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Influence of molecular weight and formulation pH on the precorneal clearance rate of hyaluronic acid in the rabbit eye

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Abstract

Hyaluronic acid is a natural polymer which, due to its water retaining capability, binds to cell membranes and can therefore be considered as a putative vehicle for controlled ocular delivery. In an in vitro mucoadhesion test, the force of detachment was significantly greater for Healon® (HA-Na) compared to low molecular weight hyaluronic acid. Also, this bioadhesion was stronger for Healon® at pH 5 than at pH 7.4. The precorneal clearance of sodium hyaluronate (0.2%) was investigated at pH 5.0 and 7.4 by employing gamma scintigraphic imaging of the ^{111}In-labelled biopolymer. Protonation of the macromolecule did not result in any increase in ocular mucoadhesion as the mean residence time at pH 5.0 was not significantly longer than at pH 7.4. The effect of molecular weight of hyaluronic acid on the corneal retention was also investigated. There was a statistically significant difference ($p < 0.05$) in the clearance half-life ($t_{0.5}$) and AUC of % activity remaining vs time plot for Healon® (Mol. Wt 2.2×10^6) compared to those observed for the two lower molecular weight hyaluronic acid samples (Mol. Wt 134 000 and 620 000).

Keywords: Hyaluronic acid; Mucoadhesion; Precorneal clearance; Ocular retention

1. Introduction

Conventional ophthalmic dosage forms commonly have low bioavailability. Rapid loss of the instilled solution due to lacrimal drainage through the drainage apparatus has considerable effect on the bioavailability of ophthalmic drugs (Chrai et al., 1973). This results in short contact time between drug and cornea, leading to reduced drug availability. In order to increase the corneal contact time, the viscosity of ophthalmic solutions are often enhanced by the incorporation of viscolysers, e.g., methylcellulose, hydroxypropylcellulose, and polyvinylalcohol (Adler et al., 1971; Chrai and Robinson, 1974; Patton and Robinson, 1975; Saettone et al., 1984).

Numerous studies have shown that intraocular penetration of drugs is very limited (Barza et al., 1983; Rubinstein et al., 1983). Camber et al. (1987) demonstrated that the addition of sodium hyaluronate to pilocarpine resulted in increased retention of the drug in the tear fluid and in a 2-fold increased concentration in the cornea and aqueous humour. Also, it has been demonstrated that hyaluronic acid can prolong the duration of

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action of local anaesthetics up to 500% (Hassan et al., 1985) by forming molecular complexes that delay absorption (Aberg et al., 1987).

Recently, sodium hyaluronate (Healon®) has been used in anterior segment surgery, having a molecular mass of 2170 kDa and a viscosity of 20 000 cPs at 3.84 s^{-1} shear rate (Iwata et al., 1984). It consists of polymerised glucuronic acid and *N*-acetylglucosamine (Bothner and Wik, 1989). At concentrations of 0.2 and 0.3% sodium hyaluronate solutions exhibit high static, and zero shear viscosities but undergo dramatic reductions in viscosity with increasing shear rate (Bothner and Wik, 1989). Sodium hyaluronate is a non-irritating substance with high water binding capacity, viscous flow and pseudoplastic behaviour, and as such it has potential as a mucoadhesive polymer in ocular drug delivery.

The development of vehicles having the capability of adhering to the conjunctival tissues and/or to its mucin coating constitutes an interesting alternative approach to the improvement of the bioavailability of ophthalmic medications. Saettone et al. (1985, 1986) have shown that some low viscosity ophthalmic preparations based on hyaluronic acid (HA) are capable of enhancing the bioavailability of pilocarpine and that some vehicles based on this natural polymer show strong bioadhesive properties in vitro (Saettone et al., 1987). These results concur with an earlier report of Park and Robinson (1984), who observed, using a fluorescence in vitro technique, a strong binding of HA to isolated human conjunctival cells. Camber et al. (1987) and Gurny et al. (1987) confirmed the positive influence of HA vehicles on the miotic effect of pilocarpine in rabbits and in humans. Sodium hyaluronate has been shown to increase the tear film stability and reduce subjective symptoms such as grittiness and burning (Mengher et al., 1986). HA (0.1%) solution has been shown to coat the corneal epithelium for at least 12 h (Polack and McNiece, 1982). Recently, Snibson et al. (1990) used gamma scintigraphy to evaluate the residence time of HA (0.2 and 0.3%) in 'dry eye' patients and normal subjects. The AUC of % activity remaining vs time and the $t_{0.5}$ of HA was greater in the patient group due to the reduced dilution and washout effects of reflex and basal lacrimation. The potential of sodium hyaluronate as a carrier for intraocular gentamicin has also been investigated by Moreira et al. (1991).

Different techniques have been used to quantify the precorneal residence time of vehicles. Staining of the conjunctival and corneal epithelium with an Agyrol marker was used by Bach et al. (1972) for studying the ocular residence time of HPMC (hydroxypropylmethylcellulose) and PVA. Adler et al. (1971) used fluorophotometery to measure the ocular penetration of fluorescein and used these values as an index of residence time of the polymer solutions containing sodium fluorescein. Waltman and Patrowicz (1970) using a similar method showed greater penetration from an HPMC vehicle than the less viscous PVA. Others have used the observable effects of drugs such as pilocarpine (Haas and Merril, 1962), tropicamide (Saettone et al., 1980), and homatropine (Mueller and Deardorff, 1956) as an index of ocular residence time. Most of these methods, however, have limitations in that they are indirect, relatively insensitive and reflect variations in other aspects of bioavailability and pharmacological responses in addition to consideration of contact time. Lacrimal gamma scintigraphy, a technique first described by Rossomondo et al. (1972) to evaluate lacrimal drainage, has been used to study precorneal residence times of drug delivery vehicles.

The current study seeks to evaluate the effect of molecular weight and pH on the mucoadhesion of the polymer to the ocular surface of the rabbit as determined by quantitative gamma scintigraphy.

2. Materials and methods

The following materials were used as received: HA-Na (Healon® 10 mg/ml) Mol. Wt 2.2×10^6, HA1-Na Mol. Wt $134\,000 \pm 9000$, HA2-Na Mol. Wt $620\,000 \pm 50\,000$ from Pharmacia Ophthalmics AB, Uppsala, Sweden and ^{113m}In obtained by elution of a sterile 370 MBq generator with 0.04 M HCl and ^{111}indium chloride purchased from Amersham International UK. Mucin

was purchased from BDH Ltd, UK. All other reagents were of at least AnalaR grade and obtained from BDH Ltd, UK.

2.1. In vitro mucoadhesion study

The in vitro mucoadhesion of hyaluronic acid was evaluated by using the method of Durrani et al. (1994) and employing a 20% w/w mucin gel as a model mucus gel.

2.2. Labelling of HA and its invitro stability assessment

The labelling procedure was developed using 6 ml of ^{113m}In chloride in 0.04 M HCl, eluted from a 370 MBq generator (Amersham Int. UK). 30 μl of a sodium acetate solution (0.05% w/v sodium acetate in 0.4 M HCl) was added and the solution neutralized by the addition of 0.04 M NaOH. 0.4 ml of either phosphate-buffered saline (pH 5) or 1% HA was then slowly added to 0.8 ml indium chloride/sodium acetate solution and stirred for 30 min. The radiolabelled polymer or phosphate-buffered saline (PBS) solution was placed in a dialysis sack (Visking, pore size 10 kDa, 1-8/32 inch, Medicell Int., UK) which was then placed in 8 ml PBS for 4 h at room temperature. Samples of the dialysing fluid were assayed for ^{113m}In in a pre-calibrated gamma counter (LKB 1282 Compugamma CS). The labelling method was developed using ^{113m}In, although it is equally applicable to ^{111}In which was used for the in vivo studies.

2.3. Labelling of HA for in vivo studies

A small volume of each formulation was replaced by ^{111}In solution (prepared by mixing 1 ml of active ^{111}indium chloride with 30 μl of acidic sodium acetate solution (0.05% w/v)) so that a 20 μl drop contained 1 MBq of activity. The solution was stirred for 30 min before ocular administration.

2.4. Precorneal clearance study

Each formulation (0.2% HA in PBS) was tested in a cross-over manner in a group of five male New Zealand White rabbits (2.5–3.5 kg) with a minimum washout period of 3 days. The rabbit was positioned 5 cm from the 3.5 mm aperture of the pinhole collimator of the gamma camera. 20 μl of the radiolabelled solution was instilled directly onto the corneal surface and the eye manually blinked to distribute the solution over the cornea. Time frames of 20 s were used for the first 5 min increasing in length to 2 min frames for the last 14 min. Regions of interest were created around the images of the cornea, the inner canthus and the lacrimal duct. Graphs of activity remaining in each region vs time were plotted after correction for background to assess the clearance from the corneal surface.

Table 1
In vitro evaluation of hyaluronic acid bioadhesion to mucus at pH 5 (a) and (b) pH 7.4 ($\pm$SE, $n = 4$)

Test material	Weight required for detachment (g)	Force (N) ($\times 10$)	Force/area (N/cm^2)
(a) pH 5.0			
HA-Na	59.95 $\pm$ 11.29	5.8 $\pm$ 1.10	2.32 $\pm$ 0.40
HA2-Na	21.31 $\pm$ 04.32 [a]	2.0 $\pm$ 0.42 [a]	0.80 $\pm$ 0.16 [a]
HA1-Na	17.92 $\pm$ 08.02 [a]	1.8 $\pm$ 0.78 [a]	0.72 $\pm$ 0.31 [a]
(b) pH 7.4			
HA-Na	34.22 $\pm$ 5.74 [b]	3.35 $\pm$ 0.56 [b]	1.34 $\pm$ 0.22 [b]
HA2-Na	20.41 $\pm$ 3.36 [a]	2.00 $\pm$ 0.33 [a]	0.80 $\pm$ 0.13 [a]
HA1-Na	8.69 $\pm$ 2.22 [a]	0.80 $\pm$ 0.20 [a]	0.40 $\pm$ 0.08 [a]

[a] Statistically significant from HA-Na ($p < 0.05$).
[b] Statistically significant from HA-Na at pH 5.0 ($p < 0.05$).

3. Results

3.1. In vitro mucoadhesion

Table 1a and b shows the force required for the detachment of the three different formulations of hyaluronic acid at pH 5 and 7.4, respectively, from the mucus. A statistically significant difference ($p < 0.05$, ANOVA) for the force of detachment was found between HA-Na and the low molecular weight hyaluronic acids at both pH values. For HA-Na, there was greater mucoadhesion at pH 5 compared with pH 7.4. At pH 7.4, HA2-Na was more mucoadhesive than the HA1-Na; this effect was not observed at pH 5.

3.2. In vivo precorneal clearance study.

After 4 h of dialysis, 97–98% of ^{113m}In remained associated with the HA (0.2%), thus demonstrating the stability of the radiolabel. Substitution of ^{111}In for ^{113}In resulted in similar labelling efficiency and stability.

Ocular drainage of the solutions of HA was a biphasic phenomenon (Fig. 1a–c). For the high molecular weight HA (Mol. Wt 2.2×10^6) 50% of the 0.2% solution was cleared approx. 700 s post-instillation. For the lower molecular weight HAs, the clearance was more rapid; for HA2-Na (Mol. Wt 620000) a $t_{0.5}$ of 100 s was observed, whereas for HA1-Na (Mol. Wt 134000) the $t_{0.5}$ was 72 s.

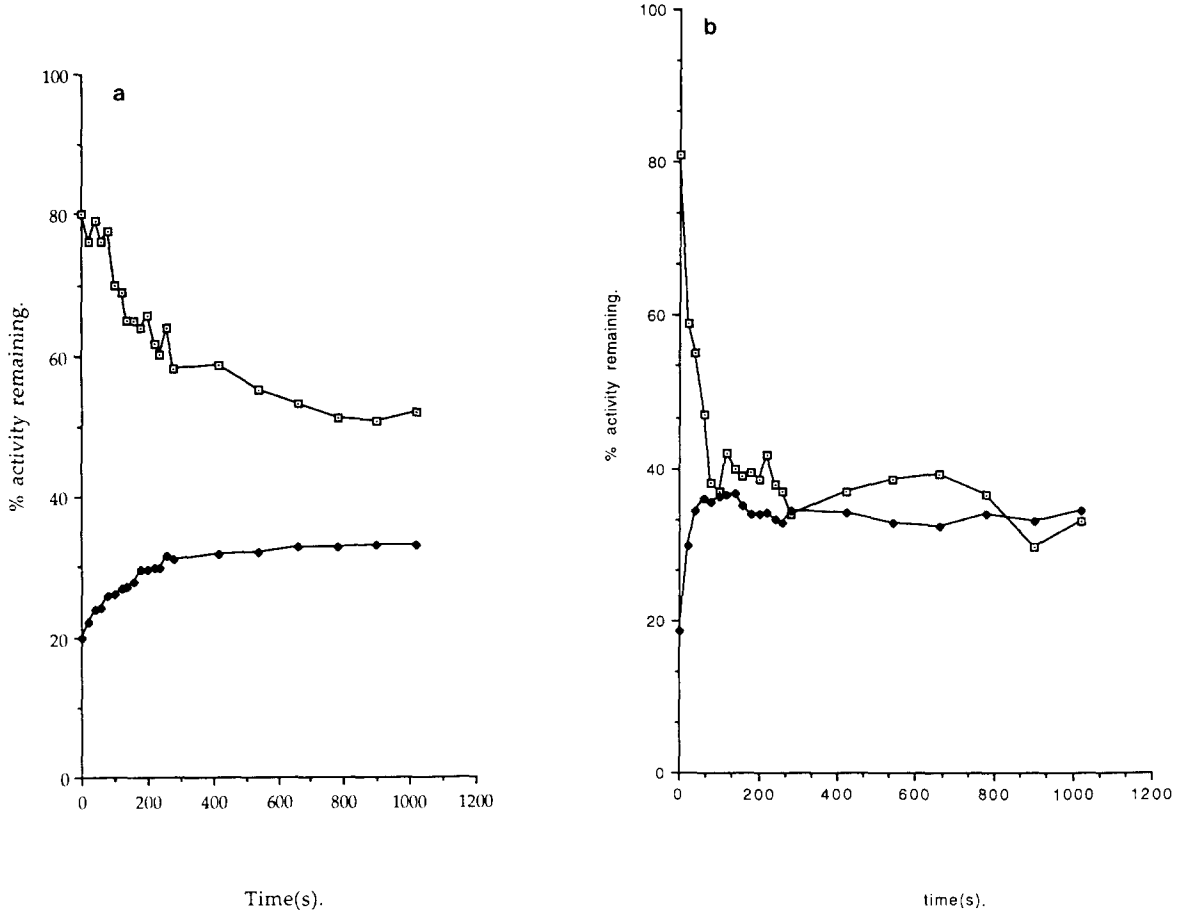

Fig. 1. Precorneal clearance of the three different molecular weight fractions of hyaluronic acid at pH 5. (a) HA-Na, (b) HA2-Na, (c) HA1-Na. Regions of interest: cornea (□); inner canthus (♦).

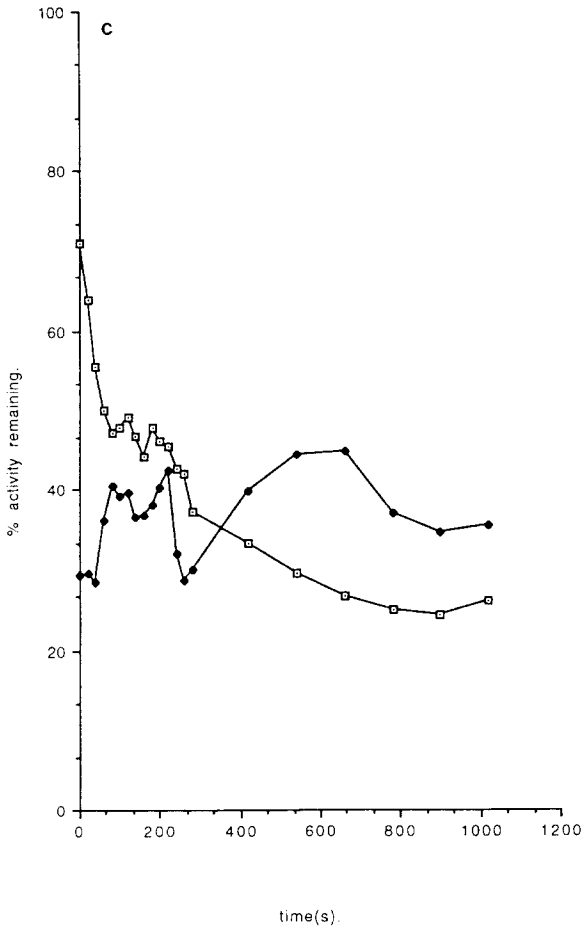

Fig. 1 (continued).

Less than 35% of the solution of HA_2-Na and HA_1-Na remained in contact with the corneal surface at the end of the study (67 min).

High molecular weight HA therefore resides for a substantial period on the corneal region compared to low molecular weight HA as assessed by the determination of half-life. The area under the curve (AUC) of % activity remaining vs time provides another index of residence time. The AUC of high molecular weight HA was also statistically significant from the AUC of the other two HA solutions, i.e., HA2-Na and HA1-Na ($p < 0.05$, ANOVA). The AUC of HA2-Na was not statistically significant from that of HA_1-Na ($p = 0.10$). Table 2 summarises the effect of molecular weight on the ocular clearance of hyaluronic acid. The effect of pH on the ocular retention of HA-Na (Healon®) was examined and assessed by the AUC or $t_{0.5}$ showed no dependence on pH (5.0 and 7.4).

Curve fitting was performed using the programme MINIM. The function which gave closest approximation to the clearance curves was the biexponential equation: $y = A \cdot \exp(-k_1 t) + B \cdot \exp(-k_2 t)$. In all cases, the clearance process consisted of a rapid initial phase, followed by the slower basal drainage phase. The values of the constants k_1, k_2, A and B are recorded in Table 3, which allow comparison between different formulations for different phases of the curve. No

Table 2
Summary of clearance parameters for solutions at pH 5.0 (±SE, $n = 4$)

Formulation	% activity remaining (420 s)	% activity remaining (4020 s)	AUC relative to HA-Na	$T_{0.5}$ (s)
HA-Na	58.75 ± 9.23	50.25 ± 6.84	1.00	697 ± 157
HA2-Na	37.00 ± 4.51 [a]	33.00 ± 2.74 [a]	0.56 [a]	100 ± 27 [a]
HA1-Na	33.25 ± 6.29 [a]	32.00 ± 6.23 [a]	0.51 [a]	72 ± 39 [a]

[a] Values statistically significant with respect to Ha-Na ($p < 0.05$).

Table 3
Initial (k_1) and basal phase (k_2) kinetic parameters of drainage from the corneal region for hyaluronic acid formulations

	HA-Na (pH 5.0)	HA-Na (pH 7.4)	HA2-Na (pH 5.0)	HA1-Na (pH 5.0)
A	71.89 ± 1.96	64.18 ± 4.5	71.51 ± 6.30	81.76 ± 1.55
k_1 (min^{-1})	1.81 ± 0.39	2.64 ± 0.59	2.97 ± 0.35	3.16 ± 0.33 [a]
B	30.40 ± 3.14	23.98 ± 7.5	28.90 ± 3.89	21.83 ± 3.52
k_2 (min^{-1})	0.001 ± 0.0005	0.05 ± 0.025	0.05 ± 0.005 [a]	0.19 ± 0.016 [a]

[a] Statistically significant with respect to HA-Na at pH 5.

significant differences were observed between mean values for both k_1 and k_2 for HA at both pH values. The rate of clearance of HA1-Na was slower at both phases than HA-Na, whereas HA2-Na cleared more slowly at the basal phase compared with HA-Na.

4. Discussion

Studies have shown that the addition of viscosity inducing agents to ophthalmic solutions decreases both the rate of fluid removal and the turnover from the precorneal surface (Chrai and Robinson, 1974; Patton and Robinson, 1975). Thus, the contact time between drug and the cornea will be increased, leading to better bioavailability. Commonly used viscosity enhancing agents such as HPMC exhibit Newtonian behaviour (Camber et al., 1987). HA in contrast is pseudoplastic. The viscosity of the HA solution is low at high shear rate and is evenly distributed on the corneal surface as evidenced during the clearance studies by gamma scintigraphy. Moreover, a Newtonian solution with high viscosity will not spread evenly on the corneal surface on blinking because it will retain a high viscosity. This may also result in loss of visual acuity.

The rate of clearance over the first 420 s (Table 2) show that two properties of HA, i.e., viscosity and mucoadhesion, ensure its retention on the corneal surface. During this period, most of the HA-Na (> 50%) resides on the ocular surface. After 420 s, two factors may contribute to the clearance of HA from the ocular surface, i.e., reduction in viscosity of hyaluronic acid by dilution with lacrimal fluid and the change of pH of the solution from pH 5 to 7.4.

Comparing the initial (k_1) kinetic parameters of drainage from the corneal region for HA-Na at pH 5 with that of the clearance rate of liposomes investigated by Davies et al. (1992) it was found that Carbopol 1342 coated liposomes ($k_1 = 1.23$ min^{-1}) and Carbopol 934P coated liposomes ($k_1 = 1.42$ min^{-1}) cleared more slowly than HA-Na. However, the initial clearance rate of uncoated liposomes is greater ($k_1 = 2.10$ min^{-1}) than HA-Na at pH 5.0. Similar trends were shown at pH 7.4. The second, or basal phase (k_2) of the corneal drainage is a much slower phase. The basal phase of corneal drainage depends on several processes, largely tear turn over, conjunctival absorption, and reflux between compartments.

Fitzgerald et al. (1987) determined the precorneal drainage rate constants following instillation of ^{111}In-labelled liposomes in the rabbit eye. The rate of clearance of EPC (egg phosphatidylcholine) MLVs (neutral), MLVs (positive), and MLVs (negative) were slower in the initial rapid phase than was shown for hyaluronic acid in these studies. However, the final clearance constant for positively charged MLVs ($k_2 = 0.04$ min^{-1}) was greater than that of Healon® at pH 5 ($k_2 = 0.001$ min^{-1}), but DPPC (dipalmitoylphosphatidylcholine) was retained longer than hyaluronic acid formulations, which in turn has a much smaller clearance rate constant ($k_2 = 0.02$ min^{-1}) than buffer ($k_2 = 0.11$ min^{-1}). These results show that hyaluronic acid formulations are cleared more rapidly than liposomes (Fitzgerald et al., 1987; Davies et al., 1992) from the ocular region in the initial phase but in the basal phase they remain for a longer time, which might contribute to a sustained drug action in the eye.

Viscosity alone may to a large extent explain the residence of sodium hyaluronate solution on the ocular surface, but bioadhesion or physical attachment to the ocular surface has been suggested by some researchers (Polack and McNiece, 1982; Gurny et al., 1987). Later, this was demonstrated by Saettone et al. (1989) using an in vitro experimental model. Although the presence of binding sites for sodium hyaluronate has been demonstrated on the surface of the corneal endothelium, none has been found on the epithelial surface (Madsen et al., 1989). Hazlett and Barrett (1987) employed scanning and transmission electron microscopy to demonstrate the residence of sodium hyaluronate for at least 60 min on the corneal surface of mice and suggested that this was not dependent on the presence of an intact mucus layer. The interaction therefore occurs between the polymer and either the glycocalyx or the cell membrane of the epithelial surface.

Moreover, our results show that high molecular weight hyaluronic acid resides on the ocular

surface for a long duration as compared to low molecular weight hyaluronic acid. This is in agreement with the results of Saettone et al. (1989) who reported that HA2-Na (5%) showed better residence on the corneal surface than PAA, while the low molecular weight HA1-Na (15%) showed the same mucoadhesive strength as that of PAA. The high molecular weight HA2-Na insert underwent a slower hydration and remained on the corneal surface for 60 min whereas HA1-Na hydrated immediately and disappeared rapidly from the eye. In their results, HA1-Na solution rapidly cleared due to fast dilution by the lacrimal fluid. The solution overflowed from the corneal surface into the nasolacrimal duct after 10 min. In contrast, HA-Na solution undergoes slow dilution and we observed 58% of the activity remaining on the corneal surface after 420 s.

The results of the effect of pH on the mucoadhesion of hyaluronic acid solution is also in agreement with the results of Saettone et al. (1989) who reported that increasing the pH of the ophthalmic solution decreases mucoadhesion. However, they found no statistical significant difference in values of mucoadhesion at three pH levels (i.e., 3.45, 6.50, and 7.40). In contrast, Ch'ng et al. (1985) reported in their ex vivo bioadhesion experiment with polycarbophil, a maximum adhesion at pH 6, while at lower and higher pH (2–3 and 7, respectively) the bioadhesion of the polymer to rabbit stomach tissue was significantly reduced. These authors attributed this effect to pH induced physicochemical changes in the mucous layer (altered rheology, ionization) and/or in the polymer itself (degree of swelling).

We have demonstrated in this study the relatively slow removal of HA-Na compared with the low molecular weight fractions from the precorneal region of the rabbit eye. However, we were unable to show pH differences in vivo which were evident in in vitro mucoadhesion studies.

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