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Improved Ocular Bioavailability of Indomethacin by Novel Ocular Drug Carriers

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Abstract

The ability of different drug carriers to improve the ocular bioavailability of drugs was investigated in the rabbit eye. The assayed drug carriers were suspensions of nanoparticles, nanocapsules and microparticles made of poly-ε-caprolactone (PECL) and a submicron emulsion.

Results indicated that the three submicron systems, nanoparticles, nanocapsules and emulsion, increased more than 3-fold the indomethacin concentration in the cornea, aqueous humour and iris-ciliary body at 0·5 and 1 h post-instillation. Furthermore, an increased indomethacin ocular bioavailability of 300% was observed after instillation of the submicron systems in comparison with the value obtained for a commercial solution. In contrast, the microparticles hardly increased the ocular bioavailability of indomethacin. The mechanism of interaction of the colloidal carriers with the corneal epithelium was investigated by confocal laser scanning microscopy. Confocal images indicated that submicron particles penetrate into the corneal epithelium cells by an endocytic mechanism. The similar behaviour of the three colloidal carriers suggests that any of their specific ingredients (PECL, lecithin and oil) acts as a penetration enhancer or an endocytotic stimulator. On the other hand, the favourable ocular penetration of indomethacin when encapsulated in the colloidal carriers, but not in the microparticles, led us to assume that the colloidal nature of these carriers is the main factor responsible for the increased ocular bioavailability of indomethacin.

PECL nanoparticles and nanocapsules as well as submicron emulsions are shown to be novel corneal drug carriers, thus representing a useful approach for increasing the ocular bioavailability of drugs.

Improving the poor bioavailability of topically applied ophthalmic drugs is a major challenge in ocular therapeutics. Such poor bioavailability, amounting to at best 10% of the applied dose, is largely due to precorneal clearance processes and to the structure of the cornea, which restricts the passage of drug molecules. Over the past two decades, considerable effort has been devoted to the development of new ocular drug delivery systems to enhance the bioavailability of drugs applied topically to the eye (Lee & Robinson 1986). Among these new ocular systems, the colloidal polymeric drug carriers, nanoparticles and nanocapsules, have received the greatest attention. A common feature of these systems is that their size is less than 1 μm, and one main difference is that nanoparticles are solid structures made of polymer whereas nanocapsules are oily globules coated with a polymer. Certainly, the polyalkylcyanoacrylate (PACA) nanoparticles and nanocapsules have been shown to improve the corneal penetration of hydrophilic and lipophilic drugs (Diepold et al 1989; Marchal-Heussler et al 1990; Losa et al 1991). However, the present potential of the PACA carriers is limited due to the observed fact that they cause a disruption of the corneal epithelium cells membrane (Zimmer et al 1991). More recently, the capacity of poly-ε-caprolactone (PECL) nanocapsules of increasing the ocular penetration of lipophilic drugs while reducing the systemic absorption was reported for drugs such as metipranolol (Losa et al 1993) and betaxolol (Marchal-Heussler et al 1992). An important advantage of these nanocapsules is that, as observed by confocal fluorescence microscopy, they are taken up by corneal epithelium cells without causing damage to the cell membrane (Calvo et al 1994).

In spite of these promising observations, the factor responsible for the positive behaviour of PECL nanocapsules is not yet clear. In an attempt to elucidate this aspect, in the present work, we have compared the ability of different carriers to improve the ocular bioavailability of drugs. With this purpose in mind, we developed different drug carriers: nanoparticles, nanocapsules and microparticles made of PECL, and a submicron emulsion, all containing indomethacin. This drug was chosen because of its favourable lipophilic character. Also, indomethacin is a potent anti-inflammatory drug that inhibits the synthesis of prostaglandins from arachidonic acid and it is known that this drug is effective in decreasing the intraocular irritation after cataract extraction (Sawa & Masuda 1976) and in cystoid macular oedema (Miyake et al 1978). However, the clinical use of indomethacin is limited due to its topical side-effects, including a burning sensation and epithelial keratitis. Therefore, the encapsulation of indomethacin into a drug carrier, which would increase its ocular bioavailability, would theoretically be of great benefit to reduce the dose and, consequently, the local side-effects inherent to this drug.

To summarize, the two main goals of the present work have been: firstly, to compare the in-vivo behaviour of several drug carriers (colloidal carriers and microparticles) and to determine their capacity to improve the ocular bioavailability of indo-

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methacin and secondly, to investigate the mechanism of interaction of the colloidal drug carriers with the corneal epithelium.

Materials and Methods

Chemicals and animals

The polymer poly-ε-caprolactone (PECL) (MW: 40 kDa) was purchased from Aldrich Chemical (Germany) and was used without further purification. The oil Migliol 840 was generously supplied by Lemmel (Barcelona, Spain). Poloxamer 188 (Synperonic F68) was a gift from ICI (Spain). The phospholipid mixture (soybean L-α-lecithin 40% phosphatidilcholine), polyvinylalcohol (MW 10-30 kDa) (PVA) and rhodamine 6G were supplied by Sigma Chemical (St Louis, MO, USA). Indomethacin was obtained as a gift from Laboratorios Cusi S.A. (El Masnou, Spain). [^{14}C]Indomethacin with a specific activity of 1·4 GBq mmol^{-1} (37·9 mCi mmol^{-1}) was purchased from DuPont NEN (Germany). Tissue solubilizer (BTS-450) and liquid scintillation fluids (Ready Organic and Ready Safe) were purchased from Beckman (Fullerton, CA, USA). Indocollyre was a gift from Laboratoires Chauvin, France. All other chemicals were reagent grade.

Male albino New Zealand rabbits, 2·5 and 3·0 kg, were used in the in-vivo cornea penetration study. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed.

Preparation and physicochemical characterization of the drug carrier systems

Nanocapsules, nanoparticles and submicron emulsions were prepared by interfacial deposition (Fessi et al 1988a), nanoprecipitation (Fessi et al 1988b) and spontaneous emulsification techniques (Yu et al 1993) respectively, as previously reported, with a few modifications. Briefly, 100 mg of polymer and 100 mg of lecithin were first dissolved in 25 mL acetone. Then, 0·5 mL of Migliol 840 oil containing 10 mg indomethacin were added to the acetone solution. This organic solution was poured, under moderate stirring, into 50 mL of aqueous phase containing 125 mg of poloxamer 188. The resulting mixed phase immediately turned milky with a bluish opalescence as a result of formation of the nanocapsules. The acetone was finally removed under reduced pressure and the colloidal aqueous suspension concentrated to the desired final volume (10 mL).

The submicron emulsion was prepared using the same procedure as the nanocapsules, omitting the polymer, and nanoparticles were prepared omitting the oil and lecithin. In the latter case, indomethacin was first dissolved in 0·5 mL of methylene chloride and added to the acetonic polymer solution.

Indomethacin-loaded PECL microparticles were prepared by a solvent evaporation method previously described (Julienne et al 1992). One hundred mg of PECL, 10 mg of indomethacin and 50 mg of the phospholipid mixture were dissolved in 1·25 mL of methylene chloride. This organic solution was then emulsified in 12·5 mL of an aqueous solution of PVA (10% w/v) using a stirring motor with a stainless-steel propeller (Ika RW20 DZM, Ika-Werk, Germany) for 5 min at 2500 rev min^{-1}. Finally, the solvent was evaporated under reduced pressure at 20°C. The resulting suspension was washed by centrifugation at 10 000 *g* for 30 min. The isolated particles were resuspended in 10 mL of an aqueous solution of poloxamer 188 (1·25% w/v).

The [^{14}C]indomethacin-loaded nanocapsules, nanoparticles, microparticles and submicron emulsion were used for the in-vivo indomethacin ocular distribution studies. These drug carrier systems were prepared by adding [^{14}C]indomethacin to the organic solution of indomethacin to attain an activity value of 50 μCi mL^{-1}. All suspensions were made isotonic with glucose (5% w/v).

Rhodamine 6G-loaded colloidal systems were used for the in-vitro study of the interaction of the colloidal particles with the corneal epithelium using laser scanning confocal microscopy. These colloidal systems were prepared as described above but by adding 50 μg of rhodamine 6G instead of indomethacin to the acetonic solution.

The mean particle size and size distribution of colloidal suspensions and microparticles were determined by photon correlation spectroscopy (PCS) and using the Coulter Counter respectively. The zeta potential values were calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry (LDA). The PCS and LDA analyses were performed using a Zetasizer III (Malvern Instruments, Great Britain).

The amount of indomethacin or rhodamine 6G encapsulated into the drug carriers was calculated by the difference between the total amount used to prepare the indomethacin or rhodamine 6G-loaded carriers and the amount of the free molecule present in the aqueous phase. For the colloidal systems, the aqueous phase was obtained following the separation of particles by an ultrafiltration-centrifugation technique (Ultrafree-MC 30 000 MW, Millipore) at 3000 g and for 15 min. The amount of free indomethacin or rhodamine 6G was determined by spectrofluorimetry.

The in-vitro release kinetics of indomethacin from the carriers were determined by a bulk-equilibrium reverse dialysis technique, as previously described (Calvo et al 1996).

In-vivo studies of indomethacin ocular distribution

A volume of 25 μL of [^{14}C]indomethacin-loaded carrier systems or [^{14}C]indomethacin control solution (Indocollyre) were administered to the cul-de-sac of the right eye of fully-awake New Zealand rabbits. After instillation animals were maintained in an upright position using restraining boxes. After 30 min, 1, 2 and 4 h rabbits were killed with an intravenous injection of an overdose of sodium pentobarbital given via a marginal ear vein. The eyes were proptosed, rinsed with normal saline, blotted dry in order to remove any adhering drug. Then, the aqueous humour was withdrawn from the anterior chamber with the aid of a 25-gauge needle fitted to a tuberculin syringe. Cornea and iris-ciliary body were subsequently dissected in situ. Preliminary studies produced undetectable levels of indomethacin in the lens and hence lens samples were generally not collected during these experiments. Each tissue was rinsed with normal saline, blotted dry and transferred to preweighed counting vials. The vials were reweighed and the weight of the tissues was calculated. The tissues were digested at 35°C until completely dissolved in 1 mL tissue solubilizer (BTS 450) and decolourized by adding 50 μL hydrogen peroxide. Ten mL of Ready Organic scintillation cocktail and 30 μL acetic acid were added to each vial. The aqueous

humour samples (100 μL) were dissolved directly in 10 mL of Ready Safe.

The samples were dark-adapted for at least 24 h in order to minimize chemiluminescence before counting in a liquid scintillation counter equipped with automatic quench correction (LS 6000 LL, Beckman Instrument, Fullerton, CA USA) for 3 min.

Data analysis

Areas under the curves (AUC 0–4 h) of indomethacin concentration in cornea and aqueous humour were calculated using the trapezoidal method. The maximum indomethacin concentrations (C_{max}) in the cornea and aqueous humour were determined from actual data points. The statistical significance of the differences was tested by analysis of variance and by a non parametric test (Kruskal-Wallis test).

In-vitro study of the interaction of the colloidal carriers with corneal epithelium by laser scanning confocal microscopy

Corneas were obtained from albino rabbits, 2·5–3·0 kg. The rabbits were killed by a marginal ear-vein injection of an overdose of sodium pentobarbital. The animal eye was proptosed within 1 h after death and a small transverse incision was made about 5 mm from the limbus and subsequently the cornea with the scleral ring was carefully cut out. Using forceps, first the lens and then the iris were carefully removed leaving the cornea as a transparent film. The cornea with the scleral ring was then mounted between two half-cells of a side-by-side perfusion apparatus which maintained corneal curvature. Glutathione-bicarbonate Ringer's solution (GBR), preajusted to pH 7·65 and at a temperature 37°C, was added first into the endothelial side (3 mL) to prevent the cornea from buckling and then the same volume (3 mL) was added to the epithelial side. A mixture of O_2 and CO_2 (95%: 5%) was bubbled in both compartments at a rate of three to five bubbles s^{-1} to provide a mixing and constant solution pH. Once the system was equilibrated, the content of the epithelial compartment was withdrawn and substituted by the rhodamine 6G-loaded colloidal systems diluted with GBR solution (2 mL of colloidal suspension or control solution plus 1 mL of GBR buffer). The contact of the encapsulated rhodamine 6G with the corneas was maintained for 2 h. After this, corneal specimens were directly mounted, epithelial side up, on a glass slide and examined by laser scanning confocal microscopy (MRC-600, Bio-Rad, Cambridge, MA, USA) without additional tissue processing.

Results

Physicochemical characterization of indomethacin carriers

Table 1 shows the mean particle size, the zeta potential and the indomethacin loading efficiency obtained for the three colloidal carriers, nanoparticles, nanocapsules and submicron emulsion, and for the microparticles. The mean size of the colloidal carriers appeared to be homogenous within the range of 0·20–0·25 μm and no statistically significant differences (analysis of variance 95% level) were observed between them. The mean size of the indomethacin-loaded microparticles was 6 μm. The zeta potential was affected by the composition of the carrier.

Nanocapsules and submicron emulsions exhibited a higher negative charge than nanoparticles or microparticles. Table 1 also shows that the loading efficiency, defined as the percentage of indomethacin encapsulated, was extremely high (more than 90% of indomethacin was encapsulated) irrespective of carrier composition. The in-vitro release of indomethacin from the systems was rapid and complete in approximately 4 h (Calvo et al 1996).

In-vivo indomethacin distribution in rabbit eye

The indomethacin concentrations attained in the cornea, aqueous humour and iris-ciliary body, at different times, following topical instillation of the indomethacin carriers and the control solution (Indocollyre), are shown in Figs 1, 2 and 3, respectively. Regardless of the preparation instilled, the highest concentration of indomethacin was achieved in the cornea, followed by the aqueous humour and the iris-ciliary body. However, the most remarkable fact from these results is that the three colloidal formulations (nanoparticles, nanocapsules and submicron emulsions) provided higher drug levels than the control solution and the microparticles. As shown in Fig. 1, at 30 and 60 min post-instillation, the concentration of indomethacin in the cornea was approximately 4 times higher for the colloidal systems than for the Indocollyre. Similar results were obtained in the aqueous humour and iris-ciliary body (Figs 2 and 3). These differences were statistically significant (analysis of variance, 95% level) during the first 2 h. On the other hand, microparticles increased the indomethacin concentration in the cornea and aqueous humour with respect to the control solution to a much lesser extent than the colloidal systems. In fact, most of these values were significantly lower than those observed for the colloidal carriers. Finally, no significant differences were observed in the drug levels attained in cornea, aqueous humour and iris-ciliary body following the administration of the three colloidal systems.

Table 1. Particle size, zeta potential and percentage of indomethacin encapsulated in the submicron emulsion and in the PECL nanocapsules, nanoparticles and microparticles.

Formulation	Particle size (μm)	Zeta potential (mV)	% Indomethacin encapsulated
Nanocapsules	0·23 ± 0·02	−41·94 ± 8·65	94·46 ± 4·30
Emulsion	0·21 ± 0·02	−42·32 ± 11·50	89·96 ± 0·84
Nanoparticles	0·21 ± 0·04	−16·33 ± 2·21	95·42 ± 2·21
Microparticles	6·5 ± 2·86	−12·74 ± 4·25	87·33 ± 3·6

Data shown are the mean and standard deviation (n = 6–10).

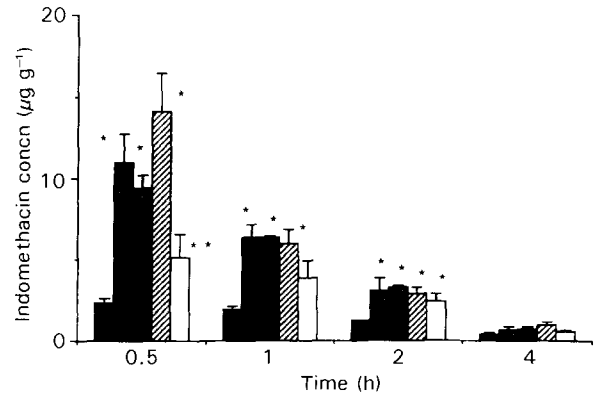

FIG. 1. Indomethacin concentrations attained in the cornea following the topical application, in rabbits, of indomethacin-loaded carriers and control solution. ■ Indocollyre, ■ nanocapsules, ■ emulsion, ▨ nanoparticles, □ microparticles. Mean values ± s.d. (n = 3–4) are shown. * $P<0.05$ compared with indocollyre; ** $P<0.05$ compared with colloidal suspensions.

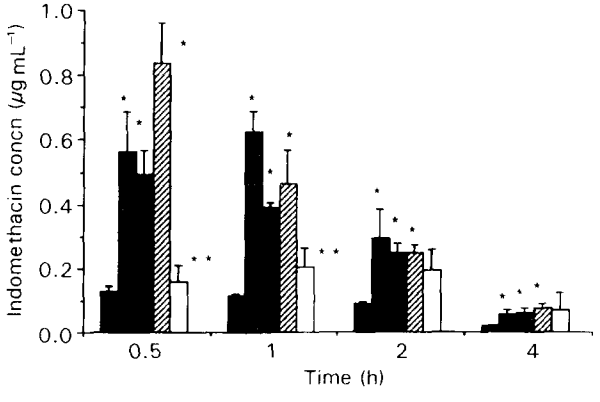

FIG. 2. Indomethacin concentrations attained in the aqueous humour following the topical application, in rabbits, of indomethacin-loaded carriers and control solution. ■ Indocollyre, ■ nanocapsules, ■ emulsion, ▨ nanoparticles, □ microparticles. Mean values ± s.d. (n = 3–4) are shown. * $P<0.05$ compared with indocollyre; ** $P<0.05$ compared with colloidal suspensions.

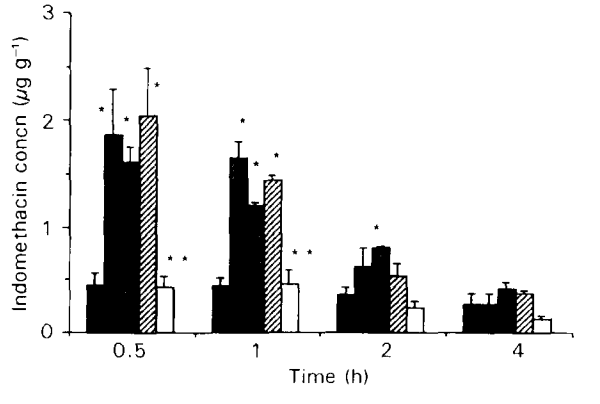

FIG. 3. Indomethacin concentrations attained in the iris-ciliary body following the topical application, in rabbits, of indomethacin-loaded carriers and control solution. ■ Indocollyre, ■ nanocapsules, ■ emulsion, ▨ nanoparticles, □ microparticles. Mean values ± s.d. (n = 3–4) are shown. * $P<0.05$ compared with indocollyre; ** $P<0.05$ compared with colloidal suspensions.

The pharmacokinetic parameters computed from the indomethacin levels in cornea and aqueous humour are summarized in Table 2. The values of the area-under-the curve for corneal concentration vs time (AUC 0–4 h), which indicates the amount of drug reaching the cornea from the lachrymal fluid, were significantly higher for the indomethacin-loaded colloidal systems and also for the microparticles as compared with the control solution. However, the extent of this increase was more pronounced for the colloidal systems than for the microparticles. The ocular bioavailability of indomethacin expressed as the amount and penetration rate of drug reaching the aqueous humour is illustrated by the parameters AUC and C_{max} and T_{max}. From the results shown in Fig. 2 it is possible to determine the AUC values, however the T_{max} values cannot be known precisely (<30 min). Nevertheless, since this time interval is very short, we could accept that the T_{max} is 30 min and, the indomethacin concentrations achieved at that time, could be considered as the C_{max} values. Taking these premises into account, it is possible to appreciate that, as indicated in Table 2, the colloidal formulations considerably increased the amount of indomethacin reaching the aqueous humour (the AUC values were 4 times, and the C_{max} values up to 7 times those corresponding to Indocollyre) although the penetration rate was not appreciably modified. In addition, the constants of elimination (k_{el}) of indomethacin from the cornea, which also illustrates the penetration rate, and from the aqueous humour (first-order elimination kinetics) were similar for all the formulations assayed. No statistically significant differences were observed when compared by a non-parametric test (Kruskal-Wallis 95% level).

In-vitro study of the mechanism of interaction of drug carriers with corneal epithelium by laser scanning confocal microscopy

To examine the interaction mechanism of the colloidal particles with the cornea, fluorescent colloidal systems were prepared. Rhodamine 6G was chosen as the fluorescent marker due to its affinity to the colloidal systems, thus avoiding the release of the marker during the experiment (Calvo et al 1994). The total encapsulation of the marker and the absence of release were necessary in order to assess that the fluorescence signal, which can be detected by confocal microscopy, is exclusively associated with the encapsulated marker. Fig. 4 shows a confocal image of the corneas incubated with rhodamine 6G-loaded nanoparticles. The confocal image displays a fluorescence signal in the form of numerous discrete endocellular granulations surrounding the cellular nucleus. These granulations have a submicron size and, therefore, can be solely attributed to the presence of nanoparticles inside the cells. In addition, it should be noted that the fluorescence signals do not appear in the intercellular junctions, a fact that suggests an endocytic mechanism of penetration. Results obtained for the submicron emulsion were very similar (not shown) and they correlate perfectly to those previously obtained for the PECL nanocapsules (Calvo et al 1994).

Discussion

From the results obtained in this study it can be deduced that the developed colloidal carriers, PECL nanoparticles, PECL nanocapsules and submicron emulsions improve the ocular bioavailability of indomethacin when compared with an aqu-

Table 2. Pharmacokinetic parameters of indomethacin concentrations in cornea and aqueous humour after topical ocular instillation of indomethacin-loaded carrier systems and control solution.

Tissue/formulation	AUC (μg min g $^{-1}$)	C_{max} (μg g $^{-1}$)	T_{max} (min)	k_{el} 10^{-3} (min $^{-1}$)	$t_{1/2}$ (min)
Cornea					
Indocollyre	292·31 ± 22·27	2·37 ± 0·21	30	8·16 ± 1·42	86·46 ± 13·69
Nanocapsules	932·56 ± 176·52[a]	10·94 ± 1·73[a]	30	9·16 ± 1·93	77·91 ± 16·28
Emulsion	907·41 ± 43·01[a]	9·37 ± 0·81[a]	30	10·15 ± 0·73	68·50 ± 4·99
Nanoparticles	1004·67 ± 70·81[a]	14·04 ± 2·34[a]	30	10·79 ± 0·35	64·25 ± 2·06
Microparticles	580·21 ± 147·48[b]	5·11 ± 1·50[b]	30	10·44 ± 1·03	66·78 ± 6·35
Aqueous humour					
Indocollyre	17·73 ± 1·68[d]	0·130 ± 0·013[e]	30	8·75 ± 0·91	79·44 ± 8·07
Nanocapsules	73·48 ± 21·19[a]	0·703 ± 0·12[a]	30	10·75 ± 1·84	65·64 ± 10·66
Emulsion	68·28 ± 6·11[a]	0·492 ± 0·076[a]	30	9·79 ± 2·34	74·03 ± 20·50
Nanoparticles	72·31 ± 9·69[a]	0·835 ± 0·125[a]	30	10·38 ± 0·68	68·51 ± 7·01
Microparticles	35·33 ± 17·77[c]	0·157 ± 0·052[c]	30	6·32 ± 5·28	95·76 ± 54·4

Data shown are the mean and standard deviation (n = 3–4). [a] Statistically significant differences from Indocollyre (analysis of variance 95% level); [b] statistically significant differences from Indocollyre and colloidal suspensions (analysis of variance 95% level); [c] statistically significant differences from colloidal suspensions (analysis of variance 95% level); [d] (μg min mL^{-1}); [e] (μg mL^{-1}).

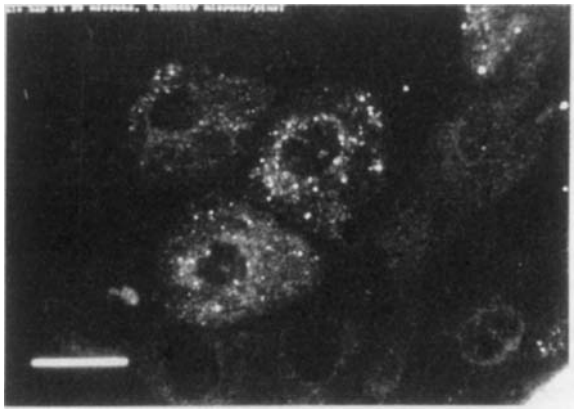

FIG. 4. Confocal fluorescence images at 5 μm from the surface of a cornea treated with rhodamine 6G-loaded PECL nanoparticles.

eous solution and with a suspension of microparticles. These findings are consistent with those observed previously by this research group for metipranolol-loaded nanocapsules (Losa et al 1993) and by other authors for betaxolol-loaded nanocapsules (Marchal-Heussler et al 1992). The final conclusion of the former workers was that nanocapsules promote corneal penetration of encapsulated drugs. Later, the enhanced penetration was explained by an endocytic uptake of nanocapsules by corneal epithelium cells (Calvo et al 1994). Despite these interesting data concerning the ocular behaviour of nanocapsules, to date, very little information has been reported on the interest of other colloidal systems, such as nanoparticles and submicron emulsions, as ocular drug carriers. Also, the reason why the nanocapsules are taken up by the endothelial cells has not been clearly understood yet. A few authors (Muchtar et al 1992; Naveh et al 1994) have reported interest in submicron emulsions for prolonging the response of antiglaucomatous drugs applied topically to rabbits. However, these authors did not study the corneal penetration of the drug included in a submicron emulsion in comparison to a reference formulation.

In a previous study we have shown that the positive behaviour of nanocapsules is common to nanoparticles and submicron emulsions (Calvo et al 1996). In addition, the fact that the three colloidal systems increased the ocular bioavailability of indomethacin to a similar extent indicates that neither the inner structure nor the specific composition of the colloidal carrier has a role in this positive behaviour. As a consequence, it could be stated that the main factor responsible for the increased ocular bioavailability of indomethacin associated with colloidal carriers is indeed their colloidal nature. This conclusion is also supported by the fact that microparticles increased the corneal penetration of indomethacin to a much lesser extent than any of the colloidal carriers. Furthermore, confocal scanning microscopy permitted examination of the interaction of the colloidal carriers with corneal epithelium and revealed that, independent of their composition, they are able to penetrate into the epithelial cells without causing damage to the cell membrane. This finding suggests that the incorporation of colloidal particles into the corneal epithelium is mediated by an endocytic mechanism. Consequently, there is important evidence that the colloidal nature of these carriers is the main factor responsible for the increased bioavailability of indomethacin.

Another primary conclusion from this study is that colloidal carriers, nanoparticles, nanocapsules and submicron emulsions, represent useful forms for improving the penetration of indomethacin to the inner part of the eye. These new carriers could allow the effective dose of indomethacin to be reduced for the treatment of intraocular inflammatory diseases.

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