International Journal of Pharmaceutics xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Gel formulations for treatment of the ophthalmic complications in cystinosis

Barbara Buchan, Graeme Kay*, Anne Heneghan, Kerr H. Matthews, Donald Cairns

School of Pharmacy and Life Sciences, The Robert Gordon University, Schoolhill, Aberdeen AB10 1FR, UK

ARTICLE INFO

Article history:
Received 18 January 2010
Received in revised form 31 March 2010
Accepted 31 March 2010
Available online xxx

Keywords:
Cystinosis
Ophthalmic delivery
Gel
Modified release

ABSTRACT

Nephropathic cystinosis is a rare autosomal recessive disease characterised by raised lysosomal levels of cystine in the cells of all organs. It is treated by regular administration of the aminothiol, cysteamine. Corneal crystal deposition is one of the most troublesome complications affecting patients and requires the hourly administration of cysteamine eye drops. In an attempt to reduce this frequency and improve the treatment, the preparation and evaluation of cysteamine containing Carbomer gel is reported. The results demonstrated that a weak gel network was formed at low shear–stress, the bioadhesion of the gel was increased with inclusion of a cysteamine derivative (e.g. mean force of 0.067 N compared to 0.107 N with compound included) and first-order release from the gel was observed. In conclusion these results offer the possibility to formulate cysteamine in an ocular applicable gel formulation.

1. Introduction

Nephropathic cystinosis is a rare autosomal recessive disease. It is characterised by high lysosomal levels of cystine. The disease, if untreated, results in death from renal failure by the second decade of life. The condition is characterised by poor growth, renal Fanconi syndrome, renal glomerular failure and impairment of other tissues and organs. If treatment is initiated just after birth this may attenuate the rate of renal failure, however, glomerular damage present at the time of diagnosis is irreversible and may result in the need for renal transplant (Levtchenko et al., 2007; Schneider et al., 2006; Thoene, 2007; Schneider, 2004).

Cystinosis arises due to a defect in the lysosomal transport mechanism for cystine. This results from mutations in the CTNS gene found on chromosome 17p13, which codes for cystinosin, a lysosomal membrane transport protein (Shotelersuk et al., 1998).

Although novel prodrug strategies are being researched (Kay et al., 2007; McCaughan et al., 2008), the main treatment for the disorder remains the administration of the aminothiol, cysteamine (Fig. 1) (as the bitartrate salt, Cystagon™). The molecule lowers intracellular levels of cystine by forming a cysteamine–cysteine mixed disulfide. The structure of the disulfide is spatially similar to the amino acid lysine and can egress the lysosome using the undamaged excretion pathway for lysine (Pisoni et al., 1995).

Cysteamine, however, possesses an offensive taste and smell and irritates the gastrointestinal tract, leading to nausea and vomiting following administration. In addition, cysteamine and its metabolites are excreted in breath and sweat. As a result of these problems, patient compliance can be poor (Levtchenko et al., 2007; Berge et al., 1977).

Cystine accumulates in all organs and tissues in the body. Corneal crystal deposition is one of the most troublesome complications affecting patients, especially as their prognosis improves and life expectancy increases. Crystals deposit in the cornea slowly through infancy until they become apparent at an age of approximately 16 months. The deposition becomes a problem when the entire peripheral stroma and endothelium have become packed, usually around the age of 6–8 years, although this varies (Dufier, 2003). Photophobia and, ultimately, blepharospasm affect the quality of life such that the slightest glimmer of sunlight can be debilitating. In addition, the crystals' accumulation over a period of years can cause corneal scars, keratitis and cataracts, as well as band keratopathies to form (Gahl and Kuehl, 2000). The oral form of the drug has no effect on depleting corneal crystals due to a lack of vasculature in the cornea, thus cysteamine must be administered topically in the form of eye drops.

Patient compliance is a major factor. The eye drops have to be administered every hour while awake in order to achieve maximum benefit. Clearly there is a need for a topical treatment to, at the very least, give some relief to patients if not slow the progression of deposition, while also allowing freedom from an 8 to 15-day dosing schedule.

Trials using topical cysteamine formulations have generated promising results, demonstrating a large depletion of corneal crystals following long treatment periods (Dufier, 2003; Gahl and Kuehl, 2000). These long treatment periods are necessary due to corneal and non-corneal barriers (Le Bourlais et al., 1998). Furthermore, novel viscous solutions of cysteamine HCl and hydroxypropylmethylcellulose (HPMC) have been reported (Bozdag et al., 2008). Of the three distinct corneal membranes, the epithelium, the

* Corresponding author. Tel.: +44 1224 262548; fax: +44 1224 262555.
E-mail address: g.kay@rgu.ac.uk (G. Kay).

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doi:10.1016/j.ijpharm.2010.03.065

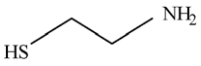

Fig. 1. Cysteamine.

stroma and the endothelium, the epithelium provides the greatest hurdle as it is lipophilic and acts as a barrier to ion transport and the absorption of hydrophilic drugs such as cysteamine bitartrate. The tight junctions of the corneal epithelium serve as a selective barrier for small molecules and prevent the diffusion of macromolecules via the paracellular route. Non-corneal absorption, on the other hand, involves drug penetration across the conjunctiva and underlying sclera into the uveal tract and vitreous humor (Nanjawade et al., 2007) and is important for the absorption of large, hydrophilic molecules. Current understanding of ocular pharmacokinetics involves mixing of the eye drops with lachrymal fluid, produced at a rate of 0.5–2.2 μL/min, resulting in a short contact time with ocular tissue (Ahmed and Patton, 1985, 1987). Subsequent drainage towards the nasolachrymal duct during blinking results in extensive elimination of the applied solution and contact times varying from 1 to 2 min (Le Bourlais et al., 1998; Gangrade et al., 1996) to 5 min (Robinson, 1989; Shell, 1984; Urtti and Salminen, 1993) have been reported. The rapid drainage rate is due to the tendency of the eye to maintain its residence volume at ~10 μL, and consequently, the overall absorption and bioavailability of a topically applied drug is typically <5% (Felt et al., 1999; Lang, 1995).

Synthetic hydrogels based on poly(acrylic acid) (PAA), commercially available as *Carbopol*®, can be obtained crosslinked with allylsucrose or allylpentaerythrol (Carbomer) for pharmaceutical application. In the dry state, PAA is highly coiled and tightly packed due to a preponderance of hydrogen bonding carboxylic acid groups along the alkyl backbone (Tamburic and Craig, 1996), but once dispersed in water the polymer swells to form a colloidal dispersion that behaves as an anionic electrolyte. When exposed to a pH above the pK_a of 4.75 (Guo, 2007), formation of carboxylate anions causes inter-chain repulsion that leads to the polymer swelling to as much as 1000-fold its original volume. The physical hydrogel formed is both transparent and bioadhesive and these attributes are highly desirable for topical ophthalmic application. In addition, the non-Newtonian pseudoplastic or 'shear-thinning' rheology inherent in PAA hydrogels facilitates the process of blinking by dramatic reductions in apparent viscosity as a function of the high external shear-stresses applied by the leading-edge and inside surface of the eye-lid (Nanjawade et al., 2007). These high shear-stresses are associated with equivalent shear rates of 0 s^{-1} at rest to 10,000–40,000 s^{-1} when blinking (Bothner et al., 1990). Pseudoplastic fluids therefore offer significantly less resistance to blinking than Newtonian liquids of equivalent consistency ('viscosity'). High apparent viscosities under 0 external stresses result in longer contact times on the surface of the eye. For topical ophthalmic formulations of cysteamine, this should result in less frequent application and better patient compliance.

2. Materials and methods

2.1. Materials

Carbomer 934 was purchased from Universal Biologicals, UK; cysteamine free base, cysteamine hydrochloride, trifluoroacetic acid, 1,8-diazabicyclo[5.4.0]undec-7-ene, potassium chloride, sodium chloride, sodium carbonate, calcium carbonate and magnesium chloride were purchased from Sigma, UK. Butoxycarbonyl-L-phenylalanine N-hydroxysuccinimide ester was purchased from Bachem, Europe. Tubing membrane 12–14,000 kDa was purchased from Visking, UK. All other chemicals were of pharmaceutical grade.

2.2. Methods

2.2.1. Preparation of a UV-tagged compound

Cysteamine does not possess a chromophore. To demonstrate release initially, a cystamine–phenylalanine conjugate (Fig. 2) was synthesised, which allowed release to be monitored by UV spectroscopy.

To a stirring solution of cystamine dihydrochloride (1 g, 0.00444 mol) in anhydrous dichloromethane (20 cm^3) at room temperature, 1,8-diazabicycloundec-7-ene (1.33 mL, 0.0089 mol) was added. The reaction mixture was then stirred continuously for 15 min at room temperature. To this was added butoxycarbonyl-L-phenylalanine N-hydroxysuccinimide ester (3.22 g, 0.0089 mol). After thin layer chromatographic (TLC) analysis confirmed that there were no starting materials left the solution was then partitioned between dichloromethane (20 cm^3) and water at room temperature. The dichloromethane extracts were then washed with water (3 cm^3 × 50 cm^3), dried with magnesium sulfate, filtered and evaporated to near dryness. The solution was applied to a silica gel chromatography column (4 cm^3 × 30 cm^3) prepared with dichloromethane. The column was initially eluted with the same solvent until all front running impurities had eluted (monitored by TLC). The eluent was then changed to dichloromethane:methanol (9:1) and the major product was eluted, this was monitored and confirmed by TLC analysis, UV visualisation at 254 nm.

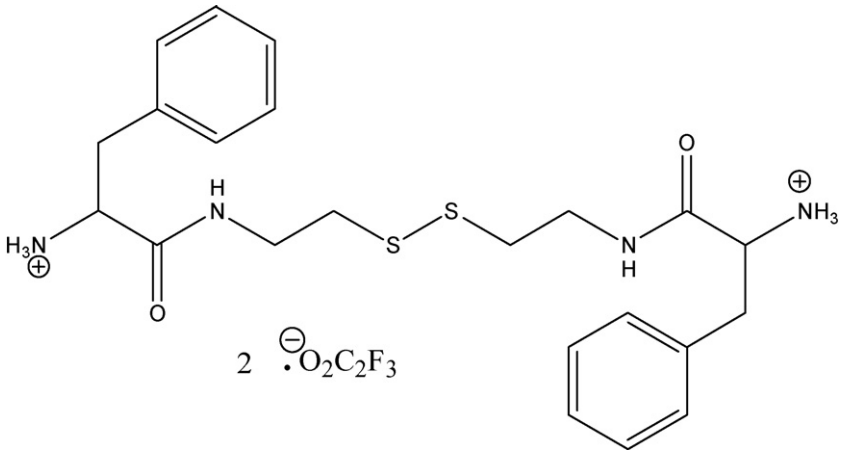

Fig. 2. Cysteamine–phenylalanine conjugate.

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Table 1
Summary of all gels tested.

Gel	Concentration of Carbomer 934 (% w/w)	No active	Phenylalanine conjugate 0.5% (w/w)[a]	Cysteamine free base 0.5% (w/w)	Cysteamine hydrochloride 0.5% (w/w)
1	0.1	•			
2	0.5	•			
3	0.1		•		
4	0.5		•		
5	0.1				•
6	0.5				•
7	1.0	•			
8	1.0		•		
9	1.0			•	
10	1.0				•

[a] Cystamine–phenylalanine required ethanol as a cosolvent to dissolve.

The protected compound was dissolved in trifluoroacetic acid (5 cm^3) at room temperature. After 3.5 h, the resulting solid was washed in ethanol (3 cm^3 × 20 cm^3), and the trifluoroacetic acid and ethanol removed by evaporation. Addition of diethyl ether gave an off-white precipitate that was filtered and dried over $CaCl_2$. The product was chromatographically homogenous by TLC [dichloromethane:methanol (9:1)].

2.2.2. Preparation of gels

All gels were made by addition of Carbomer 934 to Simulated Lachrymal Fluid (SLF), which was homogenised using a turbine mixer on low speed.

SLF was used to mimic tear fluid during tests. It was also used in the production of the ophthalmic gels to provide additional buffering capacity. The following salts were weighed out and stirred in a 1 L volumetric flask: potassium chloride 0.179% (w/v), sodium chloride 0.631% (w/v), sodium carbonate 0.218% (w/v), calcium carbonate 0.004% (w/v) and magnesium chloride 0.005% (w/v). Once dissolved, the pH of the solution was adjusted to 7.4, which is that of biological tissues, by addition of 2 M hydrochloric acid. Distilled water was added to 1 L. Gel formation followed neutralisation by addition of NaOH. The gel was then allowed to equilibrate for 24 h at 4 °C. Sorensen's Modified Phosphate Buffer (SMPB) was added, along with the active. The cystamine–phenylalanine conjugate also required ethanol as a cosolvent. The pH was maintained at 7.4. SLF addition achieved the final weight. Final gels were allowed to rest at 4 °C for 24 h until further testing commenced. Table 1 summarises all gels tested.

2.2.3. pH studies

The effect of each active on the pH of the final gel formulation was measured using a calibrated, handheld MP120 pH meter from Mettler Toledo (Columbus, OH, USA). Measurements were recorded at room temperature.

2.2.4. Rheological studies

The rheological properties of Carbomer 934, and Carbomer 934 with various actives (cystamine–phenylalanine conjugate, cysteamine hydrochloride and cysteamine free base) were studied using an Advanced Rheometer AR1000 from TA Instruments (New Castle, DE, USA). A 60 mm, 2° angle cone geometry was used, with a truncation value of 65 μm. All measurements were made at 34 °C. Shear–stress (Pa) was measured at shear rates of between 0 and 600 rad s^{-1}.

Oscillatory measurements were also performed to characterise the linear viscoelastic behaviour (Chen et al., 2002; Chhabra and Richardson, 1999), and relate the rheological parameters to molecular structure (Gunasekaran and Mehmet, 2000), using a linear mode and a frequency of 1–10 Hz, and 20 sample points. The sample volume was approximately 1.5 mL. All tests were performed in triplicate.

2.2.5. Bioadhesion studies

Bioadhesion was quantified using a Texture Analyser (Stable Micro Systems, TA-AT2i), which measured the force required to remove the gel from an area of bovine cornea. Fresh bovine eyes were collected immediately after slaughter, and washed with deionised water. The whole cornea was then excised and washed in SLF at room temperature. Prior to testing, the corneas were placed on a tissue to remove excess fluid. Cyanoacrylate glue was then used to attach a cornea to a 2 cm^2 stainless steel plate. Care was taken not to allow the glue to come into contact with the upper surface of the tissue. Immediately after this, the steel plates were attached (in pairs) to the Texture Analyser, one positioned directly above the other. Each gel sample was placed between the cornea samples and held together for 60 s; the force required to separate the plates was then measured [contact force of 0.05 N, contact time 60 s, probe speed 0.5 mm/s]. The force was plotted against distance; the area under the curve (AUC) being equal to the work of adhesion (W_{ad}). The statistical significance was determined using a Mann–Whitney test. Each individual test was undertaken nine times.

2.2.6. Dissolution studies

A 100 mL round-bottomed flask with sidearm was held in a water bath, heated to 34 °C (Ooi et al., 2007). To the sidearm, a condenser was attached. 50 mL SLF was added to the flask, and stirred magnetically using an IKA RET basic hotplate stirrer (Staufen, Germany). The dialysis membrane, containing 7 mL of gel and tied in a rod shape (length 2.23 cm; radius 1 cm, average of 3 measurements) to exclude air bubbles, was added at time 0. The medium was sampled every 2 min for the first 10 min, every 5 min for an hour, and every 15 min after the first hour. Samples were analysed at 256 nm, the λ max for phenylalanine conjugate, using an UV spectrometer from Unicam (Winsford, Cheshire, UK). All experiments were carried out under sink conditions and triplicates were obtained for each experiment.

3. Results and discussion

3.1. pH studies

It has been reported that the ocular surface can tolerate a pH range of 6.6–7.8. Beyond this range patients can experience stinging or discomfort (Dalton et al., 2008; Carney and Fullard, 1979). When formulated at a pH between 4 and 6, PAA solutions act as *in situ* forming gels (pH-dependent). When inserted into the eye, these PAA colloidal dispersions show a sol to gel transition as the pH is raised above the pK_a of the free acid groups to that of the

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Table 2
The effect of different actives on pH of the Carbomer 934.

Concentration of Carbomer 934 (% w/w)	Active	pH upon addition
0.1	Phenyalanine conjugate[a]	6.7
0.5	Phenyalanine conjugate[a]	6.2
0.1	Cysteamine hydrochloride	6.7
0.5	Cysteamine hydrochloride	6.0
1.0	Phenyalanine conjugate[a]	7.0
1.0	Cysteamine free base	7.4
1.0	Cysteamine hydrochloride	7.2
1.0	–	3.5

[a] Phenylalanine required ethanol as a cosolvent to dissolve.

eye, pH 7.4. In our studies, the hydrogels were initially neutralised using sodium hydroxide and then the actives were added in SMPB (pH 7.4). These gels are considered 'preformed' as opposed to *in situ*. The inclusion of phenylalanine conjugate, cysteamine hydrochloride or cysteamine free base to neutralised Carbomer 934 gels was to cause changes to the pH-dependent on the initial concentration of polymer (Table 2). All samples qualify as preformed gels according to the definition used for an *in situ* gel, with the exception of 0.5% (w/w) Carbomer 934 and cysteamine hydrochloride (pH 6.0).

3.2. Rheology studies

Continuous flow measurements with a 1% (w/w) Carbomer 934 gel and Carbomer 934 gels containing cysteamine compounds are represented in Fig. 3. This indicates an increase in consistency with addition of phenylalanine conjugate and cysteamine hydrochloride, and a slight decrease with inclusion of the cysteamine free base. As all of the samples are at the same pH due to the use of SMPB, these differences must be due to strengthened network interactions for the conjugate and the hydrochloride, and decreased interactions for the free base. The use of ethanol as a cosolvent decreased viscosity and clarity of the gel.

A 1% (w/w) concentration of Carbomer 934 was considered necessary to produce the consistency required for a functional eye gel as the initial 0.1% (w/w) gels produced were too fluid with viscosities akin to that of water. It has been hypothesised that a viscosity of 12–15 mPa s is optimal for ophthalmic delivery (Rupenthal, 2008), demonstrated through *in vivo* work with rabbits.

All samples display pseudoplastic flow behaviour as may be expected for PAA hydrogels of this concentration. An initially high increase in shear–stress as a function of shear rate ('apparent viscosity') followed by a more gradual or constant increase can also be interpreted as an 'apparent yield stress'. Polymers that form weak gel networks often display an apparent yield stress at low shear

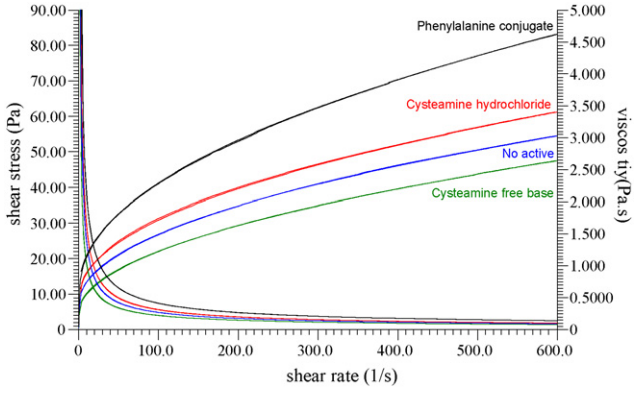

Fig. 3. Continuous flow curves for Carbomer 934 gels containing different cysteamine compounds at 34 °C.

Table 3
Oscillatory data for Carbomer 934 gels.

Gel	G'	G''	Tan δ
Carbomer 934 no active	70	6	0.09
Carbomer 934 cysteamine HCL	119	10	0.09
Carbomer 934 cysteamine–phenylalanine conjugate	65	6	0.10
Carbomer 934 cysteamine	43	4	0.09

rates and the elastic component of the viscoelastic behaviour can be quantified using oscillatory measurements. To measure this component, a small stress, σ (mPa) applied at a frequency, ω (rad s^{-1}) will create a reactionary strain, γ (mPa) with a smaller amplitude and associated phase lag, δ (°) due to the non-ideal nature of the gels' viscoelastic properties.

Results from the oscillatory measurements described in Section 2.2.4 and Table 3 indicate very little effect of cysteamine hydrochloride, conjugate and free base on the loss tangent, suggesting the same degree of elasticity for all Carbomer 934 gels studied. A degree of elastic behaviour at low shear–stress reflects the weak gel properties of these formulations. This propensity to form a gel under conditions of 0 to low shear is desirable for increased residence on the ocular surface. The oscillatory results presented clearly indicate that the addition of cysteamine actives to Carbomer 934 gels does not appear to destroy these weak gel properties. The results for the gel where ethanol was used as a cosolvent were comparable to those with no active.

As expected, Carbomer 934 was found to be pseudoplastic (shear-thinning) in agreement with other studies (Llabot et al., 2007). Upon addition of different actives, the consistency was altered (Fig. 3 and Table 4).

3.3. Bioadhesion studies

PAA has been shown to have excellent bioadhesive properties in comparison to other gels as reported by Slovin and Robinson (1993) and its ability to increase ocular residence time, relative to traditional eye drops, has also been reported (Thassu et al., 2007). It should be noted however that the negative charge on the corneal epithelium (De la Fuente et al., 2010; Calvo et al., 1997; Fitzgerald et al., 1987) would be expected to provide a natural barrier to adhesion of a highly anionic polymer such as PAA. In practice, the significant yield stress of PAA gels, particularly at a concentration of 1.0% (w/v), would appear to counteract this effect causing the gel to remain on the surface of the corneal epithelium despite microadhesion being weak or non-existent. The mean force required to remove a gel sample from the surface of bovine corneal tissue for the 1% (w/w) Carbomer 934 gel was 0.067 N and the AUC, indicative of the 'work of adhesion' (work done), was 0.205 N mm. This work of adhesion was 16% greater than the gel containing cysteamine hydrochloride and 5% greater than that containing cysteamine–phenylalanine conjugate. Reference to the peak force of removal, however, revealed that this was 37% less than that of the cysteamine containing gels, which both gave a mean value of 0.107 N ($p < 0.01$) (Table 5).

Table 4
Viscosity coefficient values for Carbomer 934 gels containing different cysteamine compounds.

Gel active	Viscosity coefficient, η' (Pa s)
No active	4.6
Cysteamine HCl	5.8
Cysteamine	3.2
Phenylalanine conjugate	7.0

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Table 5
Results of bioadhesion assay.

Carbomer 934	Force (N)	AUC
Tissue vs plain gel	0.067[a]	0.205[a]
Tissue vs gel with cysteamine HCL	0.107[a]	0.177[b]
Tissue vs gel with cystamine–phenylalanine conjugate	0.107[a]	0.196[a]

[a] $p < 0.01$.
[b] $p < 0.05$.

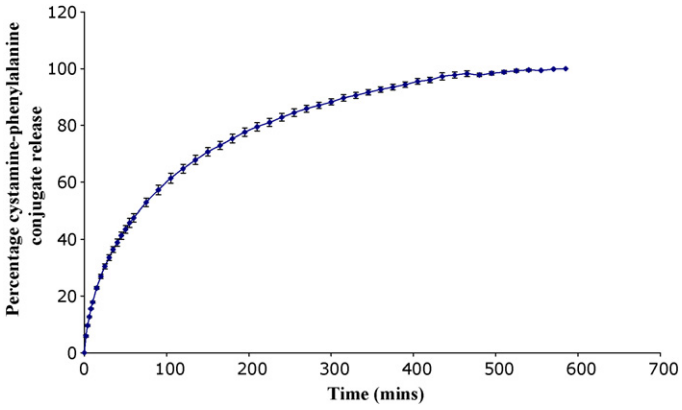

Fig. 4. Percentage cystamine phenylalanine conjugate released from Carbomer 934. Results represent the mean of all measurements $n = 3$; error bars represent standard error of deviation.

It has been reported that pH, ionic strength, molecular weight and chain flexibility all affect the bioadhesive properties of PAA gels (Park and Robinson, 1987; Rossi et al., 1995) however, it appears that the mechanisms of polymer attachment to mucosal surfaces are still not fully understood (Salamat-Miller et al., 2005). There is speculation that bioadhesion is the net effect of many physicochemical factors that include attractive forces such as hydrophobic interactions, hydrogen bonding and van der Waals attraction; physical entanglement of polymer chains via diffusional processes and electrostatic interaction of an attractive or repulsive nature all contribute to the bioadhesive process. It appears that the inherent rheological properties of ophthalmic gels may also have a decisive role to play with respect to improved residence on the eye. The precise reasons for the apparent bioadhesiveness of PAA gels requires more study.

3.4. Dissolution studies

To allow quantification of the dissolution results, the A_1[1] of the phenylalanine conjugate was determined. Thus, 100% release would be quantifiable, Fig. 4.

The release of cysteamine–phenylalanine conjugate from the gel was analysed by the Higuchi method (Table 6). The area of each membrane rod was calculated to be 20.32 cm^2.

Table 6
Results of the Higuchi model analysis on the Carbomer 934 gels.

Carbomer 934 cysteamine–phenylalanine conjugate	Higuchi model
Sample time (min)	k_H
2	0.05
35	0.08
75	0.08
240	0.07
420	0.06
540	0.04

The release could be categorised as first-order. This is indicative of reversible interactions between the polymer matrix and the active and uninterrupted drug release from the gels.

Where the constant administration of eye drops is the routine method of drug delivery, as is the case with cystinosis, the development of a controlled release formulation is desirable. These results indicate that this may be achieved using Carbomer 934 as a vehicle for ophthalmic delivery. However, demonstration that cysteamine HCl can be released from this system in a manner analogous to that of the phenylalanine conjugate needs to be undertaken.

4. Conclusion

The results presented in this paper demonstrate that the formulation of cysteamine hydrochloride as a gel for ophthalmic delivery is achievable. All the gels tested formed weak gel networks at 0 to low shear-stresses, desirable properties for increased residence time on the ocular surface. A net bioadhesion and first-order release of the active from the sample matrix was also apparent. Furthermore, the addition of cysteamine did not destroy the gel properties. These results offer the possibility of a gel formulation of cysteamine, which would considerably enhance the quality of life for cystinotic patients with ocular complications.

Acknowledgments

The authors gratefully acknowledge financial support from the Cystinosis Foundation, UK and TENOVUS, Scotland.

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Please cite this article in press as: Buchan, B., et al., Gel formulations for treatment of the ophthalmic complications in cystinosis. Int. J. Pharm. (2010), doi:10.1016/j.ijpharm.2010.03.065