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Bioadhesive Microspheres for Ophthalmic Administration of Acyclovir

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Abstract

The bioavailability of acyclovir to the ophthalmic epithelium is low and when the drug is administered in ophthalmic ointment it must be applied every four hours. An emulsification technique has been used to prepare acyclovir-loaded chitosan microspheres with the aim of promoting the prolonged release of drug and increasing its ocular bioavailability.

The microparticulate drug-delivery systems obtained have been characterized for their morphology and physicochemical characteristics by in-vitro dissolution tests and in-vivo ocular administration to rabbits. The results show that the microspheres obtained are always quite small—the diameters of 90% of the particles are ≤ 25 μm (i.e. $d_{90\%}$ never exceeds 25 μm) and physicochemical characterization shows that the drug is homogeneously dispersed in an amorphous state inside the microspheres. The in-vitro dissolution profile of acyclovir from chitosan microspheres is slower than that for the raw drug. Results from in-vivo ocular administration of acyclovir-loaded microspheres to the rabbit eye show prolonged high concentrations of acyclovir and increased AUC values.

The microparticulate drug-carrier seems a promising means of topical administration of acyclovir to the eye.

Acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine, is a synthetic analogue of purinic nucleosides with antiviral activity. It is active above all against herpes simplex virus types I and II (HSV1 and HSV2) and against varicella-zoster virus. The Epstein-Barr virus and cytomegalovirus are susceptible to acyclovir to a lesser extent. The activity of acyclovir is a result of its intracellular conversion by viral thymidine kinase, a process which is selective for infected cells (Elion 1982; Laskin et al 1982).

Acyclovir is successfully employed in the symptomatic treatment of localized or systemic HSV infections, including some ocular pathologies such as herpes simplex keratitis. This infection is usually caused by type I virus and is a common cause of blindness in developed countries. Topical ophthalmic administration of acyclovir has been thoroughly evaluated in clinical trials (Collum et al 1980; Lalau et al 1982), and the drug has proved as effective as other antiviral drugs (e.g. idoxuridine, trifluorothymidine) with the advantage of a faster healing time (approximately 4 days). Tolerance to topical ointment (4%) is extremely good and comparable with alternative therapy for HSV corneal disease (Grant 1987). The bioavailability of topically applied ophthalmic drugs is often quite poor (10% of applied dose) owing to several factors; precorneal clearance processes and corneal structure, which limit passage of drug molecules, play an important role. Because of the polar characteristics of the molecule, the bioavailability of acyclovir to the ophthalmic epithelium is very low (Hughes & Mitra 1993). Moreover, it has quite a short plasma half-life (3 h) and when administered as ophthalmic ointment it must be applied, in the lower conjunctival sac, every 4 h, 5 times daily.

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The frequency of topical administration is undoubtedly an inconvenience in acyclovir ophthalmic therapy. This could be overcome by a drug-delivery system promoting prolonged release of drug and so increasing its application intervals.

Norley et al (1987) prepared acyclovir-containing liposomes targeted with palmitoyl-derivatized monoclonal antibody to glycoprotein D of HSV. The drug-loaded immunoliposomes were tested on culture corneas infected with HSV1 and effectively inhibited virus replication in the infected cornea, with enhancement of the therapeutic effect of acyclovir compared with the free drug.

The aim of our study was the preparation and in-vitro and in-vivo evaluation of polymeric microparticulate systems loaded with acyclovir; such drug-delivery systems are envisaged as prolonging the therapeutic activity of acyclovir and increasing its ocular bioavailability.

For this purpose we have chosen a biodegradable, biocompatible polymer with bioadhesive properties. The drug-release rate should be modified by embedding the drug in the polymeric micro-matrix, the bioadhesive properties of the polymer should favour the permanence of a drug delivery system at the site of administration (lower corneal sac). The polymer employed, a chitosan [(1 → 4)-2-amino-2-deoxy-β-D-glucan], is a hydrophilic cationic polymer obtained by *N*-deacetylation of chitin, with a structural formula analogous to that of cellulose. It has low toxicity (oral LD50 in rats is 16 g kg^{-1}) and its biodegradability and biocompatibility have been well established (Hirano et al 1990). Mucoadhesion is one of several properties that make this polymer a useful pharmaceutical excipient. Leher et al (1992) suggest that the mucoadhesive properties of chitosans are probably a result of ionic interaction between the positively charged amino groups in chitosan and the negatively charged sialic acid residues in mucus.

This study involves the preparation of acyclovir-loaded chitosan microspheres, their characterization and their in-vitro and in-vivo evaluation.

Materials and Methods

Acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine, was purchased from Sigma (St Louis, MO). Chitosan [(1 → 4)-2-amino-2-deoxy-β-D-glucan], Mr 750 000, viscosity (1% in 1% acetic acid solution) 800–2000 mPa.s, was purchased from Fluka (Buchs, Switzerland). All solvents were of analytical grade unless otherwise specified.

Microsphere preparation

Acyclovir-loaded chitosan microspheres were prepared by a multiple emulsion technique previously developed in our laboratory (Genta et al 1997). Acyclovir (0·2% w/v) was dissolved in a solution of chitosan (2%) in HCl (0·1 M) containing 20% methanol. This solution was emulsified in the continuous phase prepared from light mineral oil containing 2% Span 20 (dispersed to continuous phase ratio of 1:15). Emulsification was performed at 34°C under continuous stirring at 9500 rev min^{-1} with an Ultra Turrax (Ika Labortechnik, Staufen, Germany) model T25 homogenizer. Subsequent solvent evaporation was performed at 70°C and under reduced pressure while stirring at 50 vibrations s^{-1} with a vibromixer (Vibromixer E1, Chemap, Volketswil, Switzerland). These conditions were maintained for 5 h to accomplish solvent evaporation.

The microspheres obtained were rinsed twice with petroleum ether, centrifuged, filtered through a 2-μm stainless steel filter, and kept in desiccator for 24 h before characterization (Table 1, batches 1–3).

The microspheres were cross-linked either chemically with glutaraldehyde or tripolyphosphate, as reported in the literature (Jayaakrishnan & Jameela 1989; Bodmeier et al 1989) or by heat-treatment (Lim & Wan 1995).

Glutaraldehyde was employed as a 'reference' cross-linking agent because it is a compound frequently used in the literature; this compound could not be used in microspheres intended for ophthalmic administration because it is irritating to eye tissues as reported by a technical note of Sigma, Aldrich, Fluka Chemical Division (1996). Table 1 lists the types and the amounts of the different cross-linking agents used in the preparation of the different batches of microspheres.

Chemical cross-linking with glutaraldehyde was performed by addition of a 25% w/v aqueous solution to the emulsion prepared as described above. Subsequent steps were performed as previously described (Table 1, batches 10–12).

Table 1. Cross-linking agents used in the preparation of the microspheres.

Batch number	Cross-linking agent	Ratio of cross-linking agent to chitosan (w/w)
1–3	–	–
4–6	Heat	–
7–9	0·4% (w/v) aqueous solution of tripolyphosphate	1:20
10–12	25% (w/v) aqueous solution of glutaraldehyde	1:1

Chemical cross-linking with tripolyphosphate was performed by addition of a 0·4% w/v aqueous solution to the emulsion prepared as previously described. Subsequent solvent evaporation was performed at 60°C under reduced pressure and continuous stirring (Table 1, batches 7–9).

Microsphere cross-linking by heat-treatment was achieved by dry heating of the microspheres in an oven at 150°C for 16 h (Table 1, batches 4–6).

Microsphere characterization

The shapes and sizes of microspheres from all batches were evaluated by scanning electron microscopy (CX Temscan; Jeol, Tokyo, Japan). The dried microspheres were sputter-coated, under an argon atmosphere, with a thin layer of Au/Pd, and photographed. Differential scanning calorimetry (DSC) was performed with a Mettler (Greifenser, Switzerland) model DSC 12E calorimeter connected to a Haake (Karlsruhe, Germany) D8-G thermal cryostat. The temperature range was between 40 and 300°C and the scanning rate 10°C min^{-1}. Granulometric analysis in the 2–50-μm size range was performed by the light-blockage method with an HIAC/ROYCO model 3000 instrument (AM Instruments, Desio, Italy) equipped with a HC60 sensor. The results are averages from five analyses. X-ray diffractometry was performed by means of a Philips (Eindhoven, The Netherlands) model 1050 PW 1730 powder diffractometer equipped with a graphite crystal monochromator (Cu-Kα radiation).

The microsphere drug content was determined by dissolving samples of acyclovir-loaded microspheres in HCl (0·1 M); determination of acyclovir was by HPLC analysis (Pramar et al 1990) with a Varian (Milan, Italy) model 9010 chromatograph equipped with a Varian Varichrom UV 2550 variable wavelength UV-Vis detector, operated at 252 nm, and a 250 mm × 5 mm i.d. × 5 μm LiChrosorb RP-18 reversed phase column. The mobile phase was KH_2PO_4 (0·01 M)-acetonitrile (97:3, v/v) at a flow-rate of 1·8 mL min^{-1}. Analysis of each batch of microspheres was performed in triplicate.

In-vitro dissolution tests were performed by soaking samples of microspheres with pH 7·2 buffered isotonic vehicle in closed Erlenmeyer flasks stirred by means of a shaker incubator thermostated at 37°C. The amount of acyclovir released after fixed time intervals was determined by HPLC analysis performed as for analysis of drug content. The data obtained were used to construct plots of log (percent drug released) against log (time) and the slopes (k) of the plots were calculated from the fitted linear regression lines.

In-vivo evaluation

A freshly prepared formulation containing non-cross-linked chitosan microspheres loaded with acyclovir, batches 1–3, (260 mg mL^{-1}) equivalent to 0·52% acyclovir was prepared in a pH 7·2 buffered isotonic vehicle. Acyclovir suspension containing an equivalent amount of acyclovir was prepared in the same vehicle.

Studies were performed on female New Zealand Albino rabbits (Charles River, Calco, Italy), 2·0–2·5 kg, free from any signs of ocular inflammation or gross abnormality. Animal procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research. The animals were allowed to move their heads freely and their eye movements were not restricted. Between

experiments the rabbits were housed singly with free availability of food and water.

Two groups of 12 animals were treated with the acyclovir-loaded chitosan microsphere formulation or with acyclovir suspension. Aqueous acyclovir levels were monitored 15, 30, 60, 120, 240 and 360 min after a single instillation of 50 μL of each formulation into the conjunctival sac. Two animals (four eyes) were used for each time point for each acyclovir formulation tested; paracentesis was, therefore, performed in both eyes of each rabbit. Before paracentesis the rabbit was anaesthetized with ketamine HCl (ketalar) 25 mg kg^{-1}. After paracentesis the rabbits were not reused. Aqueous humour (150–200 μL) was withdrawn through the limbus, by means of a syringe with a 26-gauge needle, and frozen at −20°C for subsequent assay. The aqueous humour samples collected (150 μL) were treated with a solution of $ZnSO_4$ $7H_2O$ (2%; 150 μL) and the mixture was vortex-mixed for 1 min and then centrifuged at 6000 rev min^{-1} for 10 min (Centrifugette 4206; ALC, Milan, Italy). The resulting supernatant was filtered through a 0·2-μm PTFE membrane (Spartan-3, Schleicher Schuell, Keene, NH) by means of a gas-tight syringe. The filtrate was analysed by HPLC.

Chromatography was performed with a Varian Star 9010 solvent pump and a Varian Star 9050 variable wavelength UV-Vis detector, operated at 254 nm, on line with a computerized Varian Star 9020 workstation. Separation was performed on a 150 mm × 4·6 mm i.d. × 5 μm Hypersil ODS reversed-phase column equipped with a 10 mm × 4·6 mm × 5 μm direct-connect Hypersil ODS guard column (Alltech, Milan, Italy). The mobile phase was CH_3COONa (0·7 M; pH 6·0 with acetic acid) at a flow-rate of 1·0 mL min^{-1} and the injection volume was 100 μL. An external standard was used.

The time-course of acyclovir in the aqueous humour was characterized by measurement of several different parameters for both ophthalmic formulations studied. The time at which the drug was first detected in the aqueous humour was recorded, as was the time after which it could no longer be detected. The maximum drug concentration (C_{max}) and the time at which this sample was withdrawn (t_{max}) were also recorded. The area under the concentration-time profile from time 0 to 240 min (AUC0-240) was calculated.

Results are expressed as means ± s.d. Student's t-test was used and $P < 0.05$ used as an indication of statistical significance.

Results and Discussion

Fig. 1 shows photomicrographs of microspheres with no cross-linking and after cross-linking by heat-treatment. The non-cross-linked microspheres (Fig. 1a) are spherical in shape, and their size is equal to or smaller than 20 μm. SEM analysis of microspheres showed no differences of size and shape between non-cross-linked microspheres and those cross-linked by thermal treatment (Fig. 1b), i.e. thermal treatment did not seem to affect microparticle morphology.

Chemically cross-linked microspheres are bigger than non-cross-linked microspheres (diameter approximately 50 μm) and are less regular in shape. This type of cross-linking seems to affect the morphology of microparticulate system (data not reported).

Table 2 lists the granulometric distribution data obtained by

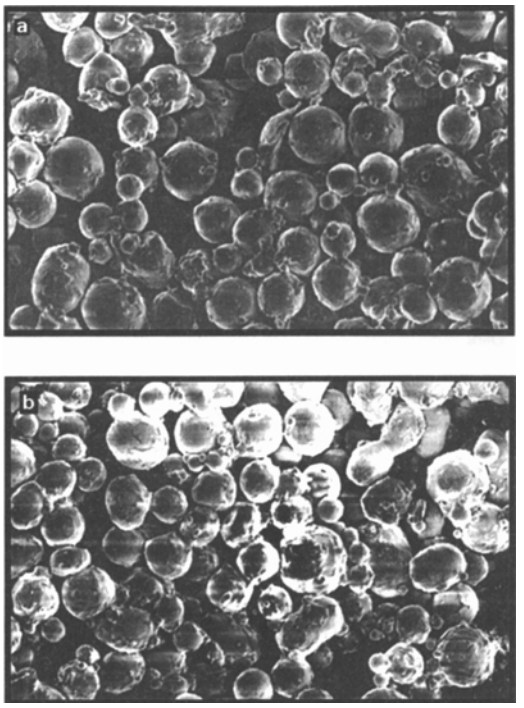

FIG. 1. Photomicrographs of acyclovir-loaded microspheres: a, non-cross-linked (magnification×500), b, cross-linked by thermal treatment (magnification×450).

HIAC/ROYCO analyses and expressed as $d_{50\%}$ and $d_{90\%}$ (figures which indicate that the diameters of 50% and 90%, respectively, of the particles are smaller than the tabulated values). These results confirm that non-cross-linked microparticles are smaller than chemically cross-linked microparticles (either glutaraldehyde or tripolyphosphate cross-linkage). Particle size is important because for ocular administration it must not exceed 25 μm: this requirement was met by all batches of microspheres. Figs 2–5 show the results obtained from physicochemical characterization.

The thermal behaviour of acyclovir is shown in the graphs presented in Fig. 2. The drug has three endothermic melting peaks at 163, 172 and 252°C (Fig. 2, curve a). Thermal treatment of the raw drug in an oven at 150°C for 16 h leads to conversion of a metastable structure with consequent disappearance of the melting peak at 163°C. (X-ray analysis of the same samples of drug (Fig. 3) also shows partial amorphization as a result of the heat-treatment (Fig. 3b)). The DSC profile of chitosan (Fig. 4a) shows an endothermic area at approximately 130°C. When chitosan is only physically mixed with the drug (Fig. 4b), shifting of the melting temperatures

Table 2. Granulometric distribution of acyclovir-loaded chitosan microspheres.

Batch number	Maximum size of 50% of particles (μm)	Maximum size of 90% of particles (μm)
1–3	2	15
4–6	4	15
7–9	5	25
10–12	10	23

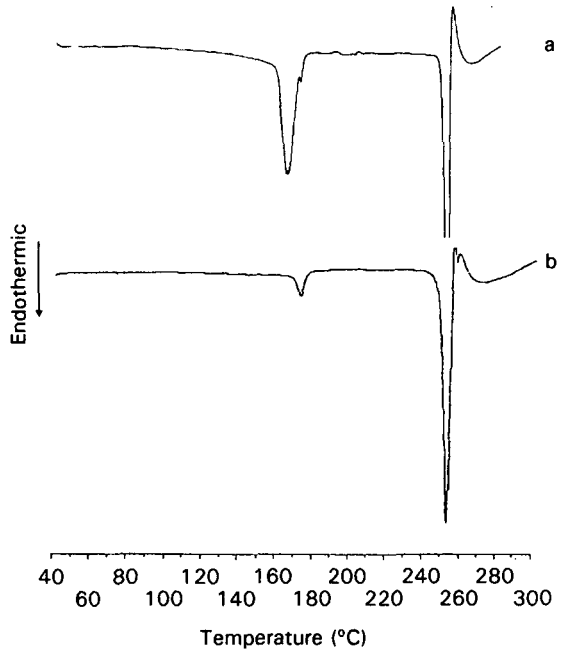

FIG. 2. DSC profiles of: a, acyclovir, b, thermally treated acyclovir (16 h at 150°C).

characteristic of polymer and drug is highlighted. The same DSC behaviour is observed both for non-cross-linked acyclovir-loaded microspheres and for those cross-linked by heating (Figs 4c, d); this is indicative of some interaction of the drug with the polymer.

Fig. 5 shows X-ray diffractograms of the samples described above. These show that the drug is still present in its lattice structure in the physical mixture (Fig. 5b), whereas it is completely amorphous inside the microspheres (Figs 5c, d). The conditions used to prepare the microspheres lead to drug-polymer interaction and cause complete drug amorphization. Moreover, this is independent of subsequent microsphere cross-linkage (data not reported).

Table 3 lists the average microsphere drug contents and the related encapsulation efficiencies. The encapsulation efficiencies are not significantly different for untreated and ther-

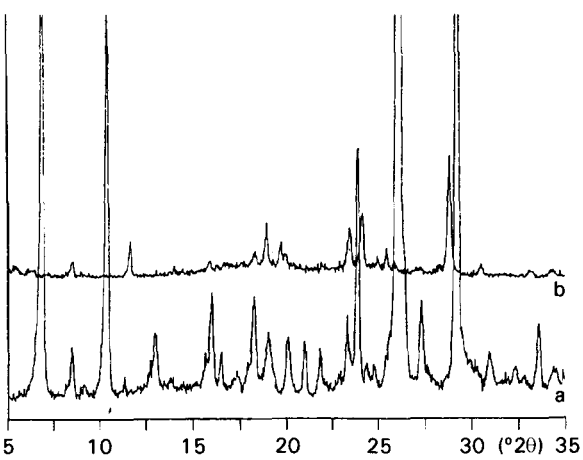

FIG. 3. X-ray diffractograms of: a, acyclovir, b, thermally treated acyclovir (16 h at 150°C).

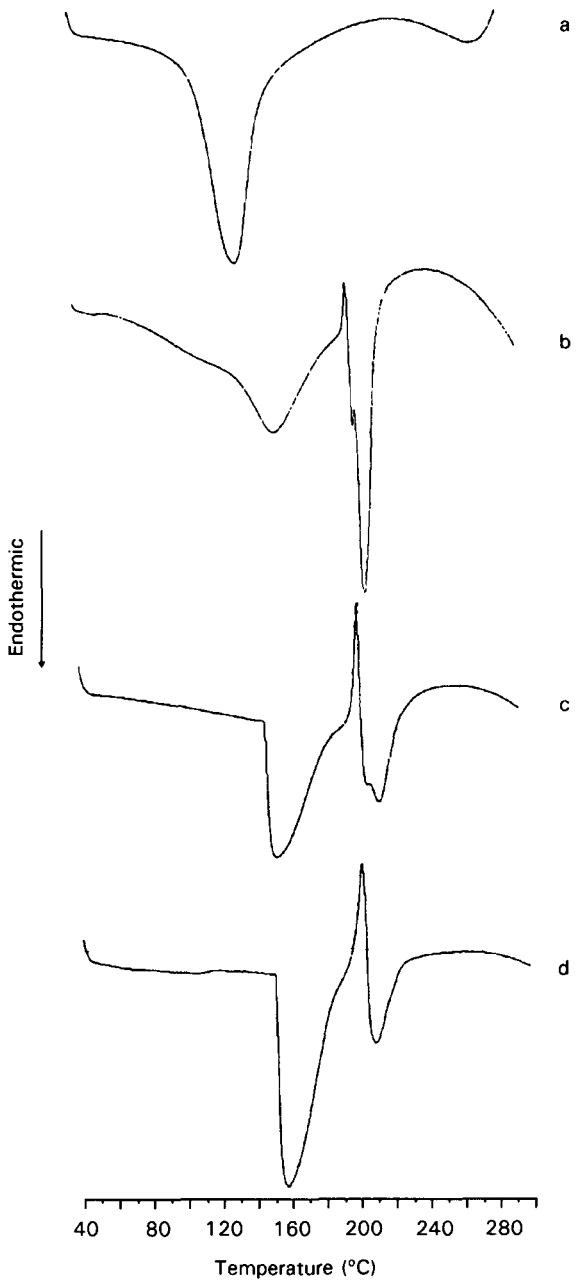

FIG. 4. DSC profiles of: a, blank microspheres, b, physical mixture of acyclovir and blank microspheres (1:40), c, batch 2, d, batch 4.

Table 3. Drug encapsulation parameters in the microspheres.

Batch number (%)*	Average drug content*	Encapsulation efficiency
1–3	2·61	28·71
4–6	2·42	26·62
7–9	2·22	25·54
10–12	1·00	14·14

*s.d. = ±5%.

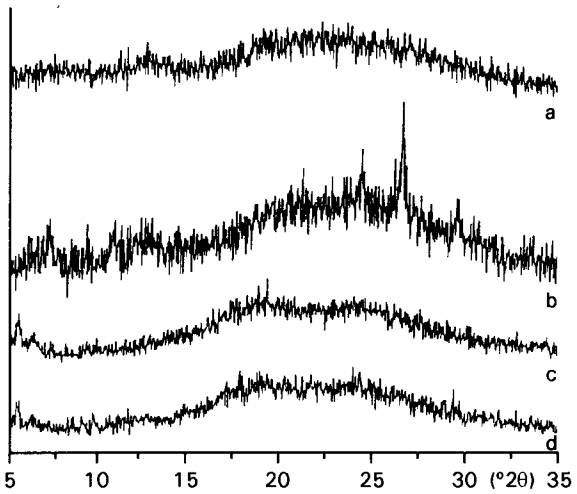

FIG. 5. X-ray diffractograms of: a, blank microspheres, b, physical mixture of acyclovir and blank microspheres (1:40), c, batch 2, d, batch 4.

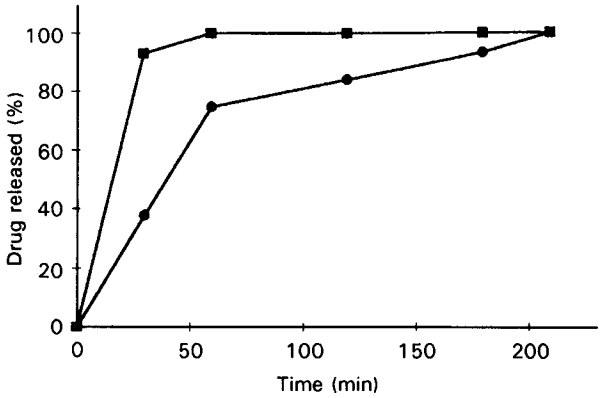

FIG. 6. In-vitro release profiles of (■) acyclovir, (●) batches 1–3.

mally treated microspheres. Tripolyphosphate cross-linkage did not affect the acyclovir content of the microspheres, whereas addition of glutaraldehyde causes a dramatic reduction of microsphere drug content.

Good production yields (>80%) were achieved for all batches of microspheres. In-vitro release studies under the experimental conditions described above show (Fig. 6) that use of chitosan microspheres slightly reduces the rate of drug dissolution (100% of drug released in 220 min) compared with raw drug (100% of drug dissolved in 60 min).

The slope of plots of log (percent drug released) against log (time) calculated for the non-cross-linked microspheres shows that acyclovir was released from chitosan microspheres according to square-root-of-time kinetics (K = 0·44; a slope of 0·5 indicates diffusional square-root-of-time release (Schwartz et al 1968)) and therefore a diffusion-controlled mechanism was operative. The amount released is proportional to the square-root-of-time in the Higuchi moving boundary model (Higuchi 1961) and in models based on a semi-infinite domain (Crank 1975). In both models the diffusion of drug through the hydrate polymer is rate-limiting.

Moreover, differences between the drug-release profiles of the non-cross-linked and cross-linked microspheres (either thermally or chemically cross-linked, data not reported) were only slight. The in-vitro results show that cross-linking cannot be used for significant control of the release of drug from the microspheres under these conditions. This led to the choice of non-cross-linked microspheres for in-vivo experimentation. No sign of ocular inflammation or discomfort was observed in the rabbits after administration of the acyclovir-loaded chitosan microspheres.

Key parameters describing the time profile of the concentration of acyclovir in aqueous humour are presented in Table 4. The mucoadhesive chitosan microsphere formulation appears to offer significant sustaining of drug release in the aqueous humour compared with the control suspension. The acyclovir levels obtained from the microsphere formulation were higher than those obtained from the suspension at all times (0–240 min) and were significantly higher at 15, 60, 120 and 240 min ($P<0.01$).

Table 5 lists the concentration of acyclovir in the aqueous humour after single instillations of acyclovir-loaded microspheres and of acyclovir suspension into the rabbit eye. With the microsphere dosage form acyclovir was first detected in the aqueous humour 15 min after instillation, whereas for acyclovir suspension the first appearance of the drug was after

Table 5. Concentrations of acyclovir in aqueous humour after single instillation of acyclovir-loaded microspheres and acyclovir suspension into rabbit eye.

Time (min)	Microspheres ($\mu g\ mL^{-1}$)	Suspension ($\mu g\ mL^{-1}$)
15	0·7067 ± 0·30*	n.d.
30	0·2288 ± 0·10	0·130 ± 0·04
60	0·8422 ± 0·20*	0·110 ± 0·05
120	0·7430 ± 0·12*	0·350 ± 0·03
240	0·8620 ± 0·25*	n.d.
360	n.d.	n.d.

Values are mean ± s.d. of results from four eyes. *$P < 0.01$, significantly different compared with suspension control. n.d., not detectable.

Table 4. Key parameters describing the aqueous humour pharmacokinetics of acyclovir after single instillation of acyclovir-loaded microspheres and acyclovir suspension into rabbit eye.

	Microspheres	Suspension
Shortest time drug was detectable (min)	15	30
Length of time when drug was still detectable (min)	240	120
Maximum concentration of drug ($\mu g\ mL^{-1}$)	0·862	0·350
Time when maximum drug concentration was measured (min)	240	120
Area under the aqueous humour concentration–time profile from 0 to 240 min ($\mu g\ mL^{-1}$ min)	172·19	39·37

30 min. Furthermore the microsphere dosage form ensures effective levels of drug in the rabbit aqueous humour for up to 240 min ($0{\cdot}86 \pm 0{\cdot}25$ μg mL^{-1}). In the group treated with the suspension the aqueous levels of acyclovir were undetectable after 120 min ($0{\cdot}35 \pm 0{\cdot}03$). The aqueous $AUC_{0 \rightarrow 240}$ values were significantly ($P < 0{\cdot}001$) greater for the microsphere formulation than for the suspension ($172{\cdot}19 \pm 41{\cdot}55$ μg mL^{-1} min compared with $39{\cdot}37 \pm 2{\cdot}17$ μg mL^{-1} min), with a 4·37-fold increase in AUC value.

These in-vivo results are particularly interesting because they show that the acyclovir-loaded microparticulate system based on chitosan increases drug bioavailability to the eye compared with the raw drug. Moreover, good results are obtained in prolonging drug residence time in the eye by administration of the acyclovir-loaded microspheres, probably because of the mucoadhesive properties of chitosan (Leher et al 1992).

Because the in-vitro results (Fig. 6) show that chitosan microspheres slow down the rate of release of acyclovir compared with the free drug, they suggest that the prolonged high concentrations of acyclovir in the eye after administration of microspheres depend not only on the mucoadhesive properties of chitosan, but also on the efficacy of the polymer in slowing down the rate of release of the drug.

Conclusions

The microparticulate drug delivery system proposed in this work, based on chitosan, a natural mucoadhesive polymer, seems to hold promise for topical administration of acyclovir to the eye. The AUC value was greater than that for the free drug. This is of utmost importance for acyclovir because its poor ocular epithelium permeability results in very low ocular bioavailability.

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