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Research paper

Ophthalmic delivery systems based on drug–polymer–polymer ionic ternary interaction: In vitro and in vivo characterization

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Abstract

Aim of the work was to develop mucoadhesive eyedrops containing tetrahydrozoline hydrochloride (TZ), a decongestant drug, and based on a ternary interaction drug–polymer–polymer. The anionic polymers assessed were the anionic hyaluronic acid (HA) and polyacrylic acid (PAA), the cationic chitosan (HCS) and the polyelectrolyte gelatin (G). Formulations based on the ternary systems TZ/G/HA, TZ/HCS/HA, TZ/G/PAA and TZ/HCS/PAA at the stoichiometry ratios between cationic and anionic polymers and containing a 10 and 20 fold excess of the anionic polymers were prepared. The formulations were characterized for in vitro mucoadhesive and release properties. The ex vivo/in vivo residence properties were assessed for the formulations that combined the better in vitro mucoadhesive and release properties. The physical stability of the formulations selected was determined following steam sterilization and storage at 25 and 40 °C.

The synergistic effect of G with HA and PAA improves the mucoadhesion of the formulations while the interaction of HCS with HA and PAA is likely to produce higher neutralization of the anionic polymer charge and minor chain flexibility resulting in a limited mucoadhesion improvement. Both G and HCS participate to control drug release.

The selected formulations demonstrate to possess consistency (viscosity) sensitive to the ions of the medium, and probably for this reason the ex vivo/in vivo residence properties could not directly correlated to mucoadhesion and to drug release control properties. However, the formulations are able to maintain levels of TZ detectable until 20 min after the instillation in rabbits, while TZ was not detectable since 3 min after instillation of the drug solution.

The physical stability, following steam sterilization and storage, the low viscosity combined with good residence time in conjunctival sac make the TZ/G/20HA the more promising formulation.

Keywords: Tetrahydrozoline hydrochloride; Hyaluronic acid; Polyacrylic acid; Chitosan; Gelatin; Mucoadhesion properties; Drug release properties, Ex vivo/in vivo residence properties; Physical stability; Steam sterilization

1. Introduction

The ophthalmic inflammation is correlated with allergic reactions and is accompanied with itching, burning and watery eyes. The histamine mediates a variety of physiological and pathological responses and plays a key role in allergy, in particular in itching and in vasodilatation that causes the swelling of the conjunctiva [1].

Decongestant drugs (ocular vasoconstrictor), often combined with antihistaminic, are able to relieve the symptoms of this pathological conditions [2].

The conventional drug delivery systems for ophthalmic administration are affected by heavy drawbacks: the precorneal drug loss due to the lachrymal flow and the palpebral blinking. As a consequence, the standard treatments consist of frequent instillation of formulation, which can create toxicity due to the nose-lachrymal drainage, problems correlated to the poor patient compliance and sometimes incorrect therapy management.

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The ophthalmic availability can be improved increasing the precorneal residence of the formulation. The improvement of the ocular permanence can be achieved with the employment of the mucoadhesive polymers that prolong the precorneal residence time by interacting with ocular mucin layer. However, the success of the mucoadhesive approach depends on the capability of the formulation to retain the drug included into the vehicle, for example by using polymers capable to interact with the drug.

Mucoadhesive polymers were employed to design viscous eyedrops, gels or inserts and microparticle systems [3,4]. The polymer physico-chemical properties, that are recognized to be useful for mucoadhesion, are the presence of groups able to form hydrogen bound, strong ionic charge (anionic or cationic), high molecular weight, chain flexibility and good spreading properties.

Hyaluronic acid (HA) is an anionic biocompatible, non-immunogenic and biodegradable glycosaminoglycan. HA is widely distributed in the extracellular matrix of connective tissues and is present in the aqueous and vitreous humour of the eyes where it acts as a lubricant and/or shock absorbing fluid; moreover it is able to modulate fribroblast proliferation and inflammatory response. Furthermore, it presents good tolerability [5] and mucoadhesive properties [6].

Chitosan (HCS) is a non-toxic, biocompatible and biodegradable cationic polysaccaride characterized by good mucoadhesive properties [7,8]. It has been recently proposed for topical use in ophthalmology and in mucosal site-specific systems [9].

Gelatin (G) is an amphoteric polyelectrolite with a net charge depending on the pH and the type of gelatin (from porcine or bovine collagen by acidic (Gelatin A) or alkaline (Gelatin B) hydrolysis, respectively). Gelatin has good mucoadhesive properties and is well tolerated after ophthalmic administration [10].

Polyacrylic acid (PAA) is an anionic mucoadhesive polymer widely employed in viscous eyedrop formulations [11,12].

Given these premises the aim of the present work was to develop mucoadhesive eyedrops based on tetrahydrozoline hydrochloride (TZ), a decongestant drug widely used in the treatment of allergic conjunctivitis [13]. Ternary interactions drug–polymer–polymer were investigated where the ionic interactions between the cationic drug and the anionic polymers were exploited to control drug release at the administration site. Moreover, the ionic complexation between the anionic and the cationic or amphoteric polymer was investigated to modulate the drug release and the mucoadhesion of the formulation to the conjunctiva (the district involved in allergic conjunctivitis).

The stoichiometry of the ternary interactions TZ/G/HA, TZ/G/PAA, TZ/HCS/HA and TZ/HCS/PAA was studied. Systems based on the stoichiometric ratios and containing 10 or 20 fold excess of the anionic polymer (HA or PAA) were prepared. The formulations were characterized for the viscosity, mucoadhesive and release properties by means of in vitro approaches. The formulations that combined the better mucoadhesive and release properties were subjected to 'wash away' measurements and were administered to albino rabbits to evaluate the ex vivo/in vivo residence properties, respectively. The physical stability of the selected formulations was assessed after steam sterilization and storage at 25 and 40 °C.

2. Experimental part

2.1. Materials

Tetrahydrozoline hydrochloride (TZ) was kindly obtained by Sims SpA (Reggello, I). The following polymers were used: hyaluronic acid (HA) (Hyalectine® MW 693 kDa, Fidia SpA Abano Terme, I); polyacrilic acid (PAA) (Carbopol EX214, high MW BF Goodrich, Cleveland, USA); chitosan hydrochloride (HCS) (Seacure Cl 213, medium MW, Pronova, Drammen, N) and gelatin (G) (Gelatin A, 75–100 bloom, Sigma, Milan I).

2.2. Methods

2.2.1. Assessment of the drug–polymer complex stoichiometry

The stoichiometry of the TZ/anionic polymer interaction was assessed in distilled water by means of a dialysis equilibrium as described by Bonferoni et al. [14,15].

Dialysis bags (cut off = 12–14 kDa, Emanuele Mires, Milan, I) were filled with 10 ml of a 0.5% w/v anionic polymer solution. The bags were closed and put in 40 ml of TZ solution where they were maintained under agitation at 37 °C until equilibrium was reached (24 h). The dialysis membrane did not allow the polymer to get out but allowed the drug to diffuse into and eventually to interact with the polymer. Different initial drug concentrations outside the dialysis bags were tested ranging between 0.5 and 5 mM. After the equilibrium was attained, the final drug concentration outside the dialysis bag was assayed by means of a spectrophotometrically detection at 254 nm wavelength (Lamba 25, Perkin–Elmer, Milan, I). The data were interpreted according to the following equation [15,16]:

$$r = nx/(K_d + x)$$

where r, amount of drug bound (μmol/mg of polymer) at the equilibrium; x, concentration of drug unbound (mM) at the equilibrium (as measured outside the dialysis bags); K_d, constant of dissociation (mM); n, maximum binding capacity of the polymer for the drug (μmoles/mg of polymer).

2.2.2. Assessment of the polymer–polymer complex stoichiometry

2.2.2.1. Viscosimetric measurements. The polymers were completely hydrated in distilled water by gentle stirring at room temperature. Mixtures (30 ml) containing a fixed amount of G or HCS (0.5% w/w) and increasing amounts of the anionic polymers (HA and PAA) were also prepared at different ratios ranging from 1/0.25 to 1/2. The interaction products were removed by means of the centrifugation at 2000 rpm for 10 min and the viscosity of the supernatants was measured. The polymer/polymer ratio where a minimum of viscosity was observed could be considered the stoichiometric composition of the complex. Viscosity measurements were performed by means of a rotational rheometer (Bohlin CS Rheometer, Bohlin Instrument Division, Metrics Group Ltd, Cirencester, UK). A coaxial cylinder combination (C25) was used as a measuring system. The apparent viscosity was measured after application for 1 min of 100 s^{-1} shear rate. All measurements were carried out at 37 °C, after a rest time of 3 min [17].

2.2.2.2. Turbidimetric measurements. Turbidimetric measurements were effected employing a spectrophotometer UV/VIS (Lamba 25, Perkin–Elmer, Milan, I) at 420 nm wavelength. The turbidity of 0.1% w/v G or HCS polymer solutions (30 ml) prepared in distilled water was evaluated after successive additions of fixed amounts (1 ml) of each anionic polymer hydrated in the same media at the same concentration [17]. A maximum turbidity value corresponded to the stoichiometric polymer/polymer ratio.

2.2.3. Preparation and characterization of the formulations

Formulations based on the stoichiometric ratios previously assessed and with a 10 fold and 20 fold excess of the anionic polymer were prepared according to the Table 1. Table 1 shows the % (w/w) composition of the formulations prepared. The amount of TZ was chosen as the maximum amount compatible to an OTC product: 0.1% (w/w) following the indication of the Italian Ministry of Health. Four formulations G/HA, G/PAA, HCS/HA and HCS/PAA are based on the stoichiometry of the drug/polymer/polymer ternary interaction products. The formulations G/10HA, G/10PAA, HCS/10HA and HCS/10PAA are based on the amount of the anionic polymers 10 folds the stoichiometry of the drug/polymer/polymer ternary interaction products. The formulations G/20HA, G/20PAA, HCS/20HA and HCS/20PAA are based on the amount of the anionic polymers 20 folds the stoichiometry of the drug/polymer/polymer ternary interaction products. The reference formulations HAref and PAAref have the same composition of the formulations containing the anionic polymers 20 folds the ternary interaction product stoichiometry without HCS or G. A TZ solution (0.1% w/w) (S) was prepared. The drug was hydrated in distilled water containing 5.07% w/w of mannitol (Sigma, Milan, I) to allow formulation isotonicity with the lachrymal fluid [11]. G or HCS were added to the TZ solution and kept under gentle stirring up to complete hydration. Subsequently, HA or PAA were added under vigorous stirring. The pH of the formulations was adjusted to 7.0 by means of NaOH 0.2 N.

Table 1
Composition % (w/w) of the formulations prepared

Composition	G/HA	G/PAA	HCS/HA	HCS/PAA
At the stoichiometry	G/HA* 0.1%TZ 0.03% HA 0.16% G	G/PAA* 0.1% TZ 0.04% PAA 0.55% G	HCS/HA* 0.1% TZ 0.03% HA 0.03% HCS	HCS/PAA* 0.1% TZ 0.04% PAA 0.09% HCS
Anionic polymer 10 fold excess	G/10HA* 0.1% TZ 0.3% HA 0.16% G	G/10PAA 0.1% TZ 0.44% PAA 0.55% G	HCS/10HA* 0.1% TZ 0.3% HA 0.03% HCS	HCS/10PAA 0.1% TZ 0.44% PAA 0.09% HCS
Anionic polymer 20 fold excess	G/20HA * 0.1% TZ 0.6% HA 0.16% G	G/20PAA 0.1% TZ 0.88% PAA 0.55% G	HCS/20HA* 0.1% TZ 0.6% HA 0.03% HCS	HCS/20PAA 0.1% TZ 0.88% PAA 0.09% HCS
Reference formulations	HA 0.1% TZ 0.6% HA	PAA 0.1% TZ 0.88% PAA	HA 0.1% TZ 0.6% HA	PAA 0.1% TZ 0.88% PAA
Solution	S 0.1%1% TZ			

Formulations (*) characterized by pH values lower than 7 were buffered by means of NaOH 0.2 M at pH 7.00.

Each formulation was subjected to viscosity measurements by means of a rotational rheometer (Bohlin CS Rheometer, Bohlin Instrument Division, Metrics Group Ltd, Cirencester, UK). A cone plate combination (CP 4/40) or a coaxial cylinder combination (C25) were used as a measuring systems depending on formulation consistency. The apparent viscosity was measured after application for 1 min of 100 s^{-1} shear rate. All measurements were carried out at 37 °C [18], after a rest time of 3 min.

2.2.4. Mucoadhesion measurements

The mucoadhesive properties of the formulations were also evaluated by means of a tensile stress tester [19,20]. Gastric porcine mucin dispersion (type II, crude, Sigma, Milan, I) was used as a model biological substrate for the ophthalmic mucosa [11].

The gastric mucin was dispersed at 8% (w/w) concentration in artificial lachrymal fluid ($NaHCO_3$ 2.2 g/l; NaCl 6.26 g/l; KCl 1.79 g/l; $CaCl_2 \cdot 2H_2O$ 73.5 mg/l; $MgCl_2 \cdot 6H_2O$ 96.4 mg/l at pH 7.5 with HCl 0.1 M) [21].

Each formulation (100 mg) was layered on a filter paper disc (area = 2 cm^2) and fixed on the movable carriage of the apparatus.

The mucin dispersion (100 μl) was spread on a filter paper disc (area = 2 cm^2) faced to the formulation on the sample-holder using a double adhesive tape.

After the contact between the formulation and the biological substrate was established, a preload of 2500 mN was applied for 3 min to allow the formation of

the mucoadhesive joints. The preload was then removed and the formulation and the biological substrate were detached forward at a constant speed (4 mm/min) up to the complete separation of the two surfaces. The experimental conditions (preload and contact time) were chosen as the minimum force and time which allow to maximize the mucoadhesive parameters and to obtain reproducible results. Both displacement of the movable carriage and force of detachment data were recorded and simultaneously collected on a personal computer.

The parameter work of adhesion (AUC) was calculated as the area under the force of detachment vs displacement curve by means of the trapezoidal rule [19,20].

Blank measurements were also performed using a filter paper disc wetted with 100 μl of artificial lachrymal fluid instead of the biological substrate. Such measurements aimed to evaluate the contribution of the cohesion of each formulation to the mucoadhesive potential.

2.2.5. Release measurements

Dialysis bags (cut off = 12–14 kDa) were filled with a fixed amount of each formulation (10 g) and put into 40 ml of artificial lachrymal fluid used as receptor phase. The receptor phase was stirred and thermostated at 37 °C. At fixed time 2 ml of the receptor phase were withdrawn and replaced with fresh fluid. The drug released was assayed spectrophotometrically as previously described. The drug release was also performed using distilled water instead of artificial lachrymal fluid in the same experimental conditions.

2.2.6. Viscosity measurements after dilution with lachrymal fluid

The formulations which showed the better mucoadhesive and drug release properties (G/20HA, G/10 PAA, G/20PAA and HCS/20HA) were subjected to viscosity measurements after dilution with lachrymal fluid to assess the influence of the diluting and washing action of the tears in vivo. In particular, the formulations were diluted 1:1 and 1:4. The same dilutions were performed with distilled water for comparison purposes, to take into account the effect of the ionic strength and the dilutions on the formulation consistency.

2.2.7. 'Wash away' measurements

A Franz diffusion cell (Permeager, USA) with a donor chamber opportunely modified, as described in [22,23], was used. Briefly, in the donor chamber a stream of buffer was maintained through two holes. The incoming buffer flux was regulated by means of an HPLC pump (model 300, Gynkotek, Munich, G). The outcoming buffer was collected in a beaker and continuously stirred.

Porcine conjunctiva, obtained from a slaughterhouse, was deprived of the connective tissue with surgical scissors and stored at −20 °C before testing [24].The donor and acceptor chambers of the cell were separated by the porcine conjunctival epithelium membrane put on a filter paper disc imbibed in the artificial lachrymal fluid (to keep the conjunctiva hydrated) and finally placed on a Parafilm® membrane (impermeable to fluids). The receptor chamber of the cell was filled with distilled water and had the only function to keep the conjunctiva thermostated. The formulations (250 mg) were layered on the mucous membrane and an artificial lachrymal fluid stream at 37 °C was fluxed at 1 ml/min over the formulation to mimic the tears washing action. 500 μl of the artificial lachrymal fluid outcoming from the donor chamber were withdrawn with the following schedule: 2, 5, 10, 20, 30 min. The drug amount 'washed away' was determined by means of a HPLC method [25]. The HPLC apparatus (Perkin–Elmer, I) was equipped with a pump (binary pump series 200), an autosampler (series 200) and a UV/visible detector (series 200). The stationary phase was a C18 column (μBondapak 10 μm, 300×3.90 mm; CPS Analitica, I). The mobile phase was 40% acetonitrile and 60% pH 7.0 buffer (10.061 g/l $Na_4B_2O_7$ solution buffered to pH 7.0 with a 13.609 g/l KH_2PO_4 solution). The flow rate was 1 ml/min and the injection volume was 20 μl. The detection wavelength was $\lambda = 254$ nm. Data acquisition and integration were carried out with Total Chrom software (Perkin–Elmer, I).

2.2.8. In vivo precorneal residence measurements

Male, New Zeland albino rabbits ranging in weight from 2.5 to 3 kg (Pampaloni Rabbitry, Fauglia, I) were used and treated as prescribed in the publication 'Guide for the care and the use of the laboratory animals' (NIH Publication No. 92–93, revised 1985). The in vivo experimental protocol was approved by the Ethical-Scientific Committee of the University of Pisa and the experiments were carried out under veterinary supervision. The animals were housed singly in standard cages, in a light controlled room (10 h dark/14 h light cycle) at 19 ± 1 °C and 50 ± 5% RH, with no restriction of food or water. During the experiments the rabbits were placed in restraining boxes: they were allowed to move their heads freely and their eye movements were not restricted. A predetermined amount of each formulation selected (50 μl) was administered in the lower conjunctival sac of one eye of each rabbit. The rabbit lachrymal fluid (1 μl) was withdrawn, by means of a capillary, from the conjunctival sac with the following schedule: 2, 5, 10, 20, 30 min, the samples were collected in microtubes and the capillary washed with an equal volume of distilled water. Then the samples were immediately frozen and stored at −20 °C until the HPLC analysis was performed. The sample was diluted with 40 μl of artificial lachrymal fluid and analysed by means of the HPLC method as previously described.

2.2.9. Physical stability of the formulations

The selected formulations (G/20HA, G/10 PAA, G/20PAA and HCS/20HA) were subjected to steam

sterilization procedure by means of an autoclave (Alpha-Junior, PBI International, I) with a sterilization cycle of 20 min at 121 °C to check the possibility to sterilize the formulations on the final packages [26]. The sterile formulations were subjected to viscosity measurements, as previously described, to evidence variation in consistency following sterilization and the parameter % viscosity variation was calculated as the percent ratio between the viscosity variation (differences between the viscosity values after and before the sterilization) and the viscosity value before the sterilization.

The sterile formulations were stored at 25 °C for 1 and 3 months and at 40 °C for 1 month. At the end of each period the formulations were subjected to viscosity measurements as previously described.

2.2.10. Statistical evaluation

Statistical differences were determined using 1-way Anova and post hoc Sheffe test for multiple comparisons and using Mann–Whitney test for the comparison between two groups (Siphar, Creteil, F). Differences between groups were considered to be significant at $P < 0.05$. In the case of the release curves the statistical comparison was performed on the 1 h values. In the precorneal residence curves statistical comparison was performed on 3, 5 and 10 min values.

3. Results and discussion

3.1. Assessment of the drug–polymer complex stoichiometry

Table 2 reports the values of maximum binding capacity n (μmol of drug bound per milligram of polymer) and K_d (constant of dissociation of polymer–drug complex) obtained for the interaction of the drug with anionic polymers HA and PAA. The values of the parameters confirm that an interaction between TZ and the anionic polymers (HA and PAA) occurs resulting in an ionic complex. In particular, HA shows a higher binding capacity for TZ with respect to PAA.

3.2. Assessment of the polymer–polymer complex stoichiometry

Table 3 reports the weight ratios corresponding to the stoichiometry of interaction obtained between oppositely charged polymers, by means of the viscosimetric and the turbidimetric methods. G mildly interacts with both HA and PAA. By means of viscosimetric measurements it was not possible to detect a minimum in the range of the weight ratios tested. The stoichiometric weight ratio is therefore below 1/0.25 for both G/HA and G/PAA. This was confirmed by means of the turbidimetric evaluations that determined the stoichiometry of G/HA and G/PAA interaction products as equal to 1/0.18 and 1/0.08. HCS interacts much more strongly with both the anionic polymers. The stoichiometry of the HCS/HA interaction products is 1/1 weight ratio as evidenced by both the viscosimetric and the turbidimetric measurements. For HCS/PAA the stoichiometry for the interaction product is 1/0.60 as determined with the viscosimetric measurements, quite close to the value obtained with the turbidimetric ones 1/0.40.

Table 2
Values of the parameters n and K_d calculated for the complex TZ/HA and TZ/PAA

	n (μmol/mg polymer)	K_d (mM)
TZ/HA	16.920	93.245
TZ/PAA	11.280	33.809

Table 3
Values of the stoichiometry of the polymer/polymer interaction product evaluated by means of the viscosimetric and turbidimetric analysis

	Stoichimetric weight ratio	
	Viscosimetric method	Turbidimetric method
G/HA	<1/0.25	1/0.18
G/PAA	<1/0.25	1/0.08
HCS/HA	1/1	1/1
HCS/PAA	1/0.60	1/0.40

3.3. Characterization of the formulations

The viscosity values of the formulations at 100 s^{-1} are reported in Table 4 (formulation compositions reported in Table 1, see Section 2.2.3). As it could be expected the viscosity of the formulations increases on increasing the anionic polymer concentration. The reference formulation HAref is characterized by a viscosity values comparable to that of G/20HA and HCS/20HA. The interaction of HA with G or HCS does not affect the HA viscosity. On the other

Table 4
Viscosity values (mPa.s) of the formulations evaluated at 100 s^{-1} (mean values ± sd; $n = 3$)

Formulations	Viscosity (mPa.s) (mean values ± sd)
HAref	12.66 ± 0.10
G/HA	3.11 ± 0.30
G/10HA	5.66 ± 0.21
G/20HA	12.43 ± 0.23
HCS/HA	1.05 ± 0.06
HCS/10HA	4.74 ± 0.07
HCS/20HA	11.55 ± 0.83
PAAref	1984.50 ± 150.95
G/PAA	1.67 ± 0.05
G/10PAA	135.00 ± 8.20
G/20PAA	1260.00 ± 51.51
HCS/PAA	3.12 ± 0.12
HCS/10PAA	128.7 ± 6.13
HCS/20PAA	1437.63 ± 22.39

hand, a clear decrease in viscosity can be observed between the reference formulation PAAref and G/20PAA and HCS/20PAA. This could be due to the higher charge density of PAA than that of HA to form ternary interaction products poorly soluble and prone to aggregation.

3.4. Mucoadhesion properties

Fig. 1 shows the values of the mucoadhesion parameter work of adhesion (AUC) obtained for the formulations based on the ternary systems TZ/G/HA (Fig. 1(a)) and TZ/HCS/HA (Fig. 1(b)) with the biological substrate (gastric mucin dispersion) (AUC mucin) and without it (AUC blank).

In all cases the AUC mucin values, calculated for measurements performed with the biological substrate, were higher than the blank ones ($P<0.05$). The increase in HA amount results in higher mucoadhesion (AUC mucin values increase more than AUC blank values). In particular, for the formulations G/HA, G/10HA and the reference formulation HAref, the AUC mucin values were not significantly different while G/20HA shows mucoadhesive properties significantly higher with respect to the other formulations and to HAref which contains the same HA amount ($P<0.05$). Such a behaviour indicates that the presence of G in G/20HA allows to improve mucoadhesion: this is probably due to a synergistic effect of G and HA to form the mucoadhesive joint. Such a synergistic effect of G and HA to form the mucoadhesive joint is probably due to the formation of a soluble polymer–polymer interaction product due to the weak charge density of G. In a previous paper, [10] G was found to improve the mucoadhesive properties of carrageenan. The improvement, observed in this case, of the mucoadhesion of HA seems more surprising, considering that HA, differently from carrageenan, has good mucoadhesive behaviours. A possible explanation can be found in the amphiphilic character of G, whose positive charge can interact both with HA and with sialic acid moieties of mucin. On the other hand, for the ternary systems based on TZ/HCS/HA, the reference formulation HAref shows the AUC mucin value higher than HCS/HA ($P<0.01$) and not significantly different with respect to HCS/10HA and HCS/20HA. Probably the interaction between HCS and HA stronger than that occurring between G and HA, produces higher neutralization of the anionic polymer charges which could weaken the mucoadhesive bond. This hypothesis could be supported by the different ionization properties between the amphiphilic G and the positively charged HCS. The different charge–charge neutralization from G/HA to HCS/HA is likely to cause a minor charge availability and polymer chain flexibility (formation of an insoluble HCS/HA interaction product) and consequently a minor capability to form mucoadhesive joint.

Fig. 2 shows the values of the mucoadhesion parameter work of adhesion (AUC) calculated for the formulations based on the ternary systems TZ/G/PAA (Fig. 2(a)) and

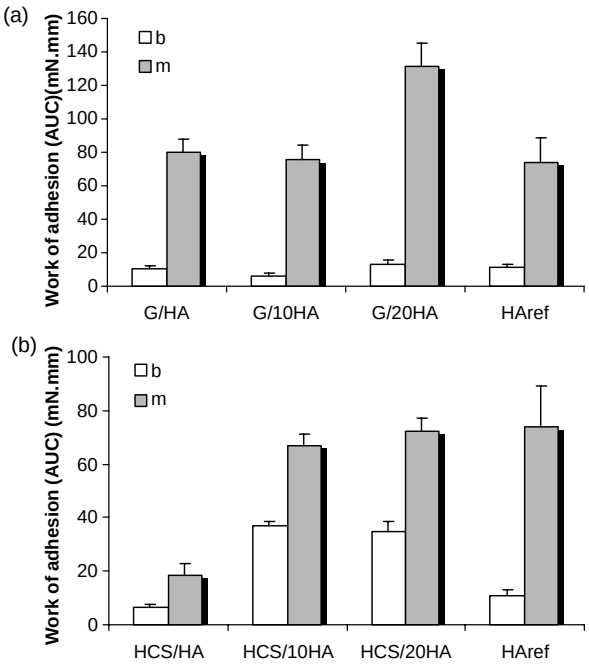

Fig. 1. Values of the mucoadhesion parameter work of adhesion AUC calculated for the formulations based on ternary systems TZ/G/HA (a) and TZ/HCS/HA (b) (mean values ± se; $n=9$) (b = values obtained from blank measurements: without biological substrate; m = values obtained with biological substrate).

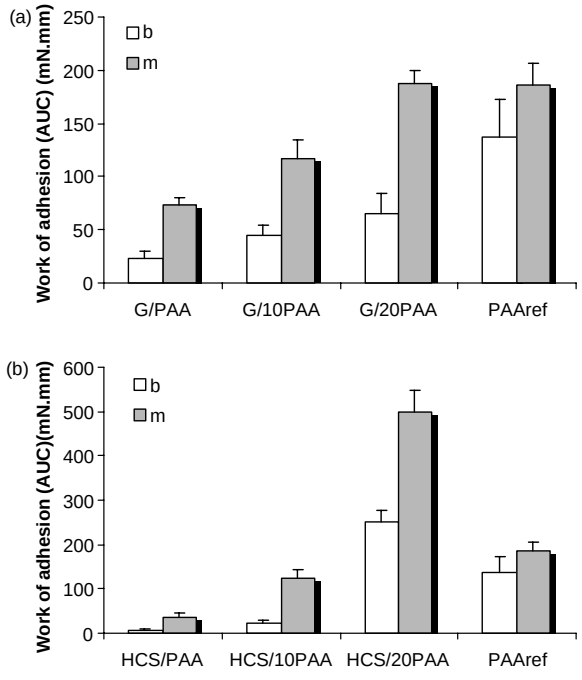

Fig. 2. Values of the mucoadhesion parameter work of adhesion AUC calculated for the formulations based on ternary systems TZ/G/PAA (a) and TZ/HCS/PAA (b) (mean values ± se; $n=9$) (b = values obtained from blank measurements: without biological substrate; m = values obtained with biological substrate).

TZ/HCS/PAA (Fig. 2(b)) with the biological substrate (gastric mucin dispersion) (AUC mucin) and without it (AUC blank).

The AUC blank values, calculated for the measurements performed without the biological substrate, are significantly lower than those carried out in presence of the mucin dispersion (AUC mucin) ($P<0.05$). For the reference formulation PAAref no differences could be seen between the AUC values calculated with and without the biological substrate ($P<0.05$) to indicate a lack of the mucoadhesive joint formation. The high ionic strength of the mucin dispersion (prepared in the artificial lachrymal fluid) probably plays a role in the poor mucoadhesive properties of the PAAref. The presence of both G and HCS, which act as counter ions for PAA, is capable to neutralize the negative effect of the medium ionic strength on mucoadhesive properties of PAA. G/20PAA shows an AUC mucin value comparable to that of PAA but a significantly lower AUC blank, while for HCS/20PAA also the absolute AUC mucin value is higher than that of PAAref ($P<0.001$).

3.5. Release properties

Fig. 3 gives the % drug release profiles vs time obtained for the formulations based on the ternary systems TZ/G/HA (a) and TZ/HCS/HA (b), the reference formulation HAref and the TZ solution S in artificial lachrymal fluid.

The TZ solution S shows the fastest % drug release in comparison with HAref and all the formulations based on both the ternary systems TZ/G/HA and TZ/HCS/HA ($P<0.05$). G/HA shows a % drug release profile similar to that of HAref. The increase in HA amount improves the control of the drug release but a synergistic effect of G can be envisaged, because G/20HA is characterized by a % drug release profiles significantly lower than that of HAref ($P<0.05$) although they contain the same HA amount. In the case of the formulations based on the ternary system TZ/HCS/HA (Fig. 3(b)) HCS/HA and HCS/10HA and HAref show % drug release profiles superimposable, while HCS/20HA is characterized by a slightly slower drug release profiles ($P<0.05$).

Fig. 4 illustrates the % drug release profiles vs time obtained for the formulations based on the ternary systems TZ/G/PAA (a) and TZ/HCS/PAA (b), the reference formulation PAAref and the TZ solution S in artificial lachrymal fluid.

The TZ solution S shows the highest % drug release profile in comparison with all the formulations based on both the ternary systems TZ/G/PAA and TZ/HCS/PAA and the reference formulation PAAref ($P<0.05$). For the formulations based on ternary system TZ/G/PAA (Fig. 4(a)), PAAref shows a % drug release profiles comparable to those of G/PAA and G/10PAA (80% of drug released in 2 h) while G/20PAA shows clearly slower drug release indicating that G participates to control drug release as in the ternary system TZ/G/HA.

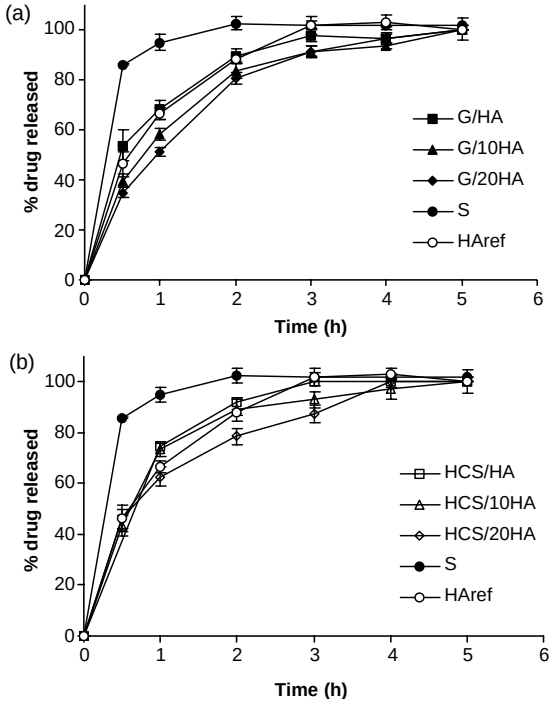

Fig. 3. % drug release profiles vs time obtained for the formulations based on the ternary systems TZ/G/HA (a) and TZ/HCS/HA (b), the reference formulation HAref and the TZ solution S in artificial lachrymal fluid (mean values ± se; $n=3$).

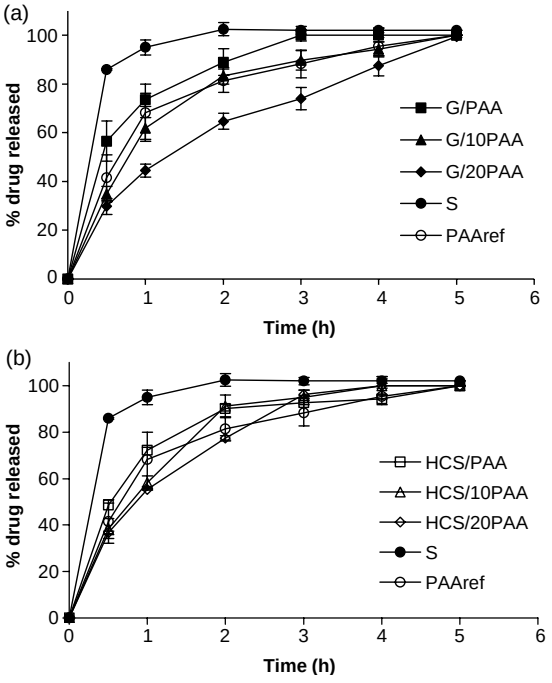

Fig. 4. % drug release profiles vs time obtained for the formulations based on the ternary systems TZ/G/PAA (a) and TZ/HCS/PAA (b), the reference formulation PAAref and the TZ solution S in lachrymal fluid (mean values ± se; $n=3$).

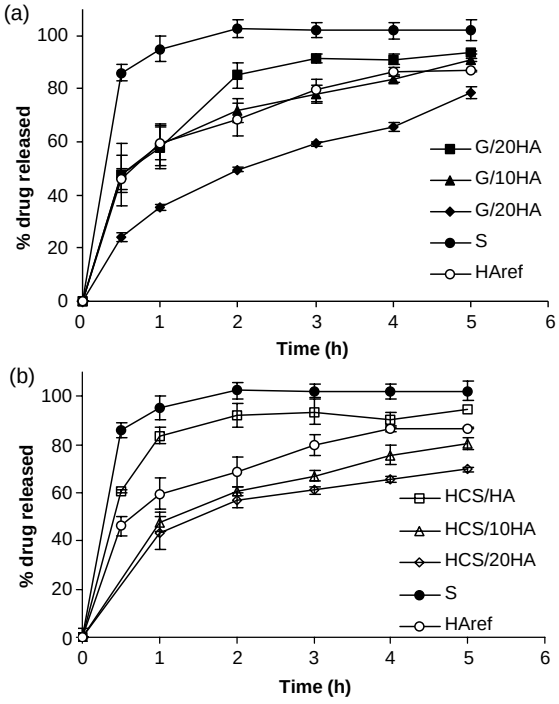

Fig. 5. % drug release profiles vs time obtained for the formulations based on the ternary systems TZ/G/HA (a) and TZ/HCS/HA (b), the reference formulation HAref and the TZ solution S in distilled water (mean values ± se; $n=3$).

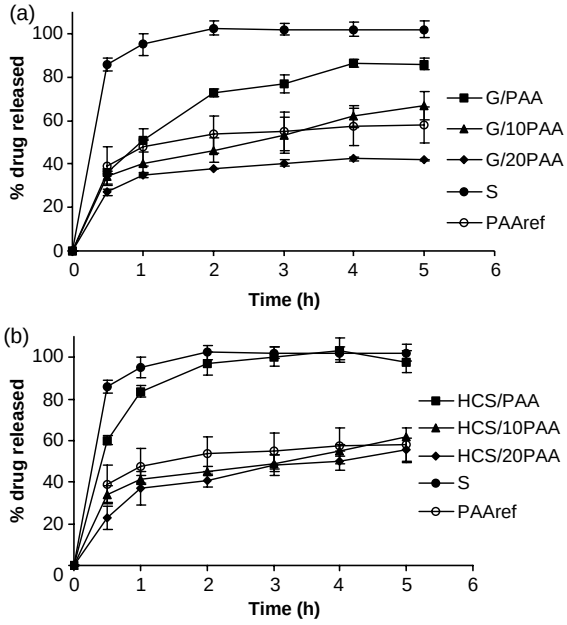

Fig. 6. % drug release profiles vs time obtained for the formulations based on the ternary systems TZ/G/PAA (a) and TZ/HCS/PAA (b), the reference formulation PAAref and the TZ solution S in distilled water (mean values ± se; $n=3$).

In the case of the formulations based on the ternary system TZ/HCS/PAA (Fig. 4(b)), all the profiles are similar to that of the reference formulation PAAref. This behaviour could be due to the poor solubility properties of the HCS/PAA interaction products besides to the insolubility of HCS at pH values close to neutrality.

To investigate to what extent the drug release from the ternary systems depends on the ionic strength of the receptor phase, the drug release test was also performed using distilled water instead of the artificial lachrymal fluid.

Fig. 5 illustrates the % drug release profiles vs time obtained for the formulations based on the ternary systems TZ/G/HA (a) and TZ/HCS/HA (b), the reference formulation HAref and the TZ solution S in distilled water. Fig. 6 illustrates the % drug release profiles vs time obtained for the formulations based on the ternary systems TZ/G/PAA (a) and TZ/HCS/PAA (b), the reference formulation PAAref and the TZ solution S in distilled water.

S always shows drug release profile higher than those of the formulations based on the ternary systems and the reference formulations (Figs. 5 and 6).

For all the ternary systems the increase in the anionic polymer concentration improve the ability to control drug release. In particular, the formulations based on the ternary systems TZ/G/HA and TZ/HCS/HA show drug release profiles which slowly but continuously increase in function of the time (Fig. 5(a) and (b)) after the initial burst. On the other hand, the formulations based on the ternary systems TZ/G/PAA and TZ/HCS/PAA show drug release profiles that reach the plateau values: G/20PAA and HCS/20PAA released 40 and 50% of TZ in 5 h, respectively (Fig. 6(a) and (b)).

The formulations at the stoichiometry of the ternary systems with HCS (HCS/HA and HCS/PAA) (Figs. 5(b) and 6(b)) are characterized by release profiles superimposable to S. For these formulations the HCS/HA and HCS/PAA interactions together with the poor solubility properties of HCS at pH close to neutrality result in the displacement of the drug TZ in the drug–polymer complex. G/20HA, G/10 PAA, G/20PAA and HCS/20HA, that combined better mucoadhesive and drug release properties, were selected for further characterization concerning viscosity after dilution with lachrymal fluid, ex vivo and in vivo retention and viscosity after steam sterilization and storage.

3.6. Viscosity measurements after dilutions

Table 5 gives the viscosity values (mPa s) (at 100 s^{-1}) of G/20HA, G/10 PAA, G/20PAA and HCS/20HA after dilution 1:1 and 1:4 with artificial lachrymal fluid and, for comparison purposes, with distilled water. When the dilution medium is the artificial lachrymal fluid, the viscosity values are always lower than those obtained after the same dilution with water to indicate that the ionic strength of the medium interferes with the formulation consistency. The greater differences in viscosity after dilution with distilled water and artificial lachrymal fluid, can be observed in particular for the formulation G/20PAA.

Table 5
Viscosity values (mPa.s) of G/20HA, G/10PAA, G/20PAA and HCS/20PAA as such (1:0) and after dilution with distilled water or artificial lachrymal fluid (1:1 and 1:4) evaluated at 100 s^{-1} (mean values ± sd; $n = 3$)

Dilution	with	Formulations			
		Viscosity values (mPa.s)			
		G/20HA	G/10PAA	G/20PAA	HCS/20HA
1:1	Distilled water	6.81 ± 0.003	11.55 ± 0.16	414.95 ± 8.30	6.20 ± 0.07
	Artificial lachrymal fluid	3.32 ± 0.03	8.37 ± 1.12	11.95 ± 0.96	3.25 ± 0.07
1:4	Distilled water	4.53 ± 0.16	14.24 ± 3.37	37.52 ± 1.02	6.80 ± 2.84
	Artificial lachrymal fluid	2.15 ± 0.21	5.00 ± 0.39	3.99 ± 1.15	1.87 ± 0.02

While the initial viscosity of the formulations G/10PAA and G/20PAA is much higher than that of G/20PAA and HCS/20HA (Table 4), after especially 1:4 dilution, the four formulations show values quite similar and in particular, no statistically significant differences were found between G/20HA, G/20PAA and HCS/20HA.

3.7. 'Wash away' properties

Fig. 7 illustrates the amount of drug 'washed away' vs time profiles for the G/20HA, G/10 PAA, G/20PAA and HCS/20HA formulations subjected to an artificial lachrymal fluid stream of 1 ml/min over excised pig conjunctiva in modified Franz diffusion cell. The TZ solution S is rapidly 'washed away' after 5 min. G/20HA shows an intermediate profile while G/10 PAA, G/20PAA and HCS/20HA are characterized by lower and comparable profiles to indicate a greater resistance to the artificial lachrymal fluid stream taking into account also the effect of lachrymal fluid on sample viscosity especially of G/10 PAA, G/20PAA. The 'wash away' behaviour indicates that all the tested formulations are able to interact with the conjunctival mucosa which they are placed on, to achieve good residence properties.

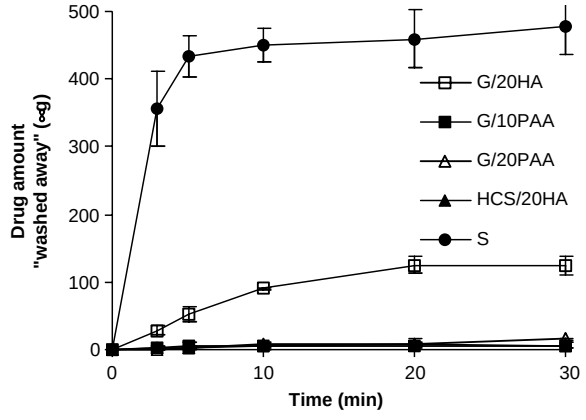

Fig. 7. Drug amount (μg) 'washed away' vs time profiles obtained for the formulations G/20HA, G/10PAA, G/20PAA and HCS/20PAA and the TZ solution S subjected to a artificial lachrymal fluid steam of 1 ml/min (mean values ± sd; $n = 3$).

3.8. In vivo precorneal residence properties

Fig. 8 shows the TZ concentration (μg/ml) vs time profiles in the rabbit lachrymal fluid following the in vivo instillation of 50 μl of each formulation and of the TZ solution S in the conjunctiva.

The TZ solution S is not detectable in the rabbit lachrymal fluid since after 3 min: this behaviour indicates that the tear stream and the blinking cause very quickly (less than 3 min) the complete elimination of the drug from the conjunctiva and the ophthalmic surface. The formulations G/20HA, G/10PAA, G/20PAA and HCS/20PAA remain in the conjunctiva for a time period much longer than S, as for all of them the drug concentration is low but still detectable after 20 min from the instillation. In particular, G/20HA shows the highest TZ levels at least at early times, followed by HCS/20PAA, G/20PAA and G/10PAA, even if the biological variability does not allow to detect statistical differences in the TZ concentration (μg/ml) in the rabbit lachrymal fluid vs time profiles.

3.9. Physical stability

Table 6 represents the effect of the steam sterilization and of aging on the viscosity values (mPa.s) of G/20HA, G/10PAA, G/20PAA and HCS/20PAA after sterilization, the values of the parameter % viscosity decrease and

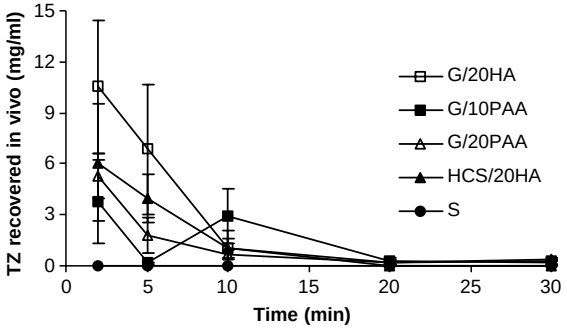

Fig. 8. TZ concentration (μg/ml) in rabbit lachrymal fluid in vivo following instillation of 50 μl of G/20HA, G/10PAA, G/20PAA and HCS/20PAA and the TZ solution S (mean values ± se; $n = 6$).

Table 6
Viscosity values (mPa.s) of G/20HA, G/10PAA, G/20PAA and HCS/20PAA at zero point ($t=0$) before sterilization and after sterilization, the values of the parameter % viscosity decrease and the viscosity values (mPa.s) after 1 and 3 months of storage at 25 °C and 1 month at 40 °C evaluated at 100 s^{-1} (mean values ± sd; $n=3$)

	Viscosity values (mPa.s)				
	$t=0$ after sterilization	% viscosity variation	1 month $T=25$ (C	3 month $T=25$ (C	1 month $T=40$ (C
G/20HA	12.09 ± 0.02	−2.79 ± 0.82	12.27 ± 0.02	12.39 ± 0.01	12.24 ± 0.06
G/10PAA	306.70 ± 9.73	26.67 ± 0.14	306.70 ± 9.73	363.40 ± 2.00	306.45 ± 3.46
G/20PAA	2063.50 ± 161.48	25.63 ± 0.01	2436.50 ± 18.55	2622.00 ± 72.21	2306.00 ± 88.26
HCS/20HA	12.58 ± 3.03	−83.46 ± 13.41	–	–	–

the viscosity values after 1 and 3 months of storage at 25 °C and 1 month at 40 °C evaluated at 100 s^{-1}.

G/20HA shows a decrease in viscosity following sterilization of about 3% and the viscosity values before and after sterilization were not significantly different. This means that the sterilisation procedure does not affect the consistency of the formulation. Both the formulations based on the ternary system TZ/G/PAA (G/10PAA and G/20PAA) show an increase in viscosity of about the 25% due to sterilization: nevertheless both the formulations remain suitable for the ophthalmic administration. The increase in viscosity is probably due to the peculiar properties of polyacrylic acid. In literature Bindal et al. [26] and Weyenberg et al. [27] described the polyacrylic acid behaviour following sterilization by means of steam. In particular, Weyenberg et al. described that the increase in temperature is likely to result in better hydration of the polymer dispersed: the mutual repulsion of ionised carboxylic groups of the polyacrilic acid polymer chains produces more stretched polymer backbones and those hydroxylic groups form also stabile hydrogen bonds with water molecules through hydrophilic interactions [27]. Moreover, it is likely that such hydrogen bonds between the polyacrylic acid and gelatin probably became stronger for the same reason and are able to confer more consistency to the formulations with respect to the unsterilized formulations [26,27]. The heat effect on polymer MW could be probably more evident for gelatin than polyacrylic acid but it was surely masked by the probable deepest polymer–polymer interactions by means of weak bond and without polymer precipitations.

On the contrary, for HCS/20PAA the sterilization process causes a 80% decrease of viscosity and the onset of a remarkable precipitation. This could be due to the presence of HCS which more strongly interact with HA at high temperature.

The storage of G/20HA and G/20PAA for 3 month at 25 °C and 1 month at 40 °C does not produce any significant change in viscosity. G/10PAA presents viscosity values not significantly different for 1 month of storage time either at 25 °C and 40 °C while after a storage time of 3 months at 25 °C a slight but significant increase in the viscosity value was detected ($P<0.01$).

4. Conclusions

The anionic polymers HA and PAA showed good capability to interact with the drug TZ giving soluble drug/polymer complexes; moreover they were able to form polymer/polymer complexes with G and HCS, with a stoichiometry depending on the polymers involved. G mildly interacted with both HA and PAA resulting in just a slight turbidity, while HCS interaction with the anionic polymers was stronger with a marked opalescence.

The presence of G (G/20HA, G/10PAA and G/20PAA) allows to improve the mucoadhesion with respect to the reference formulations HAref and PAAref. The synergistic effect of G and HA in G/20HA is able to form the mucoadhesive joint. In the case of G/10PAA and G/20PAA, G, that acts as counter ions for PAA, makes it less sensitive to the ionic strength of lachrymal fluid in the mucoadhesion test: this is a possible explanation of the much better mucoadhesive behaviour of G/10PAA and G/20PAA with respect to PAAref. The stronger interaction between HCS and HA in HCS/20PAA is likely to produce an higher neutralization of the polymer charge and a minor flexibility of the polymer chain impairing/reducing mucoadhesiveness with respect to HAref.

Furthermore, G (G/20HA, G/10PAA and G/20PAA) as well as HCS (HCS/20PAA) contributes to improve drug release control. The ternary system capability to control TZ release is particularly evident when the medium employed is distilled water, confirming the relevance of the ionic-exchange mechanism.

Both mucoadhesion and drug release tests indicated G/20HA, G/10PAA, G/20PAA and HCS/20PAA as candidates worthy of deeper ex vivo/ in vivo evaluation. All these formulations resulted to be sensitive to the ions of the medium, so that after dilution in artificial lachrymal fluid the initial differences in consistency became no more relevant. This effect can explain the results of the ‘wash away’ characterization: the formulation resistance to the buffer stream (simulating the lachrymation) was likely to be a complex result of the viscosity and of the mucoadhesion of the formulations.

The in vivo evaluation confirms that the formulation even if characterized by different consistency, are able to maintain levels of TZ detectable until 20 min after

the instillation while the drug solution was not detectable since 3 min after instillation. The lack of significant differences in the in vivo residence properties is probably due to the decrease of viscosity following the dilution in a saline medium like the lachrymal fluid. The action of the tear dilution is probably more ready in vivo with respect to the ex vivo test because of the mechanical stress of blinking that allows to spread the formulation on the conjunctiva forming a film more sensitive to the tear ionic strength. This moreover induces a faster removal of the formulation. For these reasons the drug permanence in vivo was not directly comparable with the ex vivo ('wash away' properties) performances although a similar rank order could be observed between the formulations in the two tests.

Furthermore, the good physical stability following steam sterilization and storage, together with the low viscosity which helps instillation makes G/20HA, based on the ternary system TZ/G/HA, the formulation more promising for a industrial development and scaling up.

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