lipophilic ($\log p = -0.47 \pm 0.02$, according to Zlotos et al. [18]), its partition coefficient is not so far from 1 as to assume transport by the sole paracellular route.

The potential importance of TMC as an effective enhancer of transcorneal drug absorption warrants an investigation of its mechanism of action. In this light, the aim of the present work was to investigate the relative importance of the TMC effects on the transport of topically applied drugs across the tight junctions between corneal cells (paracellular route) and across the corneal cell membrane (transcellular route). To this purpose, the TMC effects on the intraocular penetration of two markers of the respective permeation routes were studied using the rabbit, which is an animal model widely accepted as representative of the human eye. The aminoglycoside antibiotic tobramycin and the antiinflammatory corticosteroid dexamethasone were selected on a log *P* basis and in virtue of their extensive use in ophthalmology. The partition coefficient for tobramycin could never be determined, because of the drug insolubility in *n*-octanol [19], therefore, tobramycin was taken as the tracer of the paracellular route. A log *P* value of 1.95 was reported for dexamethasone [20], which justifies considering this drug a tracer of the transcellular route. It was reported that tobramycin did not reach measurable concentrations in the aqueous humour following instillation of 0.3% eyedrops six times at the frequency of one drop every 15 min [21]. These findings entailed the use of a drug administration system granting an intraocular availability higher than that allowed by eyedrops. In fact, the drugs were administered to the rabbit eyes via erodible ocular inserts based on poly(ethylene oxide), in order to maximize the drug concentration in the aqueous humour. It is known, indeed, that prolonged-release inserts increase the drug ocular bioavailability with respect to the traditional eyedrops [22–24]. A comparison between the drug concentration vs. time profiles in the aqueous resulting from application of a TMC-releasing insert and of a TMC-free reference could be a simple means of evidencing in vivo the corneal permeability enhancing effect of TMC. Indeed, drug transport from the precorneal area into the aqueous essentially occurs via the cornea, as the conjunctiva plays the role of a conduit for drug clearance into the systemic circulation or for drug transport to the deep tissues of the eye [25]. However, it must be considered that the intraocular pharmacokinetics depend not only on the corneal permeability, but also on the factors that determine the precorneal drug availability, such as the residence time of the release system at the application site and the release pattern and rate. Thus, the TMC effect on the concentration vs. time profile in the aqueous can be ascribed to a modification of the corneal permeability only if the presence of this polymer in the insert does not modify the other above-mentioned factors. In order to prepare insert formulations apt to the purpose, the effects of different TMC fractions on the release mechanism and rate of each tracer were studied in vitro, assuming that the TMC fraction(s) that did not substantially alter these properties would also leave them unchanged when the inserts were applied in vivo.

2. Materials and methods

2.1. Synthesis and characterization of TMC

TMC was prepared from a chitosan obtained from crab shells (Sigma). The molecular weight (MW) and deacetylation degree of chitosan were determined by capillary viscometry and IR spectroscopy, respectively, according to literature methods [26]. TMC was synthesized by two reductive methylation steps, using the procedure described by Sieval et al. [27], with some modifications [17]. Commercial chitosan was converted into chitosan hydrochloride microparticles by an already reported spray-drying technique [5]. Sodium iodide (Fluka), 15% aqueous sodium hydroxide solution and methyl iodide (Fluka) were added to the chitosan hydrochloride suspension in 1-methyl-2-pyrrolidinone (Fluka) at 60 °C, in the proportions and sequence indicated by Sieval et al. [27]. The polymeric material was isolated from the reaction mixture by precipitation with excess ethanol and centrifugation, then it was washed in sequence with ethanol and ether. The product was subjected to a second methylation step after which the polymer salt was converted into the chloride form and purified by dialysis [17]. The solution was finally lyophilized to obtain TMC. This was qualitatively characterized by ^{1}H NMR in D_2O, using a 200 MHz spectrometer (Bruker AC 200). The quaternization degree (QD) of TMC was assessed by an already described method based on elemental analysis of nitrogen and titration of the non-quaternized amino groups [17]. In one case, the product obtained from a single methylation step was isolated, purified, and its QD was determined as described above, in order to verify whether it was higher than the minimum potentially sufficient to produce a transcorneal permeability enhancement [17].

2.2. Preparation of TMC microspheres

TMC microspheres were prepared by spray-drying a 0.1% w/v aqueous TMC 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). Drug-containing TMC microspheres were obtained by spray-drying 0.1% w/v aqueous TMC solutions containing

drug concentrations calculated to obtain the designed w/w payloads of 7.5% and 15%. The diameter of all microspheres, observed by an optical microscope, was <2.5 μm. Following preparation, the microspheres were stored in a desiccator under calcium chloride. The actual payloads of tobramycin sulphate or dexamethasone were determined as described in Section 2.7, after dissolution of exactly weighed microsphere amounts in pH 7.4, 0.0375 M phosphate buffer. Each analysis was carried out in triplicate.

2.3. Preparation of inserts

Commercial powders of tobramycin sulphate, dexamethasone (both from Sigma) and poly(ethylene oxide) MW 900 kDa (PEO) (Polyox® WSR-1105, gifted by Union Carbide Italia S.r.l.) were passed through a 106 μm sieve before processing. One-gram powder amounts composed of 98.5% PEO and 1.5% tobramycin sulphate, or 98.5% PEO and 1.5% dexamethasone, or PEO and different fractions of drug-loaded TMC microspheres were thoroughly mixed with a spatula, then 20-mg aliquots of each mix were compressed by a hydraulic press (applied force, 9800 N) into flat-faced inserts of 6 mm diameter and 0.8–0.9 mm thickness. The inserts containing TMC microspheres loaded with tobramycin sulphate were formulated with 10.6% and 21.1% microspheres containing 14.2% and 7.1% drug, respectively. The inserts containing microspheres loaded with dexamethasone were formulated with 10.3% and 20.5% microspheres containing 14.6% and 7.3% drug, respectively. The virtual PEO–TMC wt proportions were 9:1 and 8:2 and in all cases the nominal drug fraction and dose in the inserts were 1.5% and 0.3 mg, respectively. For the in vivo tests, PEO–TMC (9:1) inserts prepared with TMC microspheres loaded with tobramycin sulphate or dexamethasone were used. For the observation of the behaviour and tolerability of inserts in the rabbit eyes, inserts containing sodium fluorescein (Aldrich) as a tracer were prepared using PEO into which 0.5% w/w tracer had been dispersed by wetting the PEO powder portionwise with the appropriate volume of a 0.05% w/v sodium fluorescein solution in absolute ethanol, while mixing and letting the solvent evaporate, then vacuum-drying to a constant weight. For comparison, conventional eyedrops, viscosized with 5.5% w/v poly(vinyl alcohol), 97.5–99.5 mol% hydrolysed, MW 72 kDa (Fluka), containing 0.3% w/v dexamethasone or tobramycin sulphate, were prepared. The dexamethasone drops contained a dispersion of drug particles (<1.5 μm) obtained by spray-drying a 0.1 mg/ml drug solution, under the process conditions described in the preceding section.

2.4. Measurement of drug release and insert erosion kinetics

An already described technique was used [23]. Briefly, each insert was tightly inserted into a 3-mm deep cylindrical cavity, of exactly the same diameter as the insert, drilled at the centre of a 4-mm thick Teflon disc. Two discs, each containing an insert, were immersed, with the exposed insert surface in upward position, into 50 ml of pH 7.4, 0.0375 M isotonic phosphate buffer, thermostated at 35 °C and stirred under controlled hydrodynamics. To determine the drug release kinetics, at intervals samples of dissolution medium were analysed for the drug as described in Section 2.7. Sink conditions were maintained in the dissolution medium throughout the release experiment. To determine the insert erosion kinetics, after a pre-established elution time each disk was withdrawn, dried and weighed, and the dissolved insert wt fraction was computed. This procedure was repeated for different elution times.

2.5. Determination of drug–polymer interactions

The tendency of each drug to interact in solution with the PEO–TMC polymer system was investigated by a previously described method, based on the dynamic dialysis technique [17]. Drug flux through a porous cellulose membrane (Spectra/Por®, molecular weight cutoff, 3500 Da, Spectrum Laboratories Inc., Rancho Dominguez, CA) under quasi-steady state conditions was measured at 35 °C in the presence or absence of the polymers under test in the donor phase (phosphate buffer, pH 7.4, 0.0375 M). A 0.6% w/v total concentration of PEO and TMC in the 9:1 ratio, and an initial drug concentration of 0.009% w/v, i.e., below the dexamethasone solubility, were completely dissolved in the donor phase. The initial drug-PEO–TMC proportions in this phase were the same as in the inserts. Sink conditions were ensured in the receptor medium. The receptor was analysed for each drug as described in Section 2.7. The regression for the fitting of dialysis data, expressed as drug concentration in the donor vs. time, to first order kinetics was always significant ($r^2 \geqslant 0.987$, $n \geqslant 8$). This allowed calculation of the dialysis rate constant. In all cases, the dialysis data were independent of the donor phase stirring speed, which demonstrated that the membrane was the only effective diffusional barrier to drug transport from donor to receptor phase. Under the above experimental conditions, a reduction of the dialysis rate constant caused by the polymers was considered a sign and a measure of drug–polymer interactions. Differences were considered significant, on the basis of Student's t-test, at $p < 0.05$.

2.6. Animal tests

Tests of biocompatibility and residence time of inserts in the precorneal area, and measurement of drug penetration into the aqueous were carried out following the procedures described in detail in a previous paper [23]. An outline is given below.

Male, New Zealand albino rabbits of 2.5–3.0 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. For the evaluation of biocompatibility and residence time in the precorneal area TMC-free inserts or PEO–TMC (9:1) inserts loaded with tobramycin sulphate or dexamethasone were used. All of them contained sodium fluorescein as a tracer. One insert of each type was applied in the lower conjunctival sac of each eye of at least two rabbits. Following insertion, the device formed a superficial gel and adhered to the application site within 5 min. At appropriate time intervals the state of the release systems was observed, in order to assess the minimum residence time in the precorneal area. The minimum residence time is defined as the time required for fluorescence to disappear. The checking intervals were regulated on the basis of the process rate, taking care that the last interval, during which the fluorescence disappeared, was no longer than 10% of the assessed residence time value. Irritation signs, such as conjunctival/corneal oedema and/or hyperaemia were checked, as well as fluorescence at the rabbit nose, due to lacrimation. For the measurement of drug penetration into the aqueous, each of the PEO–TMC (9:1) or the reference TMC-free inserts, containing a nominal dose of 0.3 mg of tobramycin sulphate or dexamethasone, was applied in the lower conjunctival sac of one eye of each rabbit. For comparison, the same dose of each drug was applied by instillation of two eyedrops (50 μl each, instilled at a 1 min interval). After a pre-established time from administration, the rabbit's eye was anaesthetized, then 50–70 μl of aqueous humour was aspirated from the anterior chamber. At least six animals were used for each time point. The aqueous humour samples were immediately frozen and stored at −18 °C before analysis. The area under the concentration in the aqueous vs. time curve ($AUC_{0-240\ min}$) was calculated by the linear trapezoidal rule (GraphPad Prism software). The pharmacokinetics determined for the PEO–TMC (9:1) insert were compared with those for the TMC-free PEO inserts. Statistical methods reported by Schoenwald et al. [28] were used in comparing AUC values. The significance of differences was evaluated by Student's *t*-test ($p < 0.05$).

2.7. Analytical methods

2.7.1. Analysis of samples from in vitro experiments

Tobramycin samples were analysed by HPLC. The apparatus (Perkin–Elmer) consisted of Series 200 pump, 20 μl Rheodyne injector, Series 200 fluorescence detector and Turbochrom Navigator HPLC software for data integration. A Spheri-5 RP18 250 × 4.6 mm 5 μm column was used. The mobile phase (flow rate 2.0 ml/min) was acetonitrile–phosphoric acid (5 g/L) (64:36). The excitation and emission wavelengths were set at 424 nm and 476 nm, respectively. Before injection, 110 μl of 1.2 mg/ml fluorescamine in acetone was added to 300 μl of sample and the mixture was vortexed. Standard curves were constructed by analysing at least six standard tobramycin sulphate solutions in pH 7.4, 0.0375 M phosphate buffer. The standard curves produced on different days were all linear ($r^2 > 0.99$) in the concentration range from 0 to 8 μg/ml (limit of determination, about 0.2 μg/ml). The tobramycin sulphate concentration of each unknown analysed on a given day was determined via the standard curve produced on the same day. The retention time was 3.4 min.

Dexamethasone samples were analysed spectrophotometrically at 241 nm. Blank runs showed the absence of any interference with the measurements.

2.7.2. Analysis of samples from in vivo experiments

Aqueous humour samples were analysed for tobramycin by HPLC [29]. The apparatus described above was used, with a Partisil SCX 250 × 4 mm 10 μm column. The mobile phase (flow rate 2.0 ml/min) was acetonitrile–phosphoric acid (20 g/L) (70:30). The excitation and emission wavelengths were set at 424 nm and 476 nm, respectively. Before injection, 20 μl of 1.2 mg/ml fluorescamine in acetone was added to 50 μl of sample, then the mixture was vortexed and centrifuged for 10 min at 13,000 rpm. Standard solutions were prepared by adding 5 μl of tobramycin sulphate solutions in pH 7.4, 0.4 M phosphate buffer to 45 μl of drug-free aqueous. Standard curves were constructed by analysing at least four standards. The standard curves produced on different days were all linear ($r^2 > 0.99$) in the concentration range from 0 to 50 μg/ml (limit of determination, about 0.5 μg/ml). The retention time was 4.4 min. Tobramycin concentrations beyond the limit of determination were never observed in any of the in vivo samples. The use of the Partisil SCX strong cation exchange column was required because the Spheri-5 RP18 column, used for the in vitro samples, would not allow separation of the drug peak from that of the aqueous humour proteins.

Dexamethasone in aqueous humour samples was determined by the following HPLC procedure. Before injection, each sample was diluted with 1:1 v/v acetonitrile and centrifuged for 10 min at 13,000 rpm. The apparatus described above was used, with UV detection (Perkin Elmer LC 290) set at 241 nm. The mobile phase (flow rate 1.0 ml/min) was water–acetonitrile–tetrahydrofuran (64:35:1). Standard solutions were prepared by adding 50 μl of drug solutions in acetonitrile to 50 μl of drug-free aqueous, then vortexing and centrifuging for 10 min at 13,000 rpm. Standard curves were constructed by analysing at least four standards. The standard curves produced on different days were all linear ($r^2 > 0.99$) in the concentration range from 0 to 20 μg/ml (limit of determination, about 0.2 μg/ml). The drug concentration of each unknown analysed on a given day was determined via the standard curve produced on the same day. The retention time was 8.7 min.

3. Results and discussion

3.1. Synthesis and characterization of TMC

An average viscometric MW of 700 kDa and a deacetylation degree of 90.6% resulted from the analysis of the chitosan used for the synthesis of TMC. A similar analysis of a chitosan used in a previous work [19] yielded a similar deacetylation degree (89.9%) but a different MW (1490 kDa), although both products were purchased from Sigma and had the same product code. The ^{1}H NMR spectrum and the alkalimetric curve for the TMC obtained from a two-step methylation of the present 700 kDa chitosan (data not shown) were equal to the respective data for the fully quaternized TMC obtained in the previous work from a three-step methylation of the 1490 kDa chitosan. In fact, a full quaternization of the present chitosan was obtained with two methylation steps, as confirmed by five replicates of the reaction, whereas with the previous Sigma product a QD of 35% was obtained under the same reaction conditions. A single-step methylation of the present chitosan yielded a QD of 11%, which was deemed insufficient to produce a corneal permeability enhancement [17].

3.2. Preparation of TMC microspheres

The comparatively low concentration of the solution subjected to the spray-drying process was due to the low solubility of dexamethasone. The process proved to be a rapid, reproducible and efficient technique for preparation of TMC microspheres loaded with pre-established drug wt fractions. Indeed, a comparison of the actual microsphere payloads (not reported) with the designed values indicated a high entrapment efficiency (>95%).

3.3. Kinetic measurements in vitro

With either dexamethasone or tobramycin as the drug, the in vitro release and insert erosion kinetics for PEO-based inserts containing different fractions of TMC microspheres were compared with corresponding data for TMC-free inserts. This served to select the insert formulation more suitable to evidence in vivo a possible transcorneal penetration enhancing effect of TMC, by observing the effect of this polymer on the drug concentration profile in the aqueous. According to the basic rationale of the present study, the release pattern and rate of the selected insert formulation should be close to those of the reference TMC-free insert, so that the same effects of these properties on the drug concentration profile in the aqueous could be assumed for the two inserts, and the enhancement effect could be isolated. Although the in vitro and the in vivo release rates from the selected formulation are not supposed to be equal, yet an absence of substantial TMC effects on the in vitro release mechanism and rate would predict a similar absence in vivo.

For each release data series, the data points found in the interval between the first point and the one corresponding to a released fraction of 50–60% were analysed, with the aid of a computer program (GraphPad Prism), by the following equation:

$$F = F_1 + K(t - t_1)^n, \quad (1)$$

where F is the drug fraction released in time t; t_1 is the time of the first data point analysed; F_1 is the drug fraction released in time t_1; K is a rate parameter; the time exponent, n, measures the curvature of the release profile, and hence, is related to the release mechanism. By choosing a sufficiently high t_1 value, the effects of transient initial phenomena (e.g., rapid dissolution of TMC microspheres from the matrix surface) on the parameters K and n could be ruled out.

3.3.1. Data for dexamethasone

Data on dexamethasone release are shown in Fig. 1. Data analysis by Eq. (1) started from $t_1 = 0.5$ h. This value is beyond the burst effect, as demonstrated by the very good alignment of the first data point with the successive ones, appearing in the figure. The results of the analysis are shown in Table 1. The r^2 values indicate a satisfactory data fitting in all cases. As appears from the 95% confidence intervals of the time exponent, n, for the reference TMC-free insert and for the inserts containing 10% or 20% TMC, this parameter is not significantly different from 1, which corresponds to a time-constant release rate. This pattern is in agreement with a release controlled by insert

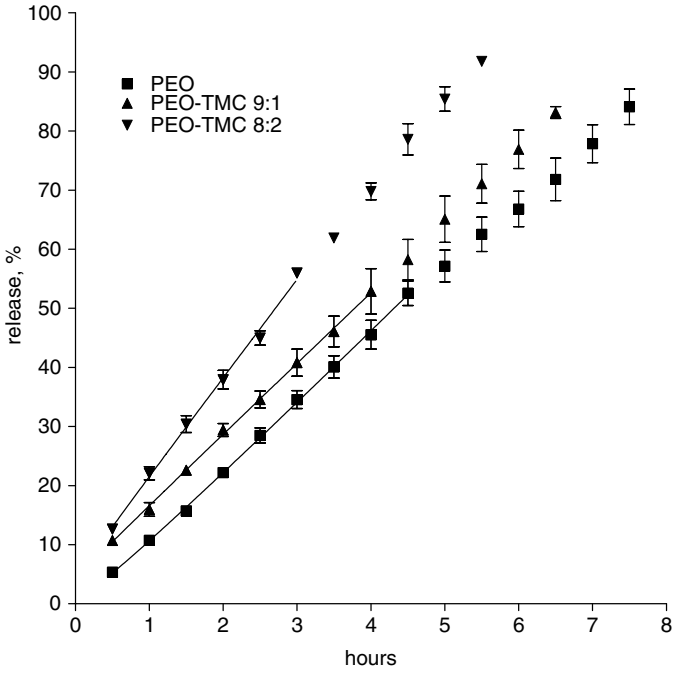

Fig. 1. Data on 1.5% dexamethasone release from PEO-based inserts containing different TMC fractions. Regression lines for the fitting of Eq. (1) to release data are reported. Each data point is means ± SD of four runs.

Table 1
Parameters obtained from the fitting of Eq. (1) to the release data for dexamethasone (DMS), presented in Fig. 1 ($t_1 = 0.5$ h), and for tobramycin sulphate (TBS), presented in Fig. 3 ($t_1 = 0.25$ h)

Drug	TMC in insert (% w/w)	$F_1 \pm$ SE (CI[a]) (%)	$K \pm$ SE (CI[a]) (% h^{-n})	$n \pm$ SE (CI[a])	r^2
DMS	0 (reference)	5.25 ± 0.42 (4.21–6.28)	11.11 ± 0.44 (10.03–12.20)	1.04 ± 0.03 (0.98–1.10)	0.999
	10	10.47 ± 0.49 (9.19–11.74)*	12.18 ± 0.55 (10.75–13.61)	0.99 ± 0.03 (0.91–1.07)	0.999
	20	12.99 ± 1.19 (9.19–16.79)*	16.91 ± 1.47 (12.22–21.61)*	0.99 ± 0.08 (0.73–1.25)	0.996
TBS	0 (reference)	12.32 ± 0.89 (9.47–15.17)	22.85 ± 1.14 (19.23–26.46)	0.90 ± 0.06 (0.71–1.09)	0.997
	10	26.14 ± 1.39 (20.16–32.12)*	24.98 ± 1.72 (17.59–32.37)	0.79 ± 0.10 (0.34–1.23)	0.992
	20	24.84 ± 1.15 (19.87–29.81)*	32.93 ± 1.41 (26.84–39.02)*	0.70 ± 0.06 (0.45–0.96)	0.997

[a] 95% Confidence interval.
* Significantly different from reference ($p < 0.05$).

erosion. In this case, indeed, when the eroding surface area is time-constant, such as in the present in vitro conditions, erosion and release occur at a constant rate. In fact, the release of poorly soluble drugs, such as dexamethasone (solubility, 0.1 mg/ml [30]), from erodible PEO matrices has been shown to be controlled by matrix erosion [31,32]. The rate parameter, K, for the insert containing 10% TMC and that for the reference insert are not significantly different, since the relevant confidence intervals are virtually superimposable. Yet, for any given time of the release process the dose fraction released from the TMC-containing insert is significantly higher than that released from the reference, as indicated by Student's t-test applied to relevant data in Fig. 1 ($p < 0.05$). However, this difference is only due to the initial burst effect, which can be ascribed to a comparatively rapid dissolution of the TMC microparticles, and of the drug contained therein, from the matrix surface. From the difference between the F_1 values relative to the insert containing 10% TMC and to the reference, found in Table 1, it can be estimated that the burst effect involved no more than about 5% of the drug dose. The rate parameter for the insert containing 20% TMC is significantly greater than that for the reference, which implies a faster erosion rate of the former. Because of the significant difference in release properties between the insert containing 20% TMC and the reference insert, the former was deemed non-compliant with the above-illustrated rationale of the in vivo tests.

The insert erosion kinetics were measured to definitely ascertain the release mechanism and the TMC effect thereon. The difference between the drug fraction released from an insert in a given time, F_R, and the fraction of the same insert eroded after such a time, F_E, can give information on the relative importance of the diffusive and erosive release mechanisms. In principle, $F_R - F_E$ is zero for a completely erosion-controlled release, while it is expected to increase with increasing contribution of the diffusive mechanism. The $F_R - F_E$ values, determined for the insert containing 10% TMC and for the TMC-free insert after various elution times, are compared in Fig. 2. A practically time-constant positive value is observed for the plain PEO insert. This can be explained as follows. At the early stages of the process PEO dissolution lagged behind drug release, because the former was preceded by polymer hydration and swelling to an extent sufficient to allow polymer chain disentanglement, whereas drug release occurred meanwhile via diffusion through the swollen polymer. Subsequently the erosion front did not reach up with the diffusion front, but rather, a constant distance between moving fronts, and hence, a constant $F_R - F_E$ value, was maintained through the time of experiment. The presence of 10% TMC did not substantially modify the $F_R - F_E$ values, and hence the release mechanism, at later times. However, it produced some initial burst of release, causing higher $F_R - F_E$ values

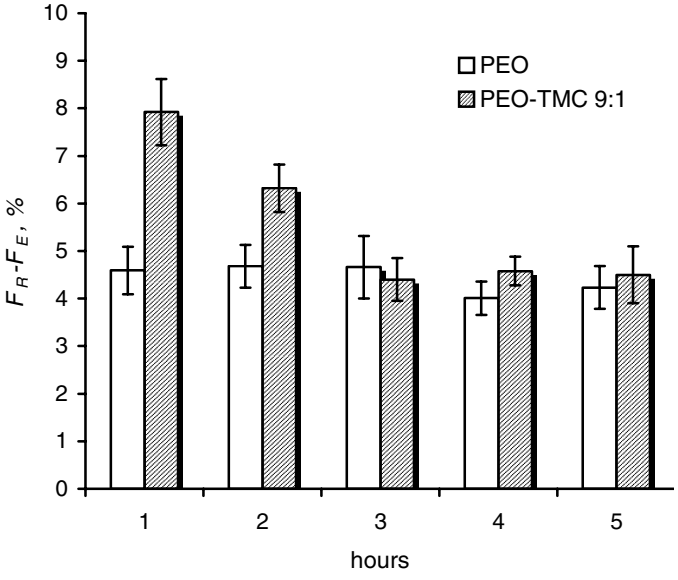

Fig. 2. Difference between the dexamethasone fraction released (F_R) and the insert fraction eroded (F_E) in a given time for PEO-based inserts not containing TMC or containing 10% TMC. Each value is the mean ± SD of four measurements.

at earlier times. Then the data in Fig. 2 substantiate the hypothesis that, apart from an initial short stage, the release from either the TMC-containing or the TMC-free insert was controlled by insert erosion. The above findings altogether indicate that the presence of 10% TMC in the PEO insert left the release mechanism and rate substantially unaltered, which was expected to be true also in the rabbit eye. Therefore, this insert was used in the experiments in vivo to evidence the enhancement effect of TMC on the corneal permeability of dexamethasone.

3.3.2. Data for tobramycin

Data on tobramycin release are shown in Fig. 3. These data, if compared with corresponding data in Fig. 1, show an apparently faster release of tobramycin compared to dexamethasone. The insert erosion rate for a given formulation is supposed to be independent of the particular drug, considering the low drug fraction in insert. Hence, the above difference in release rate could be ascribed to a substantial contribution of diffusion to the release of tobramycin, due to the high solubility of this molecule. The data in Fig. 3 were analysed by Eq. (1), following the criteria illustrated above. In the case under discussion, t_1 was given the value of 0.25 h, taking into account the higher release rate. The corresponding F_1 values for tobramycin, found in Table 1, are considerably higher than those for the same formulations containing dexamethasone, due to the different release mechanism. In all cases of tobramycin, the values of the time exponent, n, indicate some curvature of the release profile, in agreement with a significant contribution of diffusion to the release mechanism. In analogy with the dexamethasone inserts, the parameters n and K for the insert containing 10% TMC and for the reference are not significantly different, as results from the comparison between the respective 95% confidence intervals. However, the tobramycin sulphate fraction released in a given time from the system containing 10% TMC is significantly higher than that released in the same time from the TMC-free one, as Student's t-test applied to relevant data in Fig. 3 indicates ($p < 0.05$). Unlike the case of dexamethasone, discussed above, this difference is too marked to be solely ascribed to the initial burst effect but, perhaps, it is also due to some TMC effect on the release mechanism. As can be seen in Table 1, the rate parameter, K, for the tobramycin insert formulated with 20% TMC is significantly greater than the value for the reference. Therefore, in compliance with the criteria illustrated above, this insert was excluded from the in vivo tests. The diagram presented in Fig. 4 shows $F_R - F_E$ values considerably higher than those appearing in Fig. 2 for the dexamethasone inserts. This confirms the important contribution of diffusion to the release mechanism of tobramycin sulphate. The $F_R - F_E$ values increased in time until virtually constant values were attained, when insert erosion finally took complete control of the release kinetics. Fig. 4 shows that the $F_R - F_E$ values in the presence of 10% TMC in the insert are significantly higher than those for the reference insert at corresponding times. This indicates that TMC enhanced the contribution of diffusion to the overall release mechanism. Nevertheless, this effect was of limited relevance, considering the similar values of the parameters K and n characterizing tobramycin release from the insert containing 10% TMC and from the reference (see Table 1). Therefore, the insert was used for the in vivo tests.

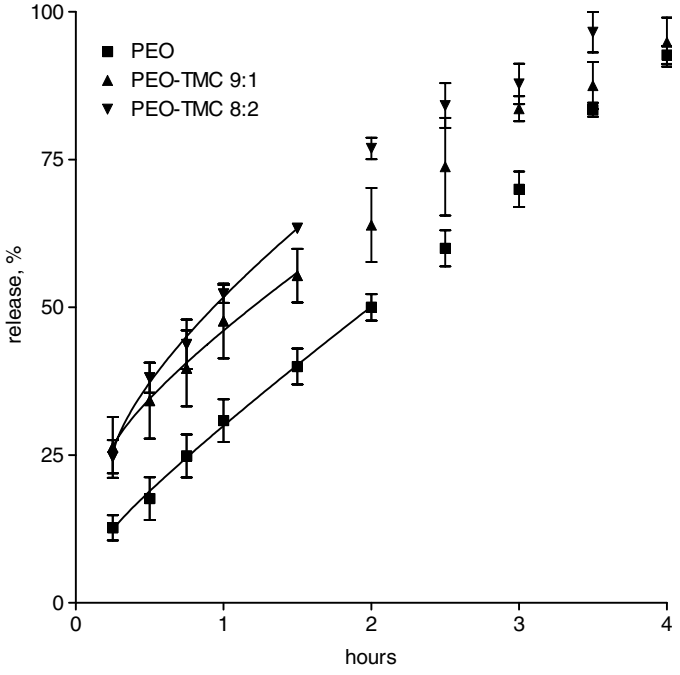

Fig. 3. Data on 1.5% tobramycin sulphate release from PEO-based inserts containing different TMC fractions. Regression lines for the fitting of Eq. (1) to release data are reported. Each data point is the mean ± SD of four runs.

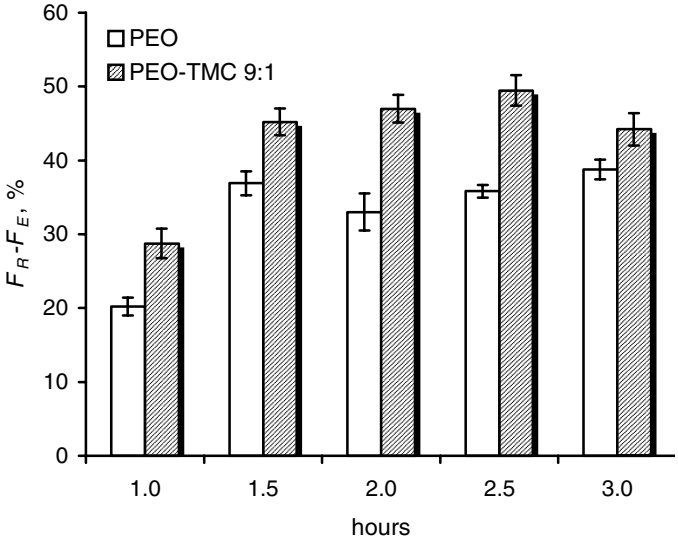

Fig. 4. Difference between the tobramycin sulphate fraction released (F_R) and the insert fraction eroded (F_E) in a given time for PEO-based inserts not containing TMC or containing 10% TMC. Each value is the mean ± SD of four measurements.

3.4. Drug–polymer interactions

Insert erosion at the application site in the rabbit eye would cause the drug and the polymers of the formulation to be at contact in the dissolved state. In this situation, drug–polymer interactions could affect the biopharmaceutical behaviour of the applied systems. The existence of such interactions was investigated by comparing the dynamic dialysis rate constants determined in the presence and in the absence of the polymers under study in the donor phase. In fact, no significant difference was found between the rate constants at comparison. This indicated the absence of significant drug–polymer interactions.

3.5. Animal tests

To investigate the TMC effect on the transcorneal penetration of dexamethasone or tobramycin, the pharmacokinetics for the TMC-free insert were compared with those for the insert containing 10% TMC. The following behaviour in the precorneal area of either insert type medicated with either drug was observed. After insertion in the rabbit eye, the insert formed a superficial gel and adhered almost instantly to the application site, then the gel gradually spread over the cornea and eroded. Fluorescence persisted in the precorneal area for about 6 h, whereas gel traces were clearly discerned after about 3 h from application. Only mild irritation signs, such as slight reddening of the conjunctiva and the eyelid rim, were observed. The similarity of the residence times in the precorneal area of the TMC-containing and the TMC-free insert, together with the similarity in release mechanism and rate, resulting from the in vitro tests, should ensure similar drug availability for intraocular absorption. Hence, differences in the respective concentration vs. time profiles in the aqueous can be ascribed to TMC effects on corneal permeability.

The dexamethasone concentration profiles in the aqueous, following administration of a 0.3 mg dose by the systems at comparison, are shown in Fig. 5, while the relevant pharmacokinetic data are listed in Table 2. The AUC values in the table show a 1.6-fold bioavailability increase produced by the TMC-containing insert compared to the reference, while the C_{max} was increased 1.8 times. These data indicate a faster intraocular dexamethasone absorption from the former insert, which can only be ascribed to a corneal permeability enhancement caused by TMC. These findings indicate that the transcellular permeation, which is the more likely intraocular penetration route of lipophilic drugs, such as dexamethasone, can be effectively accelerated by TMC. This effect could be exerted through an interaction with the glycoproteins of the mucous layer covering the cornea [33] and/or with the lipid bilayer of the corneal cell membrane. In fact, interactions of protonated chitosan with model phospholipid membranes have been reported [34,35]. The pharmacokinetic data obtained with the dexamethasone eyedrops are shown in Fig. 5 and in Table 2. A comparison of these data with those

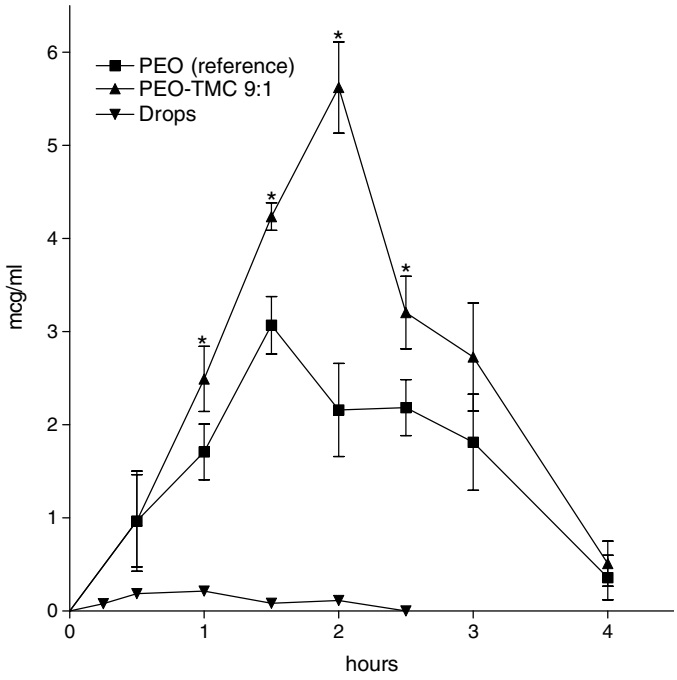

Fig. 5. Dexamethasone concentration in the aqueous humour vs. time following administration of 0.3 mg drug by TMC-containing or TMC-free PEO-based inserts, or by eyedrops. Each data point is the mean ± SE of at least six values obtained with different animals. Data points marked with * are significantly different from the corresponding data for the reference insert ($p < 0.05$). Where not shown, error bars are within the symbols.

Table 2
Pharmacokinetic data for transcorneal penetration into aqueous humour after administration of 0.3 mg dexamethasone (DMS) or tobramycin sulphate (TBS) by different vehicles

Vehicle	Drug	C_{max} ± SE (μg/ml)	t_{max} (min)	$AUC_{0–240\ min}$ ± SE (μg min/ml)
Drops	DMS	0.21 ± 0.02	60	17.1 ± 0.1
PEO[a] (reference)		3.07 ± 0.31	90	380.5 ± 32.0
PEO–TMC 9:1[a]		5.69 ± 0.49*	120	619.3 ± 32.5*
Drops	TBS	n.d.[b]	n.d.[b]	n.d.[b]
PEO[a] (reference)		n.d.[b]	n.d.[b]	n.d.[b]
PEO–TMC 9:1[a]		n.d.[b]	n.d.[b]	n.d.[b]

[a] Insert.
[b] Not detectable.
* Significantly different from reference ($p < 0.05$).

obtained with the inserts justifies the use of inserts for maximizing intraocular drug availability. On the other hand, the tobramycin concentration in the aqueous following application of either insert type or eyedrops was below the determination limit of the applied HPLC method (0.5 μg/ml). These results indicate that TMC is unable to promote tobramycin intraocular penetration to an extent sufficient to attain measurable antibiotic concentrations in the aqueous, even if the applied topical system maximizes the preconeal drug availability. Since tobramycin is likely to prefer the paracellular absorption route, it is argued that the tight junctions connecting the corneal

epithelium cells were not effectively opened by TMC. If the cornea is virtually impermeable to tobramycin, although the external cell layer might be affected by TMC, the deeper layers could remain impervious to the drug. On the other hand, dexamethasone could effectively permeate the cornea, hence the TMC action, although exerted on the corneal surface, could enhance the apparent permeability.

4. Conclusions

Introduction of 10% TMC microspheres into PEO inserts did not substantially affect the drug release pattern and rate, nor the vehicle residence time in the precorneal area. This allowed assessing the TMC effect on the corneal permeability simply by putting to comparison the concentration vs. time profiles in the aqueous obtained with the TMC-containing and the TMC-free inserts. The presence of TMC significantly increased C_{max} and $AUC_{0–240\ min}$ for the lipophilic dexamethasone, indicating an enhancement effect of TMC on the transcellular penetration pathway. On the other hand, the tobramycin concentration in the aqueous was below the limit of detection, even with the TMC-containing insert. It is known that TMC is able to enhance the paracellular penetration of hydrophilic molecules or macromolecules across cell monolayers, such as the intestinal epithelium, by opening the tight junctions between cells. Our results have demonstrated that the tight junctions of such a stratified epithelium as the cornea are not effectively opened by TMC, at least to such an extent as to allow penetration of hydrophilic drugs of the tobramycin molecular size. In the light of these results, the remarkable promotion of the transcorneal absorption of ofloxacin by TMC, observed in our previous work [17], is ascribable to a polymer action on the mucous layer covering the cornea and/or on the corneal cell membrane, rather than to an effective opening of the tight junctions.

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