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Eudragit RL100 nanoparticle system for the ophthalmic delivery of cloricromene

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Abstract

A Eudragit RL100 polymer nanoparticle system loaded with cloricromene was prepared and characterized on the basis of physicochemical properties, stability and drug release features. To investigate the ocular bioavailability of cloricromene after inclusion in the polymer matrix, the new nanoparticle system was topically administered in the rabbit eye and compared with an aqueous solution of the same drug. The nanoparticle system showed interesting size distribution and surface charge values, suitable for ophthalmic application. The results indicated that the dispersion of cloricromene within Eudragit RL100 polymer nanoparticles increased its ocular bioavailability and enhanced the biopharmaceutical profile. The new cloricromene-loaded nanoparticle system described here may be useful in clinical practice.

Introduction

Eudragit RL100 (Figure 1) is a copolymer of poly-(ethylacrylate, methyl-methacrylate and chlorotrimethyl-ammonioethyl-methacrylate) containing between 8.8 and 12% quaternary ammonium groups. Eudragit polymers are among the most commonly used materials for coating oral drug formulations to achieve controlled delivery of the active drug. They have also been widely proposed for alternative application routes, for example transdermal patches (Verna & Iyer 2000; Kotiyan & Vavia 2001; Rafiee-Tehram et al 2001).

A significant effort in the development of novel drug delivery systems for ophthalmic administration has been made in recent years. Formulations as hydrogels, microparticles and nanoparticles, liposomes and other colloid systems, as well as solid inserts and shields, or surgically applied polymeric implants have all been proposed (Le Bourlais et al 1998; Gupta et al 2000; Kawakami et al 2001; Willoughby et al 2002). Among them, nanoparticle technology is currently receiving a great deal of attention in many different pharmaceutical areas. Nanoparticles of both biodegradable and inert polymeric materials have been shown to be efficient ocular drug carrier systems (Desai & Blanchard 2000; Kawashima 2001; Merodio et al 2002). In comparison with conventional aqueous eye drop formulations, which are rapidly cleared from corneal and conjunctival surfaces, colloidal drug carriers can achieve longer residence time, allowing increased drug availability locally at the surface or inside the eye. After administration of a nanoparticle formulation to the eye the particles remain at the site of application and drug release starts by particle degradation or erosion, or diffusion depending on the biodegradable or inert nature of the polymer. Even longer residence times in the conjunctiva can be reached if the nanoparticles are coated with a mucoadhesive polymer.

Eudragit polymer nanoparticle suspensions have been reported to represent a valid carrier system for the ophthalmic release of non-steroidal anti-inflammatory drugs such as ibuprofen and flurbiprofen (Bucolo et al 2002; Pignatello et al 2002a, b). Such drug-loaded carrier systems showed good stability properties and size distribution, and a positive surface charge, which makes them potential ocular drug delivery systems. In particular, the positive surface charge (ζ-potential) of these nanoparticles can allow a

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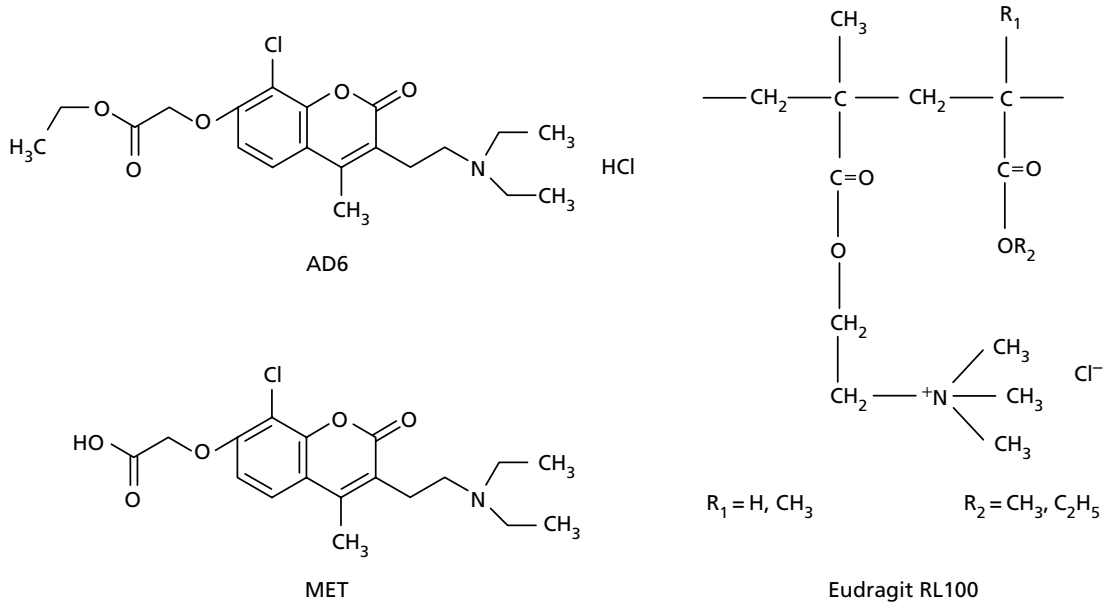

Figure 1 General structure of cloricromene (AD6), its acid metabolite (MET) and Eudragit RL100.

prolonged residence time on the corneal surface, ensuring slow drug release and higher aqueous humor drug concentrations compared with traditional aqueous eye drop formulations.

Cloricromene (8-mono-chloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy-carbonyl-methoxy coumarin hydrochloride: AD6) (Figure 1) is a synthetic coumarin derivative, which possesses antithrombotic and antiplatelet actions, inhibits polymorphonuclear neutrophil function, and causes vasodilatation (Squadrito et al 1991). Some of these properties have been ascribed to the inhibition of arachidonate release from phospholipid membranes. In fact, AD6 reduces the synthesis of the products of both cyclooxygenase and lipooxygenase pathways (Galli et al 1980; Porcellati et al 1990). This drug exerts protective effects in several models of circulatory shock, also inhibiting the induction of inducible nitric oxide synthase.

AD6 has recently been proposed for ocular applications, such as the treatment of uveitis. Bucolo et al (2003) showed that AD6 attenuates the degree of inflammation and tissue damage associated with endotoxin-induced uveitis in the rabbit eye, and protects against experimental rat uveitis, reducing the expression of adhesion molecules such as P-selectin and intercellular adhesion molecule 1. Furthermore, AD6 strongly inhibited cell infiltration, protein exudation, TNF-α production and nitrite/nitrate formation (Bucolo et al 2003).

After its administration, AD6 is extensively hydrolysed in the bloodstream and tissues to its active acid form (MET) (Figure 1). In platelets and leukocytes, AD6 is taken up as an ester and converted to its acid metabolite (Travagli et al 1989). Considering that the ester form of the drug is quite unstable in aqueous solution, forming the insoluble acid form, a delivery system able to ensure a slow and prolonged release of this agent at the intraocular level could improve the therapeutic effects of AD6.

The aim of the present study was to investigate the feasibility of this polymer matrix for the preparation of an AD6 ophthalmic drug delivery system. Eudragit RL100 nanoparticles loaded with cloricromene were prepared and characterized. The biopharmaceutical profile of the drug, as well as the time-dependent concentration in the aqueous humor after a single application of the nanoparticle or a reference drug aqueous solution in the rabbit eye was assessed. The ocular tolerability of the new formulation was also evaluated.

Materials and Methods

Animals

Male New Zealand albino rabbits (Charles River, Calco, Italy), 2.0–2.2 kg, free of any sign of ocular inflammation or gross abnormality were used. The animals were maintained on a 12-h light–dark cycle at constant room temperature and humidity. All experiments conformed to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research, and the Animal Care and Use Committee of the University of Catania approved protocols.

Materials

Eudragit RL100 polymer (Röhm) was a kind gift from Rofarma Italia S.r.l. (Gaggiano, Milan, Italy). AD6 and MET were obtained from Bausch & Lomb Oftal (Catania, Italy). All the other reactants were of analytical grade or higher.

Preparation of the nanoparticles

Drug-loaded RL nanoparticle formulations were prepared as previously described (Pignatello et al 2002a, b) using an adaptation of the quasi-emulsion solvent-diffusion method originally described by Kawashima et al (1989). Briefly, a total amount of 100 mg of drug and polymer, in a 1:1 drug to polymer weight ratio, was co-dissolved in 2 mL of ethanol at room temperature. The solution was then slowly injected (0.5 mL min^{-1}) into 50 mL of sterile distilled water, containing 0.02% (w/v) Tween 80 (as a hydrophilic emulsifier) and 0.1% (w/v) benzalkonium chloride, under constant high-rate stirring (24 000 rev min^{-1}; Ultra Turrax T25, IKA, Germany), in a cylindrical vessel maintained at low temperature by means of an iced-water bath. Stirring was kept at the same rate for 15 min and then reduced to about 300 rev min^{-1} for 8–12 h at room temperature to allow complete evaporation of the solvent. The suspension was stored in a screw-top amber glass container in the refrigerator (+4°C) until use.

In-vitro drug release

Drug release from the nanoparticles was evaluated in triplicate over 4 h by a dialysis system consisting of a Spectrapor membrane (cut off: 3500 Da), loaded with 5 mL of nanoparticles and soaked in isotonic phosphate-buffered saline (PBS, pH 7.4), at room temperature and under slow magnetic stirring. At regular time intervals, 1-mL samples of the external medium were withdrawn and immediately replaced with the same volume of fresh buffer.

To determine the eventual limiting effects of the dialysis membrane on drug dissolution, separate experiments were run in duplicate with a solution in the same PBS buffer of pure AD6 at the same drug concentration as present in the polymeric system. Similarly, an aqueous solution of the powdered drug (250-μm sieve) was loaded into the dialysis bag and the test was carried out (in duplicate) as usual. In both cases, only a limited delay in drug dissolution into the receiving medium was observed (Figure 2). The amount of drug released was determined by UV analysis at $\lambda = 321$ nm (Shimadzu UV-1601; Shimadzu Italy srl, Milan, Italy).

Size analysis and ζ-potential measurement

Mean particle size was determined by means of photocorrelation spectroscopy using a Zetamaster connected to a personal computer working with the photocorrelation spectroscopy software (version 1.27) for data collection and elaboration (Malvern Instruments Ltd, Worcs, UK). A solid-state laser was used as the light source, with a nominal power of 4.5 mW and a maximum output of 5 mW at 670 nm. The photon correlation spectroscopy measurements were carried out at a scattering angle of 90°. As a correlation function, a third-order cumulant fitting with a dilation of 1.20 was applied to obtain mean particle diameter and polydispersity. Samples were suitably diluted with high-performance liquid chromatography (HPLC)-grade water and placed in quartz cuvettes. At least 3×10 measurements per sample were performed.

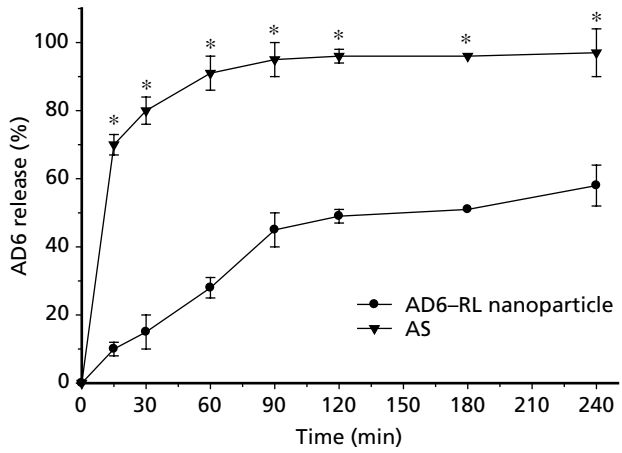

Figure 2 In-vitro dissolution pattern of cloricromene (AD6) from a Eudragit RL100 nanoparticle suspension and a drug aqueous solution (AS). Data are presented as mean ± s.d., n = 4. *$P < 0.01$ compared with nanoparticles (Student's t-test).

Electrophoretic mobility and ζ-potential distribution were measured with the Zetamaster particle electrophoresis analyser set-up, equipped with a 5-mW HeNe laser (633 nm). Parameters were set as follows: strobe delay −1.00, on time 200.00, off time 1.00. A Smoluchowsky constant F(Ka) of 1.5 was used to achieve ζ-potential values from the electrophoretic mobility data. A suitable amount of the sample (50–100 μL) was diluted to 5 mL with HPLC-grade water, filtered through a 0.45 polyamide filter and injected into the electrophoretic cell of the instrument where a ±150-mV potential was present.

Aqueous humor collection

Drug formulations (the polymer nanoparticles at a 0.1% w/v AD6 concentration, pH 7.2, 320 mOsm L^{-1}, or a 0.1% w/v AD6 solution in 0.1 M PBS, pH 7.4, 300 mOsm L^{-1}) were instilled (50 μL) in the conjunctival sac of rabbit eye and aqueous humor was collected after 30, 60, 120 and 240 min. The rabbits were anaesthetized by an intramuscular injection of 35 mg kg^{-1} ketamine hydrochloride and 5 mg kg^{-1} xylazine hydrochloride (RBI, Milan, Italy). Aqueous humor was collected by a 26-G needle attached to a tuberculine syringe. The needle was introduced into the anterior chamber of the eye through the cornea and about 150 μL aqueous humor was withdrawn. All samples were stored at −20°C until HPLC analysis.

Sample preparation and HPLC assay

AD6 and MET concentrations in rabbit aqueous humor were measured by HPLC under the conditions previously developed and validated in our laboratory (Maltese & Bucolo 2002). Briefly, a C_{18} reversed-phase column (Hypersil ODS) with UV detection at 318 nm was used. The mobile phase consisted of acetonitrile–water containing 1% triethylamine, pH 3.5 adjusted with orthophosphoric

acid. An acetonitrile gradient was necessary to achieve a good separation within 13 min.

Aqueous humor (100 μL) was treated with an equivalent volume of 0.6% perchloric acid in CH_3CN in order to precipitate proteins. The sample was vortex-mixed for 1 min and centrifuged at 12 200 g for 5 min. The supernatant was filtered through a 4-mm HPLC syringe filter with 0.2-μm nylon membrane (Alltech, Milan, Italy) and injected onto the HPLC system.

The recoveries of AD6 and MET from aqueous humor (all over the range 98–103%) were determined by comparing the peak areas obtained from the direct injection of standard solutions of compounds with those found by extraction from spiked aqueous humor.

Pharmacokinetic analysis

AD6 and MET concentrations in aqueous humor were plotted against time after instillation. Peak drug concentration (C_{max}), time to peak value (T_{max}) and area under the concentration–time curve (AUC) were obtained. Graphs and data were processed by SigmaPlot 2000 software (SPSS Inc., Chicago, IL, USA). The AUC was calculated by the trapezoidal rule.

Ocular tolerability

The potential ocular irritancy or damaging effects of the formulation were evaluated according to a modified Draize test (McDonald & Shadduck 1977) using a slit-lamp (model no. 4179-T; Sbisà, Florence, Italy). Observation of the ocular tissue condition was performed after 10 min, 6 h and 24 h after the end of the last instillation. The formulations (RL nanosuspension or a drug aqueous solution) were instilled into the right eye every 30 min for 6 h (12 treatments). The congestion, swelling and discharge of the conjunctiva were graded on a scale from 0 to 3, 0 to 4 and 0 to 3, respectively. Iris hyperaemia and corneal opacity were graded on a scale from 0 to 4. The mean values from five or six treated eyes were used for each formulation.

Statistical analysis

Data are expressed as mean ± s.d. Statistical comparisons were made by the Student's t-test or Mann-Whitney U-test used for interval and ordinal variables, respectively. Statistical significance was accepted at a level of $P < 0.05$.

Results

By using the quasi-emulsion solvent-diffusion technique we obtained AD6-loaded RL nanoparticles with a mean particle size of approximately 80 nm (n = 5). The relatively high registered polydispersity index (0.719) was owing to the presence of two particle populations, one with a mean size of 62.9 nm (86.5% of the particles) and the other one with a mean size of 201.4 nm (11.7%). This size remained constant for at least 8 months after preparation while the formulation was stored in a refrigerator (+4°C). The nanoparticles had a surface charge (ζ-potential) of +27.3 ± 1.4 mV, close to the value shown by blank RL nanoparticles (Pignatello et al 2002a), which was also maintained during storage (up to 8 months). Moreover, differential scanning calorimetry experiments carried out on freeze-dried nanoparticles indicated that the drug is uniformly dispersed within the polymer matrix, since no endothermic peak associated with drug crystal fusion was observed (unpublished results). In fact, this nanoparticle formulation belongs to a wider set of nanosuspensions in which AD6 and MET were loaded into Eudragit RS100 and RL100 nanoparticles (unpublished results). We directed our attention to the 1:1 AD6–RL system used in the present study for the in-vivo assays because of its properties and, given the amount of loaded drug, the formulation did not require any dilution before in-vivo application, thus reducing possible changes in morphological and physicochemical properties (e.g. pH and osmolarity).

Figure 2 shows the dissolution profile of the drug from the prepared nanoparticle formulation. The dispersion of AD6 inside the RL matrix allowed the modulation and slowing down of drug dissolution compared with the pure drug. A biphasic profile was observed, with faster drug leakage in the first 90 min of the test, followed by an almost linear dissolution profile with a significant amount of the initially loaded drug released after 4 h. Such behaviour could be related to the small particle size, which allows rapid diffusion of the dispersed drug into the external medium. However, the absence of a real burst phase in the first minutes of the test indicates that no drug remained on the outer surface of particles during the nanoparticle formation.

In a separate study, the chemical stability of AD6 after dispersion in the RL matrix was evaluated with the aim of optimizing such formulations (Pignatello et al 2004). The results suggested that the hydrolytic degradation of AD6 was reduced in the nanoparticles prepared with water or saline.

After the application of the AD6-loaded RL nanoparticle formulation (50% drug w/w) or the reference aqueous solution, the determination of the concentration of drugs in aqueous humor gave interesting results. The time courses of AD6 and MET concentrations in the aqueous humor following instillation of the aqueous solution formulation and of the RL nanoparticle formulation are shown in Figure 3.

The key parameters of the concentration–time curves (C_{max}, T_{max} and AUC_{0-240}) are shown in Table 1. Substantial concentrations of MET were found in the aqueous humor at each time interval, whereas no relevant amount of the AD6 ester was detected after administration of either the aqueous solution or the RL nanoparticle system, indicating rapid conversion of AD6 to the active acid form MET. The amount of MET in the aqueous humor was significantly different after the administration of the two dosage forms. The nanoparticle formulation ensured a much higher concentration of MET compared with the aqueous solution, showing a similar T_{max} value (60 min) but with a 1.7-fold increase ($P < 0.01$) of the C_{max} values

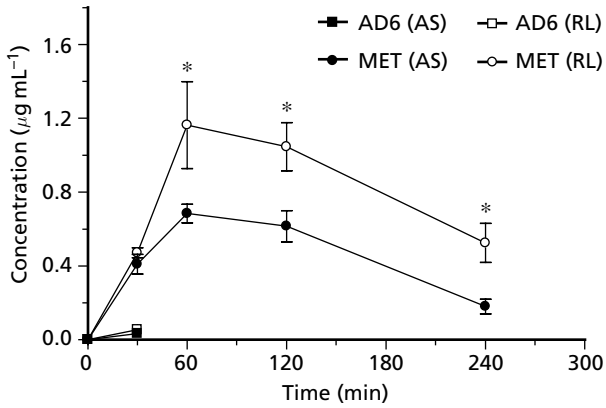

Figure 3 Concentration–time profiles of cloricromene (AD6) and its acid metabolite (MET) in rabbit aqueous humor after the application of the Eudragit RL100 nanoparticle suspension (RL) or a drug aqueous solution (AS). Data are presented as mean ± s.d., n = 5–6. *$P < 0.01$ compared with AS (Student's *t*-test).

($0.68 \pm 0.07\,\mu g\,mL^{-1}$ and $1.16 \pm 0.33\,\mu g\,mL^{-1}$ for aqueous solution and RL nanoparticle formulations, respectively) and AUC_{0-240} values ($107.7 \pm 10.32\,\mu g\,min\,mL^{-1}$ and $190.85 \pm 24.11\,\mu g\,min\,mL^{-1}$ for aqueous solution and RL nanoparticle formulations, respectively).

Topical application of the new formulation based on Eudragit nanoparticles to rabbit eyes showed no sign of toxicity or irritation to ocular tissues. Using a modified Draize test approach (McDonald & Shadduck 1977) to quantify the damage caused on eye structures, scores were equal to zero for all the examined parameters. These findings confirm the absence of ocular irritancy already shown by unloaded RS and RL nanoparticles (Pignatello et al 2002c).

Discussion

The present study demonstrated that cloricromene can be entrapped in a polymeric colloidal drug delivery system made up of Eudragit RL100, which can provide a sustained ocular drug release and an increase in the cloricromene concentrations in aqueous humor. The Eudragit RL100 nanoparticle system loaded with cloricromene showed interesting size and surface charge values, adequate for ophthalmic administration. In-vivo evaluation showed that the polymer system is able to enhance the concentration of the active drug in the aqueous humor, improving the bioavailability of the drug compared with the conventional aqueous drug solution.

The results obtained in the present study can be attributed to the increased corneal residence time of the polymer nanoparticles owing to the positive ζ-potential. Furthermore, after instillation of the aqueous solution formulation there was faster drug drainage from the cornea surface compared with the nanoparticle formulation. This latter system retained the drug for longer (Figure 2) and the amount progressively released from the nanoparticles was immediately and completely available for transcorneal absorption.

These findings are similar to our previous observations with ibuprofen and flurbiprofen (Pignatello et al 2002a, b) and indicate that the Eudragit RL100 matrix is a valid means of enhancing the bioavailability of drugs at the ocular level, modulating their local release.

Rapid drug removal from the cornea was obtained when AD6 was formulated in the aqueous solution. Conversely, when the drug was dispersed inside the polymer nanoparticles, the precorneal residence time was increased and the drug was retained in the polymer matrix for longer times and progressively released.

This carrier system showed interesting stability properties, particularly in terms of size and positive surface charge. The in-vivo pharmacokinetic profile results on cloricromene bioavailability as well as the ocular carrier tolerability prompted us to use cloricromene nanoparticles as a potential ophthalmic dosage delivery system for the treatment of ocular diseases. Further work is in progress to assess the pharmacological activity of this drug at the ocular level after inclusion in these polymer nanoparticles.

In conclusion, the present study demonstrated that cloricromene can be delivered by means of Eudragit RL100 nanoparticles, improving the ocular bioavailability of the drug. The findings suggest that the new nanoparticle system loaded with cloricromene is safe in the rabbit eye and may be used as a potential ocular drug delivery system.

Table 1 Pharmacokinetic parameters for aqueous humor after the application of a cloricromene (AD6)-loaded Eudragit RL100 nanoparticle suspension (RL) or a drug aqueous solution (AS)

Parameter	AD6		MET	
	AS	RL	AS	RL
T_{max} (min)	30	30	60	60
C_{max} ($\mu g\,mL^{-1}$)	0.031 ± 0.010	0.046 ± 0.015	0.68 ± 0.07	1.16 ± 0.33*
AUC_{0-240} ($\mu g\,min\,mL^{-1}$)	n.d.	n.d.	107.70 ± 10.32	190.85 ± 24.11*

T_{max}, time to peak value; C_{max}, peak drug concentration; AUC_{0-240}, area under the concentration–time curve between 0 and 240 min; n.d., not detectable. Values are presented as mean ± s.d., n = 5–6. *$P < 0.01$ compared with AS (Student's *t*-test).

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