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Topical use of chitosan in ophthalmology: tolerance assessment and evaluation of precorneal retention

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Abstract

The mucoadhesive polysaccharide chitosan was evaluated as a potential component in ophthalmic gels for enabling increased precorneal drug residence times. This cationic vehicle was expected to slow down drug elimination by the lacrymal flow both by increasing solution viscosity and by interacting with the negative charges of the mucus. The molecular weight (M_w) and concentration of polysaccharide were studied in four types of chitosan as parameters that might influence ocular tolerability and precorneal residence time of formulations containing tobramycin as therapeutic agent. An ocular irritation test, using confocal laser scanning ophthalmoscopy (CLSO) combined with corneal fluorescein staining, clearly demonstrated the excellent tolerance of chitosan after topical administration onto the corneal surface. Gamma scintigraphic data showed that the clearance of the formulations labelled with ^{99m}Tc-DTPA was significantly delayed in the presence of chitosan with respect to the commercial collyrium (Tobrex®), regardless of the concentration and of the molecular weight of chitosan in solution. At least a 3-fold increase of the corneal residence time was achieved in the presence of chitosan when compared to Tobrex®.

Keywords: Chitosan; Ocular tolerance; Ophthalmoscopy; Gamma scintigraphy; Precorneal retention.

1. Introduction

A wide range of antibiotics are currently available for the treatment of external ocular diseases, such as blepharitis, conjunctivitis and bacterial keratitis. Of these, aminoglycosides are probably the most widely used antibiotics in ophthalmol-

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ogy. In vitro (Traub and Raymond, 1972; Waterworth, 1972) and in vivo (Smolin et al., 1974; Laibson et al., 1981) studies have demonstrated the efficacy of tobramycin against bacterial infections caused by *Pseudomonas* and *Staphylococci*. However, tobramycin is a very hydrophilic compound and hence its topical application onto the cornea results in poor transcorneal penetration. In addition, its ocular residence time is shortened by rapid elimination from the corneal surface by the lachrymal flow. As a consequence, the standard treatment consists of frequent instillations, which can create compliance and management problems.

Since the ocular efficacy of topically applied drugs is influenced by corneal contact time, the most common method to improve drugs ocular availability is to increase precorneal residence time by using hydrogels based on natural, synthetic or semi-synthetic polymers (Ludwig et al., 1992; Unlü et al., 1992; Bernatchez et al., 1993).

Chitosan, a polycationic biopolymer obtained by alkaline deacetylation of chitin was chosen as a vehicle for ophthalmic formulations since it exhibits several favorable biological properties such as biodegradability (Struszczyk et al., 1994), nontoxicity (Knapczyk et al., 1984) and biocompatibility (Chandy and Sharma, 1990; Hirano et al., 1990). A prolonged precorneal residence time of formulations containing chitosan was attempted, not only based on its ability to increase solution viscosity but also because of its mucoadhesive properties. In fact, due to its positive charges at neutral pH, an ionic interaction with the negative charges of sialic acid residues of the mucus has been proposed as a mechanism of mucoadhesion (Lehr et al., 1992; Henriksen et al., 1996; He et al., 1998).

To our knowledge there are few reports in the literature on the use of chitosan in ophthalmology (Henriksen et al., 1996; Calvo et al., 1997; Genta et al., 1997). In addition, the residence time of viscosified solutions will be directly related to the tolerability of the polymer. For these reasons, the first objective of this work was to assess and quantify the potential ocular irritation of four different types of chitosan varying in their M_w and/or in their deacetylation degree (DD). This study was conducted using confocal laser scanning microscopy and corneal fluorescence staining after repeated topical administration to the eye. A further aim of the present study was to investigate the precorneal residence time of chitosan formulations containing tobramycin by gamma scintigraphy and to compare them with the commercial solution, Tobrex® (Alcon, Fort Worth, TX).

2. Materials and methods

2.1. Materials and animals testing

Two high purity grade chitosan salts of similar deacetylation degree ($>80\%$) were purchased from Pronova Biopolymer (Oslo, Norway) and were used without further modification: chitosan hydrochloride UPCl 110 and chitosan glutamate UPG 210. Two types of chitosan (CHITO-1 and CHITO-2) of high molecular weight (Table 1) and low deacetylation degree ($<60\%$) were a gift from Ciba Vision® (Duluth, GA, USA). Characteristics of the four types of chitosan tested are summarized in Table 1.

Tobramycin was a gift from Ciba Vision® (Duluth, GA, USA).

Technetium-99m-diethylentriaminepentaacetic acid (^{99m}Tc-DTPA) was prepared in the Department of Nuclear Medicine at the University Hospital of Geneva (Switzerland), by elution of a commercially available ^{99}Mo-^{99m}Tc generator (Mallinckrodt, the Netherlands) as pertechnetate ions. A kit (Oryx Pharmaceutica, Switzerland) was used for the preparation of ^{99m}Tc-labelled diethylentriaminepentaacetic acid (^{99m}Tc-DTPA) from the eluate.

Table 1
Characteristics of chitosan used in tolerance and gamma scintigraphic studies

Chitosan type	Deacetylation degree (%)	Molecular weight[a] (kDa)
UPCl 110	87	160
UPG 210	83	580
CHITO-1	53	1350
CHITO-2	59	1930

[a] M_w were determined by a GPC analysis adapted from the method of Lee et al. (1995).

Male albino New Zealand rabbits weighing approximately 4–5 kg and free of any ocular damage were used throughout tolerance and gamma scintigraphic studies. In vivo experiments were conducted as approved by the Animal Care and Use Committee of Geneva.

2.2. Formulation preparation and characterization

Formulations for tolerance purposes contained increasing concentrations (0.5, 1.0 and 1.5% w/v) of each polysaccharide, and were freshly prepared by dissolution of the polymer at room temperature in an isocryoscopic sterile phosphate buffer solution pH 7.4. As chemical and gamma sterilization processes are reported to degrade chitosan in solution (Rosiak et al., 1992), and as filtration of the final formulations was difficult due to their viscosity, formulations were prepared with precautionary measures to avoid microbial contamination. Final formulations were submitted to microbiological tests to verify the absence of bacterial contamination.

Formulations were prepared in a similar way for gamma scintigraphic studies. However, in addition to the polymer, they contained 0.3% w/v of tobramycin. Further adjustment with acetic acid (1% v/v in distilled water) to pH 6.0–6.2 was necessary in order to solubilize chitosan. Labeling of the formulations was achieved as described previously (Meseguer et al., 1993) by addition of 50 μl of ^{99m}Tc-DTPA to 450 μl of the formulations. The three same concentrations of each polymer were investigated as for the tolerance test.

The final pH of all the formulations was verified (pHmeter 691, Metrohm, Switzerland) as well as the cryoscopicity (Automatic osmometer type Digital/L, Knauer, Germany). Each measurement was made in triplicate. Rheological behavior of the formulations tested was investigated using a Bohlin CS rheometer, coupled with an ETO (Extended temperature Option) system of control of the temperature.

2.3. Tolerance evaluation

Twenty five μl of the solution to be tested were instilled onto the cornea of the right eye four times a day, for a period of 3 days and, once on the fourth day just before the examination of the cornea. After the last instillation, rabbits were sedated with an intramuscular injection of ketamine HCl (15 mg/kg weight body) and xylazine (3 mg/kg) (Furrer et al., 1998a). A total of 25 μl of a sodium fluorescein solution 0.5% was instilled to allow the injuried areas to be selectively marked. The eye was then rinsed for 1 min with NaCl 0.9% kept at body temperature. Finally, the cornea was observed with a confocal laser scanning ophthalmoscope (CLSO® Zeiss, Germany) modified as previously described (Furrer et al., 1998b). Briefly, a set of lenses was added to the original ophthalmoscope in order to examine the cornea instead of the retina. The CLSO was coupled to an image-processing system (Semper6, Synoptics, UK) to enable three-dimensional reconstruction from digitized frames and evaluation of the injured areas, which were represented by fluorescent zones. Each formulation was tested on three rabbits.

2.4. Scintigraphic studies

In vivo precorneal drainage of each formulation was assessed after instillation of 25 μl of radiolabeled solution onto the left cornea using a gamma camera (Toshiba GCA 602A) adjusted to detect the radiation of ^{99m}Tc (140 KeV) and fitted with a 4-mm pinhole. The activity instilled ranged between 1 and 2 MBq per 25 μl dose (Meseguer et al., 1993). A small plastic vial containing 25 μl aliquot of solution to be tested was placed near the eye of the rabbit, and used as a position tracer. The rabbit was kept on a table without a restraining box, its head being supported by the experimenter hand with its left eye in front of the collimator aperture at a distance of 6 cm.

Recording was started 5 s after instillation and frames were recorded over a period of 10 min using a 128×128 pixel matrix. Each formulation was tested on eight rabbits.

2.5. Data analysis

Individual 63 frames (36×5 s frames followed by 12×10 s frames and 15×20 s frames) were

Table 2
Characteristics of formulations based on chitosans

Chitosan type	Chitosan content (%)	pH	Osmolality (mosm/kg)	Viscosity[a] (mPa s)
UPCl 110	0.5	5.7	277.3	10
	1.0	5.6	293.3	17.4
	1.5	5.5	288	30.7
UPG 210	0.5	5.7	296	16.8
	1.0	5.5	271.3	54.5
	1.5	5.4	304	114.2
CHITO-1[b]	0.5	7.1	238	73.7
CHITO-2[b]	0.5	7.1	257	477.1

[a] Bohlin CS Rheometer®, 25°C, oscillation mode, cone-plate measuring system (1/40 and 4/40), 0.001–1.00 Hz.
[b] Formulations based on the two types of chitosan of higher Mw have been only tested at 0.5% since higher concentrations of polysaccharide were found to have viscosities that were too high to be administered onto the surface of the eye.

summed to obtain an overall picture of the distribution of the label. The final image was divided into five regions of interest (ROIs), which were, respectively, (1) the position reference, (2) the precorneal surface (3) the inner canthus, (4) the lachrymal duct and, (5) the background.

The parameters calculated were: t_{10} (remaining activity on the corneal surface at the end of the study: 10 min), $AUC^{(0,\ 10\ min)}$ (area under the curve of the percentage activity remaining in the precorneal ROI versus time) which represents the residence time of the formulation tested and, $t_{1/2}$ (half-life of elimination).

After verification that results followed a normal distribution, the parameters evaluated were analyzed by a Student's t-test (unpaired samples) for comparison purposes between the results obtained with formulations based on chitosan and the commercial solution, Tobrex®.

3. Results and discussion

3.1. Tolerance evaluation

The characteristics of the different formulations tested by confocal microscopy are summarized in Table 2. Since the viscosity values of the formulations tested were not too high, they could be easily and reproducibly applied onto the corneal surface. At the low concentrations studied (0.5–1.5%), all the formulations containing chitosan showed a newtonian behavior. It must be noted that chitosan salts (UPCl 110 and UPG 210) gave slightly acidic solutions (pH ranging from 5.4 to 5.7), whereas chitosan of higher M_w (CHITO-1 and CHITO-2) gave neutral hydrogels when dissolved. This last result was unexpected since chitosans are well known as giving transparent solutions or hydrogels up to pH 6.0–6.5. On the other hand, formulations based on CHITO-1 and CHITO-2 exhibited lower osmolality values than UPCl 110 and UPG 210.

However, in all cases pH and osmolalities were within a physiologically compatible range; as such, any irration that could be induced after instillation of solutions based on chitosan, could be expected to be due mainly to the polysaccharide itself rather than to the physico-chemical characteristics of the formulations.

Fig. 1 shows the relative area of fluorescence, representative of the percentage of corneal injury following the instillation of formulations containing chitosan in rabbits. A sodium chloride solution (0.9%) as well as phosphate buffer solution were used as controls and induced about 2% of corneal injury after instillation. When compared with the physiological solutions, administration of chitosan containing solutions resulted in slightly higher levels of irritation, scores ranging from 5 to 22% of damaged areas. A relationship can be observed between the molecular weight of chi-

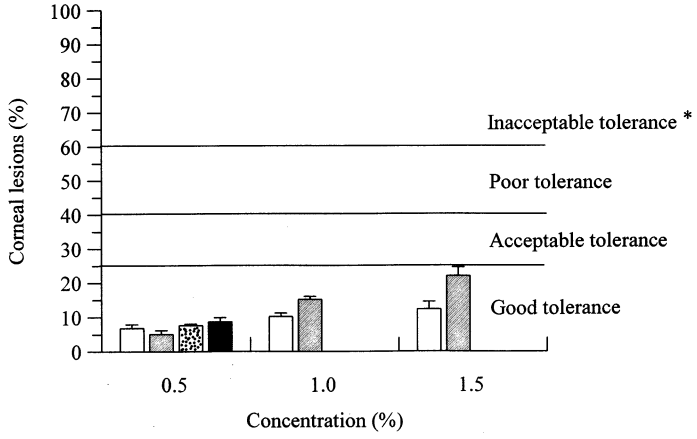

Fig. 1. Percentage of corneal lesion after instillation of increasing concentrations of UPCl 110 (□), UPG 210 (▨), CHITO-1 (▩) and CHITO-2 (■). (* Scale established by Kälin, 1994)

tosan and the degree of irritation. The higher the molecular weight tested, the greater is the calculated irritation score, except for the lowest M_w chitosans UPCl 110 and UPG 210 (but the difference of corneal lesions induced was not significant). For example, considering the lowest concentration of polymer tested (0.5%), the irritation score induced by CHITO-2 is 8.7 ± 1.2, in comparison it is $6.1 \pm 1.1\%$ for UPCl 110. The same trend can be observed by increasing the polysaccharide content from 0.5 to 1.5%. Nevertheless, independent of the molecular weight and of the concentration of chitosan tested, all of the solutions used were considered as being very well tolerated according to the previously established scale (Kälin, 1994). In fact, damaged corneal areas calculated were always inferior to 25%.

Unlike the official Draize test commonly used for tolerance evaluations, the CLSO method is limited to the cornea and do not provide informations on possible toxicity of tested chemicals on the nictitating membrane, the conjunctiva or, for example, lachrymation reactions. For this reason, observations of the cornea with the ophthalmoscope were completed with a clinical examination of the eye. This clinical evaluation was in agreement with confocal data since the usual symptoms of eye irritation, e.g. conjunctiva chemosis and/or redness, discharge, corneal swelling, were not detected.

With regards to the CLSO method used, two main advantageous points can be raised from the results obtained. The small standard deviations indicate that the method offers a good reproducibility for tolerance assessment of topically applied medication, making it possible to reduce the number of subjects tested from 6 to 3 with reliable results. In addition, the confocal method is sensitive enough to allow distinctions to be made between solutions containing the same product but at different concentrations, which are all very well tolerated; such a discrimination would not be possible with a conventional Draize test, the eye being always appearing to be healthy after administration of any of the formulations tested.

3.2. Precorneal retention time

The observation of the acquired gamma-camera images showed a good spreading over the entire precorneal area for chitosan based formulations immediately after administration, as compared to the Tobrex® commercial solution.

The curves of the remaining activity on the corneal surface as a function of time (10 min dynamic imaging) are shown in Fig. 2 and the parameters describing the precorneal drainage are summarized in Table 3.

As shown by the AUC and $t_{1/2}$ values, the presence of chitosan in ophthalmic preparations

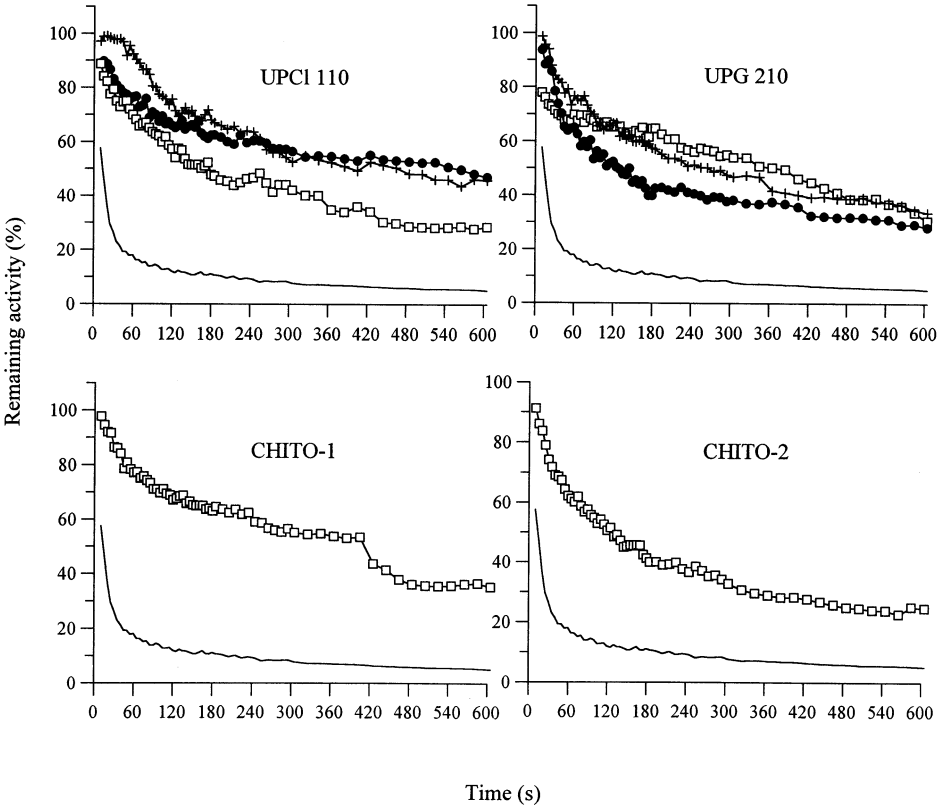

Fig. 2. Precorneal drainage of ^{99m}Tc-DTPA in formulations containing 0.3% w/v of tobramycin and various concentrations of chitosan; (□) 0.5%, (●) 1.0%, (+) 1.5% and (−) 0% (Tobrex® as reference).

always resulted in a significant increase ($P < 0.05$ and $P < 0.005$) of the mean precorneal residence time of the formulation on the corneal surface, when compared with Tobrex®. More precisely, a 3–5-fold improvement was achieved by adding chitosan in the formulations, depending on the Mw and on the concentration studied as represented by the AUC values. Similarly, $t_{1/2}$ values show that elimination of the formulations from the precorneal area was delayed in presence of chitosan, being 2.5–6.5 superior to Tobrex®. Furthermore, at the end of the study all the activity was concentrated in the lachrymal duct in the case of Tobrex® whereas about 25–50% of the activity remained associated with the cornea for solutions based on chitosan (Fig. 3).

The performances of the different types of chitosan investigated, in terms of AUC values, are compared in Table 3. Statistical analysis of these data have demonstrated that increasing the concentration of chitosan from 0.5 to 1.5% did not lead to significantly higher retention times ($P > 0.5$) of the ophthalmic preparations on the cornea, as had been expected. Furthermore, changing the molecular weight of the polysaccharide incorporated in the formulations did not significantly modify one or other of the precorneal drainage parameters evaluated ($P > 0.5$). However, there is a trend in favour of the existence of an optimal viscosity range (between 20 and 30 mPa s). In fact, formulations exhibiting such a viscosity, e.g. UPCl 110 0.5 and 1.0%, and UPG 210 0.5%, present higher AUC values, as well as higher half-life times of elimination and remaining activity on the corneal surface after 10 min (Table 3). The lack of significant influence of the concentration as well as of the molecular weight of chitosan could be interpreted as a proof, that the great

Table 3
Clearance half-life ($t_{1/2}$), remaining activity after 10 min (t_{10}) and area under the curve values (AUC $^{(0,\ 10\ min)}$) for chitosan formulations ($n = 5$–8) and for Tobrex® ($n = 6$)

Chitosan type	Concentration (%)	$t_{1/2}$ (min)	t_{10} (%)	AUC ± S.D. (% min)
UPCl 110	0.5	4.0	29	473 ± 303[a]
	1.0	10.0	47	569 ± 258[b]
	1.5	9.0	46	618 ± 152[b]
UPG 210	0.5	9.0	31	521 ± 207[b]
	1.0	7.5	28	432 ± 157[b]
	1.5	4.0	34	517 ± 307[a]
CHITO-1	0.5	7.0	35	473 ± 285[a]
CHITO-2	0.5	5.5	25	380 ± 189[b]
Tobrex®	—	1.5	5	120 ± 90

[a] $P < 0.05$, Student's t-test, unpaired samples, comparison with Tobrex®.
[b] $P < 0.005$, Student's t-test, unpaired samples, comparison with Tobrex®.

improvement of the retention times induced by the presence of the polysaccharide is not only due to the increased viscosity of the solutions but also to a saturable bioadhesive mechanism. Indeed, if the mechanism of bioadhesion of chitosan is really based on an ionic interaction with the negative charges of the mucus, as earlier reported (Lehr et al., 1992; Henriksen et al., 1996; He et al., 1998), it could explain why increasing the concentration or the M_w is not useful once all the negative sites of the ocular mucosa are occupied by the positive moieties of the polysaccharide. If this is the case, it could be proposed that a modification of the deacetylation degree of chitosan will have a greater influence on the duration of contact of the preparation with the corneal surface than the concentration or the M_w of the polysaccharide. The assumption of a possible specific ionic interaction between cationic polymers and the mucus is attractive, since it implies a quite different mechanism of bioadhesion than anionic polymers. In fact, some polyanionic polymers such as polyacrylic acids exhibit good mucoadhesive properties based on non-specific interactions with mucin, e.g. electrostatic and hydrophobic interactions, hydrogen-bonding and interdiffusion of the mucin and the polymer (Leung and Robinson, 1988).

4. Conclusion

Ophthalmic formulations based on chitosan are simple to manufacture and exhibit an excellent tolerance when topically administered onto the cornea, as demonstrated by using an objective and reproducible method. The presence of the polysaccharide significantly prolonged the corneal contact time as measured by gamma scintigraphy, when compared with the commercial solution used as control (Tobrex®). The absence of any influence of the concentration of chitosan used suggested that a concentration as low as 0.5% is sufficient to ensure a significant enhancement of the residence time of ophthalmic preparations. In addition, since increasing the M_w does not achieve better contact times with the cornea, it allows the use of low M_w chitosan giving solutions of low viscosity, enabling easy manipulation for administration.

However, these promising results require further investigation regarding tobramycin dosage in tears (Felt et al., 1997), relevance of lower concentrations of polysaccharide, influence of the presence of a preservative and of the sterilization process on the final formulations and, of course, clinical efficacy.

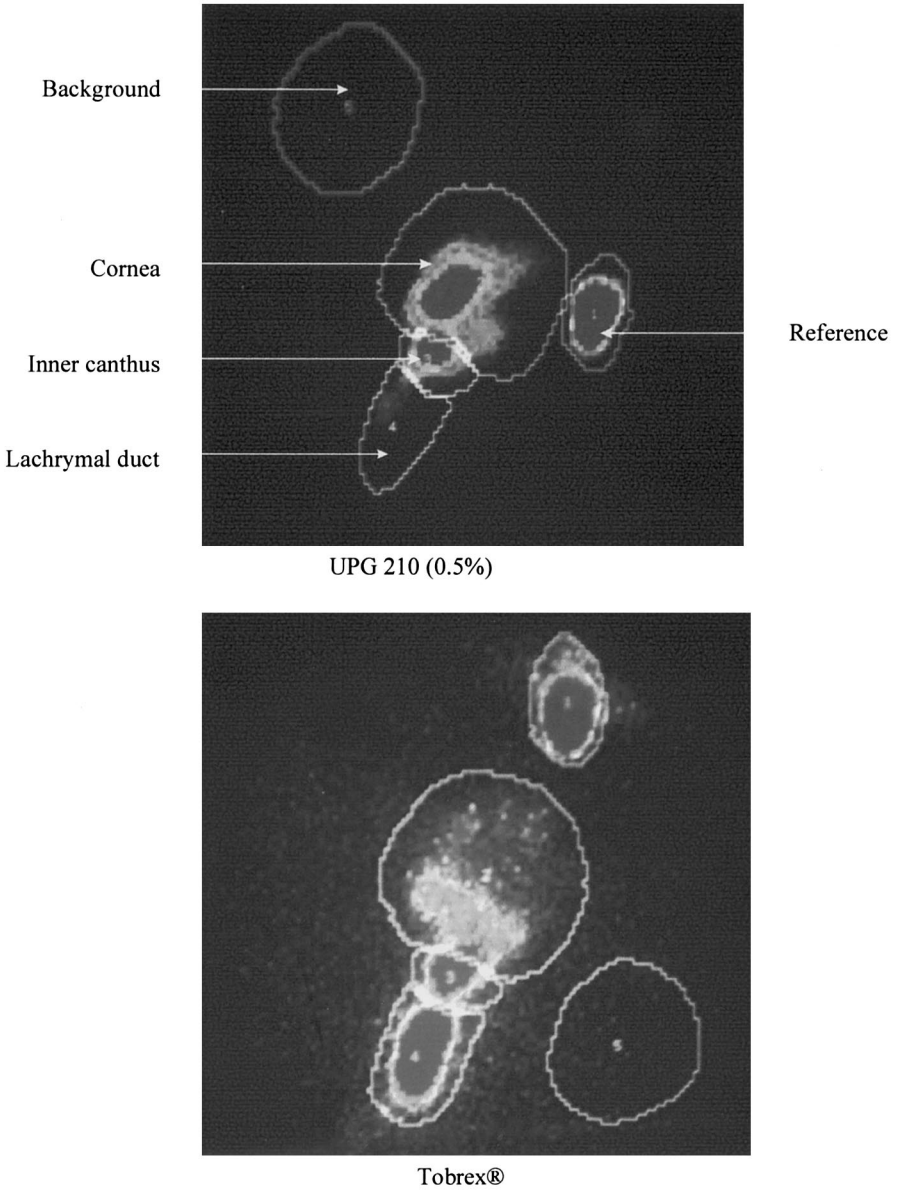

Fig. 3. Precorneal retention of a formulation based on chitosan and of Tobrex® after 10 min.

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