Pharmacokinetic and Pharmacodynamic Aspects of an Ophthalmic Pilocarpine Nanoparticle-Delivery-System

Andreas Zimmer,[1] Ernst Mutschler,[2] Günter Lambrecht,[2] Dieter Mayer,[3] and Jörg Kreuter[1,4]

Received February 2, 1994; accepted May 10, 1994

The regional pharmacokinetics as well as the pharmacodynamics of pilocarpine-loaded nanoparticles for the treatment of glaucoma were investigated and compared to a solution of this drug. Polybutylcyanoacrylate nanoparticles were prepared by an emulsion polymerization process. Formulations with different drug concentrations (2–6%) as well as different particle concentrations were investigated and analyzed for size and drug loading. Drug binding to the particles was achieved at a level of 10–18% of the total drug content. The colloidal nanoparticles were sufficiently small (diameter: 100–300 nm) for a non-irritating application to the eye. All preparations were applied to the eyes of New Zealand white rabbits which were treated with betamethasone before to create an elevated intraocular pressure (IOP). Pilocarpine concentrations, assayed from aqueous humor using gaschromatography, increased by 23% (AUC) for nanoparticle suspensions compared to aqueous reference solutions. Additionally, $t_{1/2}$ was prolonged and the elimination coefficient was significantly decreased. Pharmacodynamic effects such as miosis and IOP reduction were investigated. t_{max} values of aqueous humor concentration were observed to be in a similar time range as miosis t_{max} readings. It was found that at lower drug contents a more pronounced prolongation of miosis was achieved with nanoparticles versus a standard solution. The IOP-reduction was significantly prolonged with nanoparticles preparations; whereas maximum reduction was obtained with a reference solution after 1–2 hours, it was reached with nanoparticles at about 2–3 hours. Differences between nanoparticles and aqueous solutions were most pronounced at lower drug concentrations.

KEY WORDS: pilocarpine; glaucoma; nanoparticles; betamethasone; miosis; intraocular pressure.

INTRODUCTION

Human glaucoma is one of the most common ophthalmic diseases occurring in 2% of the population older than 40 years (1). Pilocarpine is still used by most ophthalmologists to initiate glaucoma therapy. New drugs with the same pharmacological profile, such as carbachol and aceclidine are also employed for medication (2). In addition, other classes of drugs were introduced in glaucoma therapy. Alpha-receptor mediation of IOP is achieved with dipivalylepinephrine, adrenaline and clonidine. The β-agonist isoproterenol was first shown to lower IOP almost 40 years ago (3). L-Timolol, a non-selective β-antagonist, was introduced clinically in 1978. The same pharmacological class of drug is also represented by carteolol and metipranolol. Carbonic anhydratase inhibitors, especially acetazolamide and methazolamide have been given orally to lower IOP in man for 40 years.

Despite these available therapeutics, the treatment of glaucoma needs to be improved since topical aqueous ophthalmic preparations like pilocarpine eyedrops only achieve a bioavailability between 1 and 3% and must be applied frequently (4). Unfortunately in the case of pilocarpine the major part of the drug does not penetrate into the eye, but it is lost by physiological drainage. This effect is generally described as non-productive loss (5). This loss and, subsequently, a nasal or oral absorption of the drug becomes responsible for systemic side effects like salivation, lacrimation, sweating, and nausea setting the limits for ophthalmic treatments.

Recent investigations have shown the potential of polybutylcyanoacrylate (PBCA)-nanoparticles as ophthalmic drug delivery system (6) with improved drug action. Applications were demonstrated for antiglaucoma drugs e.g. pilocarpine (7,8). Despite the fact that a prolonged drug action and an antiglaucomatous effect was observed in different glaucoma models (9), the relation between the pharmacokinetic and the pharmacodynamic response provoked by pilocarpine administered with nanoparticle preparations remained unknown.

Therefore the goal of this study was to evaluate in animals with an artificially induced glaucoma using the betamethasone model (10): I. ophthalmic aqueous humor pharmacokinetics of pilocarpine nitrate delivered by nanoparticles at different drug and nanoparticle concentrations, II. pharmacodynamic responses such as miosis and IOP-reduction, and III. possible relations between pharmacokinetic and pharmacodynamic effects.

MATERIALS AND METHODS

Nanoparticle Preparation

The particles containing pilocarpine nitrate were prepared as described earlier (9,11). Briefly, an anionic emulsion-polymerization was performed in an aqueous solution of 50 ml 0.01 M HNO_3 (preparations see Table I). Pilocarpine nitrate and poloxamer 188 were dissolved completely. The solution was stirred with a magnetic stirrer at 300–500 rpm while 2-butyl-cyanoacrylate (BCA) was added slowly. Stirring was continued for 4 h, and the resulting nanoparticle suspension subsequently was buffered with 0.1 M NaOH to pH 6. In order to complete the reaction, stirring was maintained for 30 min. The suspension was purified by filtration through a G1 glass filter (Schott, Mainz, Germany), freeze dried (Lyovac GT 2, Leybold, Germany) and stored at 4°C in a refrigerator until use. Prior to application, the nanoparti-

[1] Institut für Pharmazeutische Technologie, Biozentrum Niederursel Universität Frankfurt a.M. Marie-Curie-Straße 9, D-60439 Frankfurt a.M. Germany.

[2] Pharmakologisches Institut für Naturwissenschaftler, Biozentrum Niederursel Universtität Frankfurt a.M. Marie-Curie-Straße 9, D-60439 Frankfurt a.M. Germany.

[3] Hoechst AG, Pharma Entwicklung, Zentrale Toxikologie, Kastengrund-Hattersheim, D-65795 Frankfurt a.M., Germany.

[4] To whom correspondence should be addressed.

0724-8741/94/1000-1435$07.00/0

cles (PBCA-nanoparticles) were resuspended in a aliquot of Michaelis-acetate-buffer, pH 5.45, by sonification (Bandelin RK106, Berlin, Germany) for 30 min.

Particle Size

The size of PBCA-nanoparticles was determined by photon correlation spectroscopy (PCS) (BI-90, Brookhaven Instruments Corporation, Holtsville, USA; Laser: 5 mW, HeNe). Samples were prepared as follows: the nanoparticle suspension was diluted with distilled water (HPLC grade, particle free filtered with a 0.2 μm filter, Millipore, Eschborn, Germany) between 1:100 and 1:1000 depending on the optimum count rate of the instrument. The samples were sonicated to minimize aggregation before taking the measurements (Bandelin RK106, Berlin, Germany).

Drug Loading

After preparation the nanoparticle suspensions were filtered with an Anotop 10, 0.02 μm, disposable syringe filter (Merck, Darmstadt, Germany). The filtrate was diluted with distilled water 1:100. The pilocarpine content in the particle-free filtrate was determined by UV-spectroscopy (Beckman DU-7, Fullerton, CA, USA). The measurements were carried out relative to a blank sample prepared by the same procedure without pilocarpine nitrate. The absorption at 215 nm was used to calculate the drug content. For quantification of the drug which was bound to nanoparticles the data were compared to measurements with reference solutions containing 2, 4 and 6% pilocarpine nitrate.

Betamethasone Model

For all in vivo experiments New Zealand white rabbits, age approximately 1/2 to 1 year, weight 3–4 kg (Hoechst AG, Frankfurt a.M., Zentrale Toxikologie Kastengrund, Germany) were used. The animals were placed in restrainer boxes and a local anaesthetising pretreatment followed by dosing 50 μl of a proxymetacain-HCl solution (5,5 mg/ml) (Ophthetic, Pharm-Allergan GmbH, Karlsruhe, Germany) into the left cul-de-sac. After 5 min, 0.4 to 0.8 ml of a betamethasone : disodium-betamethasone-21-dihydrogenphosphate = 4 : 1.32 mg/ml crystal suspension (Betnesol-Kristallsuspension, Glaxo GmbH, Bad Oldesloe, Germany) were injected under the left conjunctiva (subconjunctivally) of the elevated lower lid. A visible bubble of the suspension remained forming a depot. The treatments were repeated weekly over a period of three weeks until an elevated IOP was achieved (10). Animals which did not respond to the treatments were not used in further experiments. In some cases, according to the physical conditions of the rabbits, the injections were continued for an additional week to stabilize the IOP.

Miosis Measurements

New Zealand white rabbits as specified above were used for the miosis measurements. The pupillary diameter was measured by using a video system (Panasonic NV-M7, VHS-HQ, Matsushita, Japan) under standard lighting conditions (15–20 lux), while the animals were placed in restrainer boxes (Techni Plast, Italy). The focus of the camera was adjusted to the iris and a metric measure tape (1 cm) was mounted under the eye in the same optical section.

Measurements were performed with 8 to 10 animals. 25 μl of the nanoparticle preparations as well as the reference solutions were applied with an pipette into the everted conjunctival sack of the left eye and both lids were gently pressed together in order to minimize loss of the dosage form for a few seconds.

Pupil pictures were recorded after baseline readings were employed prior each series of measurements. The pupil size was measured using the still picture frame function of a video tape recorder (AKAI, VS-767, AKAI, Egelsbach, Germany) and was calculated in relation to the measure tape.

Aqueous Humor Sampling Procedure

Dosage forms were applied as outlined for the miosis measurements. For each preparation 4 to 6 animals were used. The animals were rapidly sacrificed by injection of 1 ml T61 (Hoechst AG, Frankfurt, a.M., Germany) into the left ear vein. The anterior chamber of the eye was punctured with a cannula (26G × 12mm Nr. 18, Terumo Europ-N.V., Belgium) and the aqueous humor was withdrawn with a syringe. In general, 200–300 μl liquid was obtained and the samples were stored in a freezer at −25°C until further analytics were performed.

Pilocarpine Determination

Due to sensitivity and selectivity, capillary gaschromatography (Hewlett Packard 5890 series II, Integrator: HP 3396A, HP-1 Methyl Silicone, 5 m × 0.53 × 2.65 mm film thickness, Hewlett Packard, Böblingen, Germany) equipped with a nitrogen selective detector (NPD) was used for the pilocarpine assay.

The aqueous humor samples were processed as follows: the frozen samples were thawed and divided in two aliquots each containing 100 μl aqueous humor. Each aliquot was filled into a 3 ml extraction vial (Alltech, München, Germany). To one aliquot 50 μl of an isopilocarpine solution (1 μg/ml isopilocarpine) was added as internal standard. 600 μl sodium hydrogen carbonate solution (30 mg/ml sodium hydrogen carbonate) and 1 ml dichlormethane were finally added. Analogously, to the second aliquot 50 μl distilled water was added instead of the 50 μl isopilocarpine solution. Both samples were extracted for 1 min by shaking and the organic phases were transferred each into a clean vial. Dichlormethane was evaporated under nitrogen at room temperature until aprox. 10 μl remained. From each vial 3 μl of the residual organic phase was injected splitless.

The specified GC method was developed for the pilocarpine assay (He carrier 3.5 ml/min, splitless inject., temp. 75–250°C, rate 18°C/min). As described above each sample was divided into two parts in order to subtract the aqueous humor isopilocarpine content from the amount that was added as internal standard.

IOP Measurements

Readings were performed using a pneuma tonometer as commonly used for human ophthalmic examinations (Digilab Modular One, Pneuma Tonometer, Bio-Rad Ophthalmic De-

vision, Cambridge, MA, USA). The first IOP measurements were recorded 4 days after the last subconjunctival injection. For each preparation 6 to 10 rabbits were used. The application of the dosage form was similar to those outlined for the miosis measurements. Topical anaesthetic pretreatment with 10 µl Ophthetic (5,5 mg/ml proxymetacain-HCl) was applied prior each measurement. After baseline recordings, the IOP was measured in order to determine the pressure lowering effect of the preparations between 1/2 and 7 1/2 h. The animals were used until the IOP was on a decent level, pausing at least one to two days between each series of measurements as a minimum wash out time interval and in order to avoid major corneal damages.

Data Analysis and Statistics

Generalized equations describing the pilocarpine time profile in the anterior chamber have been published by Makoid and Robinson (12). Taking into account that the concentration in the anterior chamber is initially zero, the aqueous humor drug concentration can be described by Eq. (1). The concentration C_a in the anterior chamber at time t is given by:

$$C_a = C_A (e^{-k_{el}t} - e^{-k_a t}) \quad (1)$$

where C_A is a constant which depends on the initial drug loading of the cornea. k_a and k_{el} are absorption and elimination coefficients. For this study, only the elimination coefficient k_{el} in Eq. (1) is of interest for differentiation between the pharmacokinetics of the used preparations. The elimination of pilocarpine from the anterior chamber can be described equivalent to the elimination part of Eq. (1). A semilogarithmic relation Eq. (2) was used to calculate the drug elimination constant k_{el},

$$C_a = C_A e^{-k_{el}t} \quad (2)$$

The half life ($t_{1/2}$) was calculated from k_{el}. In this study for all data the AUC was determined using the linear trapezoid rule. In the case of the miosis a zero order decrease was observed after attaining a maximal effect. The zero order decrease constant was calculated by linear regression. According to a previously published method $\Delta_{1/2}$ was defined to be the duration of the IOP-response at half the maximal response (I_{max}) level (13).

For statistical analysis the ANOVA method was used and for further statistical purposes comparing only two groups Students *t*-test (unpaired) was performed.

Chemicals

2-Butyl-cyanoacrylate (Sichel, Hanover, Germany), poloxamer 188 (Erbslöh, Düsseldorf-Hafen, Germany), pilocarpine nitrate (Merck, Darmstadt, Germany), 0.1 M NaOH (Merck, Darmstadt, Germany), acetic acid conc. (Merck, Darmstadt, Germany), sodium acetate × $3H_2O$ (Roth, Karlsruhe, Germany), dichlormethane (LiChrosolve, Merck, Darmstadt, Germany), methanol (Merck, Darmstadt, Germany), sodium hydrogen carbonate (Roth, Karlsruhe, Germany), pilocarpine nitrate (Merck, Darmstadt, Germany) and isopilocarpine nitrate (Aldrich-Chemie, Steinheim, Germany) were used as received from the suppliers. 0.01 M HNO_3 was prepared from 0.1 M HNO_3, (Merck, Darmstadt, Germany) by diluting with distilled water. Michaelis-acetate-buffer was produced by dissolution of 5.23 g sodium acetate × $3H_2O$ and 353.0 µl acetic acid conc. in distilled water to obtain a final volume of 1000.0 ml. Distilled water (HPLC grade) was prepared by a Milli-Q_{Plus} apparatus (Millipore, Eschborn, Germany).

RESULTS

Characterization of Nanoparticles

Size. In this study photon correlation spectroscopy was used for routine particle sizing. Table I summarizes the results obtained for pilocarpine loaded nanoparticles. The mean diameter of all nanoparticle preparations was within a size-range of about 180 to 280 nm. The differences were not statistically significant (ANOVA, 95% level).

Drug loading capacity. The highest drug loading of around 18% was found for the 2% NP/c (Table I). This preparation had a PBCA-polymer/drug ratio of 4:1. All other preparations showed a drug loading of about 10% with a PBCA-polymer/drug ratio of 1:2. The differences in loading between the NP/c preparation and the other preparations are statistically significant (ANOVA, 95% level).

Pharmacokinetics

Fig. 1 depicts the pilocarpine nitrate aqueous humor levels for the nanoparticle preparations (NP, NP/c) and the aqueous solutions (AS) used as reference. The pharmacokinetic parameters obtained from rabbits treated with betamethasone, are summarized in Table II. The bioavailability was determined as the AUC of the aqueous humor concentration in the eye vs. time curve. The 2% NP/c and the 4% NP preparations showed the highest improvement of bio-

Table I. Characterization of Pilocarpine-Loaded Nanoparticles

Preparation	Pilocarpine [%]	Poloxamer 188 [%]	BCA [%][a]	Nanoparticle:Drug Ratio[b]	Diameter [nm][c]	Drug load [%][b]
2% NP	2	1.2	1	1:2	183 ± 35	10.4 ± 4.5
2% NP/c	2	1.2	8	4:1*	257 ± 66	18.3 ± 5.2*
4% NP	4	1.2	2	1:2	211 ± 47	11.8 ± 3.9
6% NP	6	1.2	3	1:2	223 ± 30	9.2 ± 4.1

[a] Butylcyanoacrylate monomer.
[b] Mean ± standard deviation (n ≥ 3). *The drug load between nanoparticle:drug ratio 1:2 and 4:1 is statistically significant different (ANOVA, 95% level).
[c] Mean ± standard deviation (n ≥ 3). Data are not statistically significant different.

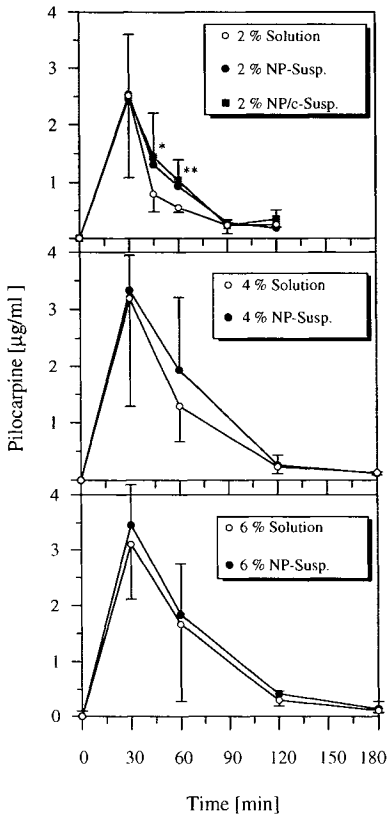

Fig. 1. Pilocarpine aqueous humor concentrations resulted from rabbits treated with betamethasone. Applied were preparations containing 2, 4 and 6% pilocarpine, solution = AS (aqueous reference solution), NP-Susp. and NP/c-Susp. = nanoparticle suspensions (see Table II). The differences between aqueous solutions and nanoparticle preparations are statistically significant for preparations with 2% pilocarpine at t_{45} and t_{60} (Student's *t*-test). Shown are mean values ± S.D. ($n \geq 4$, *$P < 0.05$; **$P < 0.01$)

availability in comparison to the corresponding aqueous reference solutions (2% AS and 4% AS). The AUC was increased by about 23%. With the 2% NP/c preparation $t_{1/2}$ was prolonged by about 48% and k_{el} was reduced by around 32%. All other nanoparticle preparations with higher drug content (i.e. 4% and 6% pilocarpine) did not show such an improvement of $t_{1/2}$ and k_{el} values relative to normal aqueous drug solutions. The statistical comparison of the AUC values showed significant differences only between the 2% AS and the 6% NP preparation (ANOVA, 95% level).

In order to investigate possible steroid-induced alterations in the kinetics, pilocarpine aqueous humor assays were also performed in untreated rabbits. As shown in Fig. 2, no statistically significant differences were found in the pilocarpine aqueous humor levels between animals treated with betamethasone (BM-Eye) and untreated animals (UT-Eye). The measurements were carried out for a 2% aqueous reference solution and the 2% NP preparations at t_{max} (see Table II) and at t_{60} min. Statistically significant differences (*t*-test) were obtained between nanoparticles (2% NP-Susp.) and the reference solution (2% solution) in glaucomatous eyes (BM-Eye) as well as in healthy eyes (UT-Eye) at 60 min.

Pharmacodynamics

Miosis. Experiments were performed for practical reasons in part with healthy, untreated rabbits with normotone IOP, because no statistically significant differences (AUC) between miosis in betamethasone-treated and untreated rabbits were observed (Fig. 3). Fig. 4 shows the pupil response after treatment with the preparations under evaluation. As outlined in Table III, the miotic response was prolonged for the 2% NP and 2% NP/c preparation compared to the aqueous reference solution. It was found that the decrease of the pupil response followed a zero order relationship. k_{dm} was decreased by nearly 40%. Correspondingly, statistically significant increased AUC values between 40% and 50% occurred. In addition, prolonged d_{max} values were observed. Similar results were found for the 4% NP and 4% AS preparations. The differences which occurred between both k_{dm} values were in the same range as those obtained with preparations containing 2% pilocarpine. However, differences between the k_{dm} values were not found for preparations containing 6% pilocarpine.

Interestingly, in the case of most nanoparticle preparations, with exception of the 6% NP preparation, t_{max} did not represent one single time point. Instead a plateau of the maximal reduction of the pupil diameter occurred for a prolonged

Table II. Pharmacokinetic Parameters of Pilocarpine Aqueous Humor Concentrations

Preparation[a]	AUC[b] (min · µg/ml)	t_{max} (min)	$t_{1/2}$ (min)	k_{el} (min)$^{-1}$[c]	C_{max} (mg/ml)[d]	r^2
2% AS	91 ± 43	30	18.7	0.037	2.51 ± 1.42	0.93
2% NP	108 ± 27	30	23.9	0.029	2.53 ± 0.42	0.97
2% NP/c	112 ± 48	30	27.7	0.025	2.48 ± 1.13	0.97
4% AS	171 ± 93	30	31.5	0.022	3.19 ± 1.90	0.95
4% NP	209 ± 87	30	31.5	0.022	3.33 ± 0.62	0.99
6% AS	189 ± 99	30	31.1	0.023	3.10 ± 0.98	0.99
6% NP	216 ± 70*	30	33.0	0.021	3.46 ± 0.72	0.99

[a] AS = aqueous reference solutions with 2, 4, and 6% pilocarpine content. NP and NP/c = nanoparticle preparations (see Tabl I).
[b] Mean ± S.D. ($n \geq 4$) * statistically significant different from 2% AS (ANOVA, 95% level).
[c] k_{el} = elimination coefficient computed from an exponential curve-fit (r^2 = correlation coefficient).
[d] Mean ± S.D. ($n \geq 4$), data are not statistically significant different.

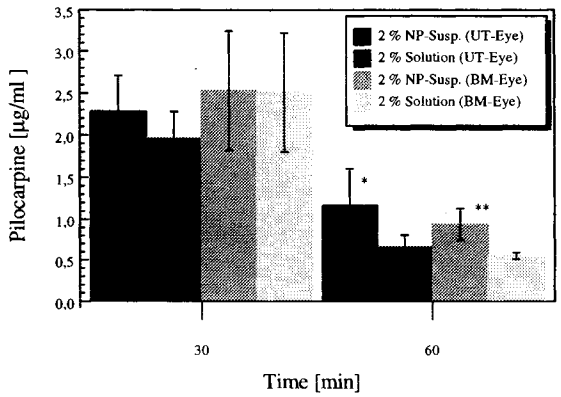

Fig. 2. Comparison between pilocarpine aqueous humor levels of preparations containing 2% pilocarpine, solution, AS = aqueous reference solution, NP-Susp. = nanoparticle suspension. Data were determined from rabbits with untreated eyes (UT-Eye) and rabbits treated with betamethasone (BM-Eye) at two relevant time points (30 = t_{max} and 60 min). No statistically significant differences occurred between the untreated eyes and eyes treated with betamethasone (Student's *t*-test; $P > 0.05$). Statistical differences were observed at 60 min between aqueous solutions and nanoparticle preparations in treated as well as untreated eyes (n = 5–6, *$P < 0.05$; **$P < 0.01$). Shown are mean values ± S.D.

time-interval, which in most cases ranged between 30 and 45 minutes. A plateau in the present study was defined as the interval between two time points when changes of the miotic effect were less or equal 0.1 mm.

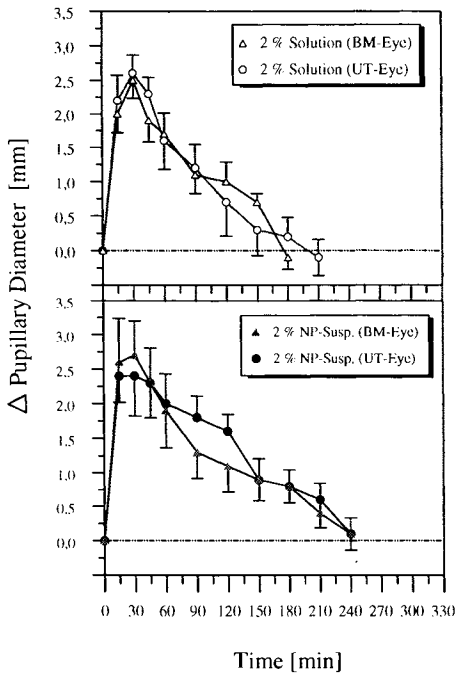

Fig. 3. Comparison between the miotic effect of preparations containing 2% pilocarpine, AS = aqueous reference solution, NP-Susp. = nanoparticle suspension. Data were determined from rabbits with untreated eyes (UT-Eye) and rabbits treated with betamethasone (BM-Eye). Pupillary diameters are shown as differences between baseline measurements and pupil diameters after pilocarpine treatment. The preparations show no statistically significant differences within each of the two groups (Student's *t*-test, n ≥ 6, $P > 0.05$). Shown are mean values ± S.D.

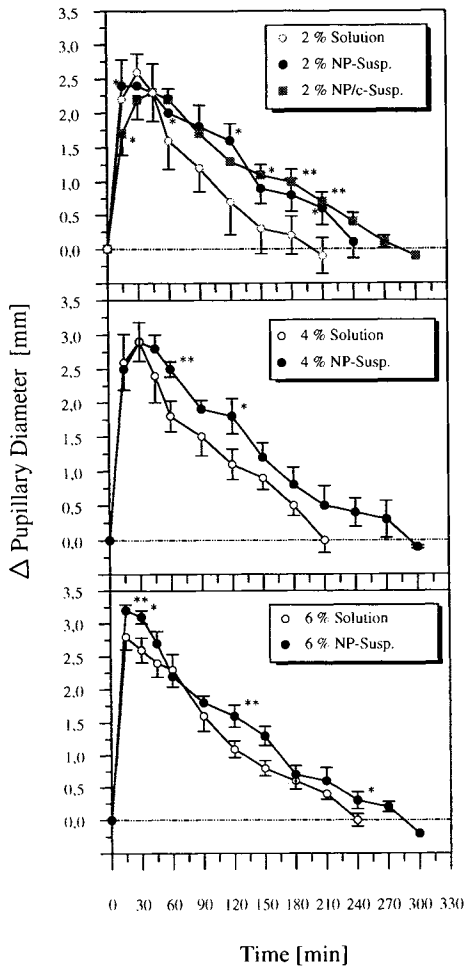

Fig. 4. Miotic effect resulted from untreated rabbits with normotone IOP. Applied were preparations containing 2, 4 and 6% pilocarpine, solution, AS = aqueous reference solution, NP-Susp. and NP/c-Susp. = nanoparticle suspensions (see Table III). Pupillary diameters are shown as differences between baseline measurements and pupil diameters after pilocarpine treatment. The asterisks visualizes statistically significant differences (Student's *t*-test) between aqueous reference solutions and nanoparticle preparations (n = 8–10, *$P < 0.05$; **$P < 0.01$). Shown are mean values ± S.D.

IOP. The results obtained in the IOP-measurement experiments are summarized in Table IV and illustrated in Fig. 5. In contrast to the pilocarpine aqueous humor profile or the miotic response it was not possible to find a general equation to describe the IOP-response. From the data of the mean curves (Fig. 5) AUC, highest pressure reduction (I_{max}), and t_{max} (time of I_{max}) and $\Delta_{1/2}$ were calculated. As shown in Table IV and also observed before, t_{max} did not represent a single time peak for the 2% NP/c and 4% NP preparations. In these cases, a plateau of the maximal IOP-reduction occurred over a prolonged period of time. This plateau which was defined as the interval between two time points when IOP changes were less than 0.5 mmHg.

An enhanced pharmacological response occurred for the 4% NP preparation in comparison to the corresponding aqueous reference solution, but again not for the 6% NP preparation. The best improvement of the IOP-profile compared to an aqueous reference solution was found for the 2%

Table III. Miotic Response Parameters

Preparation[a]	AUC[b] (min · mm)	t_{max} (min)	d_{max} (min)[c]	k_{dm} $(min)^{-1}$[d]	M_{max} (mm)[e]	r
2% AS	213 ± 117	30	185	0.0147	2.6 ± 0.53	−0.97
2% NP	312 ± 107*	15–45	253	0.0107	2.4 ± 0.84	−0.99
2% NP/c	329 ± 62*	30–60	283	0.0090	2.3 ± 0.65	−0.99
4% AS	278 ± 76	30	207	0.0144	2.9 ± 0.56	−0.98
4% NP	376 ± 78*+	30–45	276	0.0110	2.9 ± 0.38	−0.98
6% AS	308 ± 55	15	228	0.0127	2.8 ± 0.38	−0.99
6% NP	379 ± 52*+#	15	266	0.0117	3.2 ± 0.18	−0.98

[a] AS = aqueous reference solutions with 2, 4, and 6% pilocarpine content. NP and NP/c = nanoparticle preparations (see Table I).
[b] Mean ± S.D. (n ≥ 8) statistically significant different from: *2% AS, +4% AS, #6% AS, (ANOVA, 95% level).
[c] d_{max} = maximal duration of miosis.
[d] k_{dm} = pupil response coefficient computed from linear regression (r = correlation coefficient).
[e] M_{max} = highest miotic response, Mean ± S.D. (n ≥ 8), data are not statistically significant different.

NP preparation. The highest IOP-reduction in terms of AUC values was observed for the 4% NP preparation. Up to 6 h, a statistically significant prolonged IOP-reduction in comparison to the aqueous reference solutions was obtained for the 2% NP, 2% NP/c and 4% NP nanoparticle preparations and, in addition, between 1 and 4 to 5 h a low-level IOP-plateau was maintained (statistical comparison see Table IV).

DISCUSSION

Drug loading is of major importance for the performance of a colloidal drug delivery system. The most likely binding mechanism of pilocarpine to nanoparticles is given by adsorption, although covalent linkage can not be excluded totally (14). The adsorption can occur during polymerization or later onto the surface of the previously formed particles. However, since nanoparticles represent porous polymeric meshworks (15), absorption also can take place by diffusion of the drug into the particle matrix. The results demonstrate that the major drug portion (82–91%) was not bound to the particles and remained in solution. As long as the ratio between polymer (nanoparticles) and drug was kept constant, the percentage of drug loading (about 10%) was unchanged (Table I). This finding strongly suggests a constant partitioning of the drug between the aqueous phase and the nanoparticles.

As known from previous investigations (5), only 1–3% pilocarpine (corresponding to 5–15 μg) of a conventional aqueous dosage is bioavailable. Since about 50 μg pilocarpine per 25 μl dose were bound to the nanoparticles at a 2% drug concentration and supposing that the nanoparticles are eliminated from the eye much slower than an aqueous reference solution (16), a major amount of this drug portion would be absorbed by ocular tissues, leading to a higher ophthalmic bioavailability in comparison to normal eye drops. The observed prolonged drug action with nanoparticles may satisfy the requirements of Lerman and Reininger (17) who showed that releases of about 5–10 μg/h pilocarpine were sufficient to control most forms of glaucoma.

In the betamethasone model the intraocular pressure (IOP) is artificially increased with this steroid. This pretreatment could alter the pharmacokinetics of the drugs to be investigated, i.e. in the present case pilocarpine. Steroid-induced changes are likely to occur in most ocular tissues (18) because of the presence of glucocorticoid receptors.

Table IV. IOP-Response Parameters

Preparation[a]	AUC (min · mmHg)[b]	t_{max} (min)	$\Delta_{1/2}$ (min)[c]	I_{max} (mmHg)[d]
2% AS	1002 ± 582	60	90 ± 39	7.20 ± 1.60
2% NP	1455 ± 918	180	276 ± 55*	5.30 ± 1.20*
2% NP/c	1431 ± 486	60–180	312 ± 54*	5.58 ± 1.20*
4% AS	1444 ± 570	120	150 ± 28	7.60 ± 0.80
4% NP	1958 ± 828	120–240	324 ± 19*	6.12 ± 0.70
6% AS	1351 ± 756	60	204 ± 44	6.05 ± 0.80
6% NP	1514 ± 612	120	270 ± 33	5.48 ± 1.20

[a] AS = aqueous reference solutions with 2, 4, and 6% pilocarpine content. NP and NP/c = nanoparticle preparations (see Table I).
[b] Mean ± S.D. (n ≥ 6), data are not statistically significant different.
[c] $\Delta_{1/2}$ = duration of the IOP-response at half the maximal response level Mean ± S.D. (n ≥ 6), * statistically significant different from 2, 4 and 6% AS (ANOVA, 95% level).
[d] I_{max} = highest pressure reduction, Mean ± S.D. (n ≥ 6), * statistically significant different from 2 and 4% AS (ANOVA, 95% level).

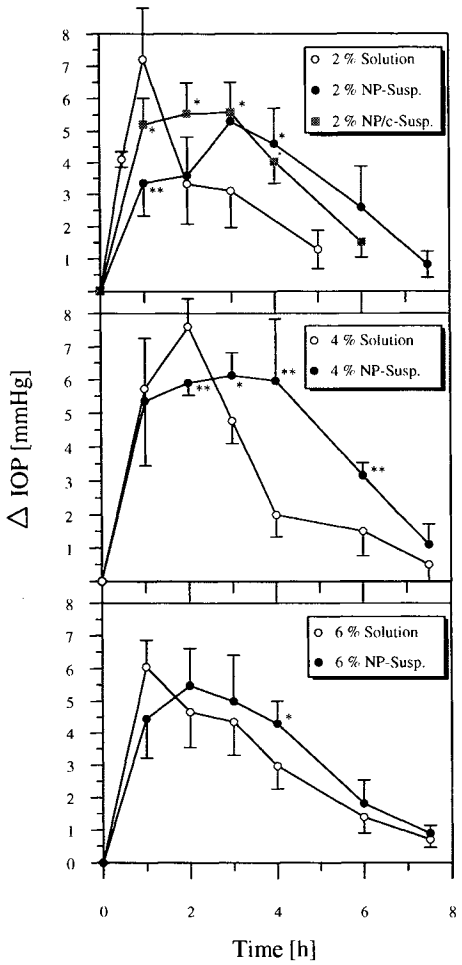

Fig. 5. IOP-lowering effect resulted from rabbits treated with betamethasone. Applied were preparations containing 2, 4 and 6% pilocarpine, AS = aqueous reference solution, NP-Susp. and NP/c-Susp. = nanoparticle suspensions (see Table IV). IOP-reductions are shown as differences between baseline measurements and IOP-values resulted after pilocarpine treatment. The asterisks visualizes statistically significant differences (Student's *t*-test) between aqueous reference solutions and nanoparticle preparations ($n \geq 6$, $^*P < 0.05$; $^{**}P < 0.01$). Shown are mean values ± S.D.

Physiological evidence for changes in ocular tissues induced by steroids includes effects on corneal thickness and metabolism, changes in the composition of the aqueous humor, and vasoconstrictive effects in the conjunctiva. However, in the present study no statistically significant differences of AUC and t_{max} between betamethasone-treated eyes and untreated eyes were observed (Fig. 2 and Fig. 3). These results emphasize that the short period of treatment of 3 weeks with betamethasone has no major effect on the pilocarpine permeation through the cornea and on the drug distribution between cornea and aqueous humor. Therefore, the betamethasone model seems to be a suitable glaucoma model to compare pharmacokinetic to pharmacodynamic effects determined as changes in the miosis and in the reduction of the IOP of various preparations employed by this study.

The best improvement of the bioavailability of pilocarpine in the aqueous humor with nanoparticles compared to an aqueous reference solution occurred with preparations containing 2 and 4% pilocarpine. The 2% NP/c preparation, which also had the highest drug loading, was most efficient to decrease the elimination coefficient, k_{el} (Table II). Surprisingly, preparations containing 6% pilocarpine showed no further increase of the bioavailability. One possible explanation for this result is that the free drug portion not bound to the nanoparticles was sufficient to saturate the corneal epithelium leading to an almost equivalent aqueous humor level. On the other hand, due to a high drug concentration of the applied dose it is very probable that the hypertonicity of the preparations and, as a result, tear flow were increased. These possible effects might reduce the half life of drug carriers as well as the half life of dissolved drug in the precorneal compartment. Therefore, a negligible enhancement of the pilocarpine aqueous humor concentration may have occurred despite a drug payload of at least 10% pilocarpine.

Miosis induced by pilocarpine more or less represents a side effect which is not desirable for therapy but has several advantages for analyzing ophthalmic drug preparations. Furthermore, a simple linear dose-response relationship between the pupil response and ophthalmic drug concentration was found (19). As depicted in this study, the decrease of the miotic response follows a zero-order process. However, a first-order decrease of the miotic response was postulated by Maurice and Mishima (20), but was not found in this study nor was it observed by other research groups (6,8). This result seems to be especially valid for drug-loaded nanoparticles, viscous solutions, and bioerodible ophthalmic inserts. The miotic response was relatively prolonged most with the 2% NP and the 2% NP/c preparation compared to an aqueous reference solution (Table III). In contrast to the regional pharmacokinetics, nanoparticle preparations with 4 and 6% drug content also showed a significant improvement over the reference solution. Generally, differences between nanoparticle preparations and aqueous reference solutions were lower with higher pilocarpine concentrations.

The present investigation with rabbits whose IOP was artificially increased by a pretreatment with betamethasone confirms earlier results by Diepold et al. (9) carried out in part with normostatic rabbit eyes. Similar to the miosis measurements of the present study, the nanoparticle suspensions with 2% pilocarpine showed an increased IOP-reduction vs. an aqueous reference solution. Additionally, the 4% NP preparation showed the highest IOP-reduction in terms of AUC values. As mentioned above, a large and important difference in IOP-reduction occurred between the pilocarpine solution and the nanoparticle preparation that was especially pronounced at low drug concentrations (2%). However, statistically significant differences as observed for the AUC values of the miosis measurements were not detected for the IOP AUC data (ANOVA, 95% level). Although with all preparations a pilocarpine aqueous humor t_{max} was obtained at about 30 min, in the case of the IOP-reduction the t_{max} values were observed to range from 60 to 120 min for aqueous solutions and from 60 to 180 min for nanoparticle preparations. These results were confirmed by statistically significant prolonged $\Delta_{1/2}$ values. In addition, the solutions caused very sharp maximal responses, whereas the nanoparticles led to a broad flat response (Fig. 5). As shown in this study, pilocarpine was almost totally eliminated from the aqueous humor after 180 min. However, at this time the

highest IOP-reduction was determined (i.e. 2% NP, 2% NP/c and 4% NP preparations). This inconsistency can be discussed in that the drug was distributed differently in the eye tissues after application of aqueous solutions and of nanoparticles. This different distribution profile may have provoked a prolonged reduction of the IOP.

As described in a previous study (21), a penetration of the nanoparticles into deeper tissue layers of the cornea and conjunctiva is very unlikely. On the other hand it was demonstrated that nanoparticles might be able to penetrate into surface structures of the surrounding tissue thus creating a precorneal depot. This may provoke a direct drug diffusion from this depot to its site of action without interference by an additional aqueous diffusion pathway (precorneal tear film). It is very likely that the trabecular meshwork represents in part this site of action. This was previously also discussed as non-corneal, alternative, or scleral pathway (8,22).

The prolonged miotic response may be explained at least in part by the slower elimination rate from the aqueous humor after application of nanoparticles. As shown in the present investigation, the situation is different for the IOP-response. Due to the nature of the physiological process of aqueous humor production and its outflow resistance, it is obvious that maximal IOP-reduction may not occur at the same time as observed for the miosis, due to a much faster physiological response of the latter effect (23). Additionally, the anterior chamber filled with the aqueous humor might not be the relevant compartment for comparing the pilocarpine concentration profile with the IOP-reduction in terms of extent and duration. The drug which is eliminated from the aqueous humor through a meshwork of channels to the systemic circulation, instead might be distributed into a more relevant compartment, for example into the trabecular meshwork. In connection with this, it should be noted that the drug concentration in the aqueous humor does not always reflect that in the surrounding intraocular tissues (24). From these tissues the drug will not be redistributed into the aqueous humor so that a true equilibrium between these compartments does not exist.

ACKNOWLEDGMENTS

The authors would like to acknowledge the financial support by the "Land Hessen", Forschungsprojekt "Muskarin-Rezeptor-Agonisten und -Antagonisten", (MAA-Projekt).

REFERENCES

1. M. Diestelhorst. Charakteristik und Diagnostik wichtiger Glaukomformen, *Pharm. Ztg.* 136(10):9–13 (1991).
2. G. Keren and G. Treiser. Effect of aceclidine (+) isomer and pilocarpine on the intraocular pressure decrease and the miosis in glaucomatous eyes. Effect on accommodation in normal eyes of young subjects, *Ophthalmologica* 180:181–187 (1980).
3. R. Weekers, Y. Delamrcelle, and J. Gustin. Treatment of ocular hypertension by adrenalin and diverse sympathomimetic amines. *Am. J. Ophthalmol.* 40:666–672 (1956).
4. S. S. Chrai and J. R. Robinson. Corneal permeation of topical pilocarpine nitrate in the rabbit. *Am. J. Ophthalmol.* 77:735–739 (1974).
5. T. F. Patton and J. R. Robinson. Quantitative precorneal disposition of topically applied pilocarpine nitrate in rabbit eyes. *J. Pharm. Sci.* 65:1295–1301 (1976).
6. R. Gurny, T. Boye T, and H. Ibrahim. Ocular therapy with nanoparticulate systems for controlled drug delivery. *J. Controlled Rel.* 2:353–361 (1985).
7. T. Harmia, P. Speiser, and J. Kreuter. A solid colloidal drug delivery system for the eye: encapsulation of pilocarpine in nanoparticles. *J. Microencapsul.* 3:3–12 (1986).
8. R. W. Diepold. *Entwicklung und Untersuchung von kolloidalen Depot-Arzneistoffträgersystemen zur Glaukombehandlung.* Dissertation, Fachbereich Biochemie, Pharmazie und Lebensmittelchemie, JWG-Universität, Frankfurt, a.M., 1988.
9. R. W. Diepold, J. Kreuter, J. Himber, R. Gurny, V. H. L. Lee, J. R. Robinson, M. F. Saettone, and O. E. Schnaudigel. Comparison of different models for the testing of pilocarpine eyedrops using conventional eyedrops and a novel depot formulation (nanoparticles). *Graefe's Arch. Clin. Exp. Ophthalmol.* 227:188–193 (1989).
10. L. Bonomi, S. Perfetti, E. Noya, R. Bellucci, and L. Tomazzoli. Experimental corticosteroid ocular hypertension in the rabbit. *Graefe's Arch. Clin. Exp. Ophthalmol.* 209:73–82 (1978).
11. J. Kreuter. Evaluation of nanoparticles as drug-delivery systems I: Preparation methods. *Pharm. Acta Helv.* 58:196–208 (1983).
12. M. C. Makoid and J. R. Robinson. Pharmacokinetics of topically applied pilocarpine in the albino rabbit eye. *J. Pharm. Sci.* 68:435–442 (1979).
13. J. Meier. Pharmakokinetische Grundlagen. In H. Sucker (ed.), *Pharmazeutische Technologie*, Georg Thieme, Stuttgart, 1978, pp. 275–276.
14. D. Leu. *Polyacrylic nanoparticles as controlled release drug delivery systems,* Dissertation Nr. 7184, ETH-Zürich, 1983.
15. J. Kreuter. Nanoparticles and nanocapsules - new dosage forms in the nanometer size range. *Pharm. Acta Helv.* 53:33–39 (1978).
16. R. W. Wood and J. R. Robinson. Ocular disposition of pilocarpine in the pigmented rabbit: site of metabolism. *Int. J. Pharm.* 29:127–135 (1986).
17. S. Lerman and B. Reininger. Simulated sustained release pilocarpine therapy and aqueous humor dynamics. *Canad. J. Ophthal.* 6:14–23 (1971).
18. B. Schwartz. Physiological effects of the corticosteroids on the eye. In B. Schwartz (ed.) *Corticosteroids and the eye.* Little Brown, Boston, 1966, pp. 753–765.
19. S. Yoshida and S. Mishima. A pharmacokinetic analysis of the pupil response to topical pilocarpine and tropicamide. *Jpn. J. Ophthalmol.* 19:121–138 (1975).
20. D. M. Maurice and S. Mishima. Ocular Pharmacokinetics. In M. L. Sears (ed.), *Handbook of Experimental Pharmacology - Pharmacology of the Eye,* Springer, Berlin, Heidelberg, New York, 1984, 69:pp. 19–116.
21. A. Zimmer, J. Kreuter and J. R. Robinson. Studies on the transport pathway of PBCA-nanoparticles in ocular tissues. *J. Microencapsul.* 8:497–504 (1991).
22. I. Ahmed and T. F. Patton. Disposition of timolol and inulin in rabbit eye following corneal versus non-corneal absorption. *Int. J. Pharm.* 38:9–21 (1987).
23. S. Mishima. Clinical pharmacokinetics of the eye. *Invest. Ophthalmol. Vis. Sci.* 21:504–541 (1981).
24. S. Miller, K. Himmelstein, and T. F. Patton. A physiologically based pharmacokinetic model for the intraocular distribution of pilocarpine in rabbits. *J. Pharm. Biopharm.* 9:653–677 (1981).