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Research paper

Preparation and evaluation of drug-loaded gelatin nanoparticles for topical ophthalmic use

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Abstract

Gelatin nanoparticles encapsulating pilocarpine HCl or hydrocortisone as model drugs were produced using a desolvation method. The influence of a number of preparation parameters on the particle properties was investigated. For the pilocarpine HCl-loaded spheres, an influence of the pH during particle preparation on the size was observed. Slightly negative zeta potential values were measured for all samples. In the case of pilocarpine HCl-loaded spheres, no influence of the gelatin type or the pH level was observed, which could be attributed to the shielding effect of ions present in the dispersion medium. When hydrocortisone was entrapped, a difference in zeta potential value between gelatin type A and gelatin type B particles was measured. A high pilocarpine HCl entrapment was established. Hydrocortisone was complexed with cyclodextrins in order to increase its aqueous solubility. The drug encapsulation was lower than in the case of pilocarpine HCl, but still amounted to approximately 30–40%. Compared to the aqueous drug solutions, a sustained release for both drugs was observed. The release kinetics of pilocarpine HCl are close to zero order, and no significant differences were measured between the various preparations. In the case of hydrocortisone, the release data suggests a difference in release rate depending on the type of cyclodextrin employed.

Keywords: Gelatin; Nanoparticles; Pilocarpine; Hydrocortisone; Ophthalmic drug delivery; Desolvation; Particle size; Zeta potential; Drug release

1. Introduction

Drugs for ophthalmic use are generally applied topically as eye drops. The bioavailability of this well accepted dosage form, however, is quite low. After instillation, conventional eye drops reside at the eye surface for only a few minutes due to the defense mechanisms of the eye, such as lachrymation, reflex blinking and increased drainage. Furthermore, for most drug molecules the cornea is a difficult barrier to permeate [1,2].

The need for an improved bioavailability has resulted in the development of drugs with adequate pharmacokinetic properties, such as prodrugs and soft drugs [3–4]. Other research efforts have been aimed at the improvement of the dosage form. The use of colloidal carriers, such as liposomes and micro- and nanoparticles has been investigated. Liposomes are removed quite rapidly from the eye surface after instillation unless they have a positive charge or are coated with bioadhesive polymers or lectins [5–7]. Micro- and nanoparticles can be administered as a dispersion in the form of eye drops. A prolonged residence time at the eye surface can be obtained by alteration of the particle zeta potential or the use of bioadhesive polymers. Moreover, an uptake of nanoparticles in the first cell layers of the cornea has been reported [8,9].

Several polymers, of natural as well as synthetic origin, have been used for the preparation of micro- or nanoparticles. The aim of the study presented was to evaluate the preparation and properties of gelatin nanoparticles loaded with a hydrophilic (pilocarpine HCl) and a hydrophobic (hydrocortisone) drug.

Gelatin was chosen because of its biocompatibility and biodegradability. Moreover, collagen, the native protein from which gelatin is derived, is present in the eye, more specifically in the stroma, the middle cell layer of the cornea, and has been extensively employed in ocular applications [10,11].

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Several methods have been developed for the preparation of gelatin particles, such as emulsification, coacervation and desolvation. The emulsification method, in which an aqueous gelatin solution is emulsified in an oily phase and subsequently precipitated by cold or heat treatment, usually leads to the formation of microparticles [12]. In order to obtain nanoparticles, desolvation methods are more appropriate [13]. A desolvating agent (e.g. a salt solution, alcohol, acetone) is added to an aqueous gelatin solution in order to dehydrate the gelatin molecules, resulting in a change in conformation from stretched to coiled, indicated by a rise in turbidity. Next, a cross-linking agent is added to harden the native particles.

In the study of the pilocarpine HCl-loaded gelatin nanoparticles the influence of the gelatin type and the pH level during particle preparation was evaluated. There are two types of commercial gelatin, often designated as type A and type B. Gelatin type A is derived from acid processed collagen, while type B is obtained by alkaline collagen treatment, resulting in a difference in isoelectric points, being 7–9 for gelatin type A and 4–5 for gelatin type B. Particles were prepared at two pH levels: 4 and 6. At pH 4, both gelatin type A and B are positively charged. At a pH level of 6, however, gelatin type A still has a positive net charge, while gelatin type B is negatively charged. The aim of the study was to evaluate whether this difference in electrical charge at different pH levels would result in a different particle size, zeta potential value, drug loading or drug release profile. To plan and execute the experiments, a full factorial experimental design at two levels was employed.

The hydrophobic drug chosen was hydrocortisone. In order to improve the aqueous solubility of the drug, complexes with cyclodextrins were prepared. Cyclodextrins can be used to optimize ocular formulations. At the ocular surface, the complexed lipophilic drug molecule is delivered to the membrane surface [14,15]. Additionally, cyclodextrins can act as penetration enhancers by increasing drug availability at the surface of the corneal or conjunctival membranes, and through the extraction of components of those membranes, such as cholesterol and triglycerides [16,17].

Two different cyclodextrins were employed: hydroxypropyl-β-cyclodextrin (HP-β-CD), a neutral molecule, and 2-hydroxy-3-trimethyl-ammoniopropyl cyclodextrin (Cat. CD), a cationic cyclodextrin derivative. The influence of the choice of gelatin type and cyclodextrin type on particle size, zeta potential value, drug loading and in vitro drug release was studied.

2. Materials

Two types of gelatin were used throughout the study: gelatin type B from bovine skin, Bloom 225, and gelatin type A derived from porcine skin, Bloom 175, both from Sigma (St. Louis, MO, USA). Pilocarpine was obtained from Federa (Brussels, Belgium), while hydrocortisone was delivered by Alpha Pharma (Zwevegem, Belgium). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from Wacker Chemie GmbH (München, Germany) and 2-hydroxy-3-trimethyl-ammoniopropyl cyclodextrin (cat CD) from Roquette (Lestrem, France). Ethanol was from Merck-Belgolabo (Leuven, Belgium). Glutaraldehyde and sodium metabisulfite were obtained from Sigma (St. Louis, MO, USA). NaOH and HCl (pro analysi) were purchased from Merck (Darmstadt, Germany).

3. Methods

3.1. Hydrocortisone–cyclodextrin complex preparation

For the hydrocortisone–cyclodextrin complexes incorporated in the nanoparticle preparations a molecular ratio hydrocortisone/cyclodextrin of 1:4 was employed. For each gelatin particle preparation 50.0 ml of a solution containing 0.25 mmol of hydrocortisone and 1 mmol of cyclodextrin was used.

During a phase solubility study, constant amounts of hydrocortisone (0.1 mmol or 36.25 mg) were put into erlenmeyers, containing 20 ml of milliQ water. To each Erlenmeyer, an increasing amount of cyclodextrin was added. The contents of each recipient were then stirred magnetically for 3 days until equilibrium (Variomag poly 15, Variomag, München, Germany). Afterwards, non-dissolved hydrocortisone was separated by subsequent filtration over a paper filter and a 0.2-μm cellulose nitrate filter (Sartorius, Göttingen, Germany). The resulting solution was then analyzed for hydrocortisone content by means of UV spectroscopy at 248 nm after dilution of the samples (U 2001, Hitachi Instruments Inc., Tokyo, Japan).

3.2. Particle preparation

The nanoparticles were prepared using a desolvation technique. Pilocarpine-loaded particles were prepared as follows. An aqueous gelatin (type A or B) solution (0.5% w/v, 100 ml) was prepared, containing 2000 g of pilocarpine HCl. The pH was adjusted either to 4 or 6 by the addition of 0.1 N HCl or 0.1 N NaOH solution. The solution was stirred at 50 °C and 100 rpm (IKA Eurostar digi-visc, IKA labortechnik, Staufen, Germany). To induce the desolvation process, ethanol was added until a permanent faint turbidity was obtained. Glutaraldehyde aqueous solution (25% v/v, 2 ml) was added to harden the particles. The preparation was then stirred for 2 h at 1200 rpm. The crosslinking process was stopped by the addition of aqueous sodium metabisulfite solution (1.2 g in 300 ml). Ethanol and part of the water were evaporated using a rotavap (Laborota 4000, Heidolph, Schwabach, Germany). The total volume of the suspension was reduced to less than 100 ml and then

distilled water was added to obtain a total dispersion volume of 100.0 ml.

The preparation procedure of the hydrocortisone-loaded particles was very similar. Gelatin was dissolved in 50 ml of the prepared hydrocortisone–cyclodextrin complex solutions to obtain a gelatin (type A or B) concentration of 0.5% w/v. The solution was stirred at 50 °C and 100 rpm. To induce the desolvation process, ethanol was added until a permanent turbidity was obtained, after which 5.0 ml of distilled water was added. Glutaraldehyde aqueous solution (25% v/v, 1 ml) was added to harden the particles. The preparation was stirred at room temperature, allowing the preparation to gradually cool down (2 h at 1200 rpm). The crosslinking process was stopped by the addition of a sodium metabisulfite solution (0.6 g in 150 ml). Ethanol and part of the water were evaporated using a rotavap, after which the total volume of the dispersion was brought to 50.0 ml.

3.3. Experimental design

The number of factors investigated for the pilocarpine HCl-loaded nanoparticles was two: the type of gelatin and the pH of the aqueous phase during preparation. An overview of the factors and their levels is given in Table 1.

The type of gelatin can be regarded as a discrete variable, while the pH level of the water phase has a continuous character. A full factorial design was set up, resulting in $2^2 = 4$ experiments. A replica of each preparation was made, leading to a total of $4 \times 2 = 8$ experiments.

The experimental design for the hydrocortisone-loaded particles is similar, but other factors were investigated, as shown in Table 1. In this case, both factors are discrete. The four experiments of the full factorial design were replicated, resulting in eight particle preparations.

To perform the statistical analysis of the data, the Statistica® software was employed (Statsoft, Tulsa, OK, USA).

3.4. Particle evaluation

3.4.1. Particle size

Particle size was determined using photon correlation spectroscopy (PCS) using a Zetasizer 3000 (Malvern Instruments, Malvern, UK). The samples were measured in suspension after particle preparation, without further dilution. Each sample was measured three times, after which the average value was used for further calculations.

Table 1
Factors investigated in the preparation of pilocarpine HCl and hydrocortisone loaded gelatin nanoparticles

	Low level (−)	High level (+)
Pilocarpine HCl-loaded nanoparticles		
Gelatin type	A	B
pH of water phase	4	6
Hydrocortisone-loaded nanoparticles		
Gelatin type	A	B
Cyclodextrin type	Cationic CD	HP-β-CD

3.4.2. Zeta potential

Zeta potential values were measured using Laser Doppler Anemometry (LDA), employing a Zetasizer 3000 (Malvern Instruments, Malvern, UK). All samples were diluted eightfold with distilled water before measuring, resulting in an optimum signal intensity. Each sample was measured 10 times, after which the average value was used for further calculations.

3.4.3. Drug loading

Binding of pilocarpine HCl or hydrocortisone to the gelatin particles was measured by centrifuging part of the particle suspension at 14,000 rpm for 2 h. A sample of the supernatant was analyzed by a validated HPLC method in order to determine the amount of non-entrapped drug.

Drug entrapment was calculated using the following formula:

$$\text{Drug entrapment (\%)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of drug used in formulation}} \quad (1)$$

3.4.4. Drug release

To study the release of the drug from the particles, a Franz type diffusion cell was used. A volume of 2.0 ml of the particle suspension was applied to the donor chamber. Samples of 1 ml were taken from the receptor compartment at specified times and analyzed by HPLC. The samples taken were replaced by an equal amount of distilled water. A dialysis membrane with a molecular weight cut off (M.W.C.O.) of 10–12 kDa (Visking 3–20/32, Midcell, London, UK) was selected to separate the donor and acceptor compartment. A 2% (w/v) aqueous pilocarpine HCl solution was used as a reference for the pilocarpine HCl loaded gelatin particles. For the hydrocortisone loaded nanoparticles, samples of the complexes with both types of cyclodextrin were used as reference.

From the results of the HPLC analyses the amount of drug released from the donor compartment was calculated. Each diffusion experiment was performed six times, and the mean values and standard deviations were calculated.

4. Results and discussion

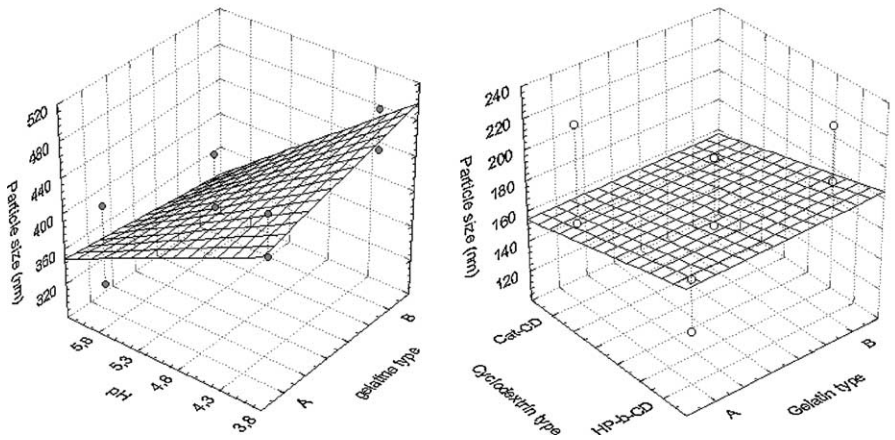
4.1. Particle size

4.1.1. Pilocarpine-loaded nanoparticles

The results of the particle size measurements are presented in Table 2. The size and significance of the effects is presented in Table 3.

Table 2
Particle sizes of pilocarpine HCl and hydrocortisone-loaded gelatin nanoparticles prepared [the results for both replicas prepared (1 and 2) are presented]

Pilocarpine HCl-loaded nanoparticles			Hydrocortisone-loaded nanoparticles		
Preparation parameters		Z ave (nm)	Preparation parameters		Z ave (nm)
Gelatin A	1	425	Gelatin A	1	154
pH = 4	2	471	Cationic CD	2	217
Gelatin A	1	400	Gelatin A	1	168
pH = 6	2	310	HP-β-CD	2	135
Gelatin B	1	456	Gelatin B	1	110
pH = 4	2	500	Cationic CD	2	157
Gelatin B	1	375	Gelatin B	1	189
pH = 6	2	312	HP-β-CD	2	224



All particle sizes of pilocarpine HCl loaded gelatin nanoparticles measured were in the nanometer range between 300 and 500 nm. The differences between the sizes of replicate preparations vary between 50 and 90 nm.

The average size for particles prepared with gelatin A was 402 nm while for particles with gelatin type B the average size was 411 nm, resulting in an effect of the factor 'gelatin type' of 9 nm. Considering the rather large variation

Table 3
Calculation of the size and significance of the effects of the preparation factors investigated on the particle size of pilocarpine HCl- and hydrocortisone loaded gelatin nanoparticles

	Effect (nm)	SS	d.f.	MS	F	*P*
Pilocarpine HCl-loaded nanoparticles						
Gelatin type	9.25	171.1	1	171.1	0.085	0.785
pH	−113.75	25,878.1	1	25,878.1	12.84	0.023
Gelatin type × pH	−20.75	861.1	1	861.1	0.427	0.549
Error		8060.5	4	2015.1		
Total SS		34,970.9	7			
Hydrocortisone-loaded nanoparticles						
Gelatin type	1.58	5.0	1	5.0	0.004	0.949
Cyclodextrin type	−19.65	772.2	1	772.2	0.716	0.445
Gelatin type × cyclodextrin type	−53.48	5720.8	1	5720.8	5.305	0.083
Error		4313.7	4	1078.5		
Total SS		10,811.7	7			

$P = 0.05$, significant effects are printed bold; SS, sum of squares; d.f., degrees of freedom.

between replicas one can conclude that the particle size is not influenced by the type of gelatin used, as is confirmed in the ANOVA analysis. The desolvation process, leading to particle formation, does not seem to be influenced by the differences between the two gelatin types, such as their differences in isoelectric points.

The effect of the pH on the particle size is clearer. At a pH level of 6, the average particle size obtained is 349 nm, compared to 463 nm at pH 4. This effect is also clearly visible on the graph included in Table 2, as the response surface has a marked slope in the direction of the pH axis. This significant difference in particle size, 114 nm between the two pH levels tested, indicates that the protonation or deprotonation of the amino or carboxylic acid residues present in the gelatin molecules influences the way the gelatin molecules fold together as particle formation occurs. Another possible explanation for the difference in particle size might be a difference in cross-linking between the two pH levels. If the cross-linking reaction would be favored at a pH of 6, more cross-links would be formed, resulting in a denser network and a reduction in particle size.

Controlling the particle size offers a possibility to regulate drug release. Smaller particles have a larger total external surface allowing for a more intense interaction with the medium in which they are dispersed, resulting in a faster drug release, as was demonstrated by Wakayima et al. for local anesthetics incorporated in poly(lactic acid) microspheres[18].

The distribution in vivo is also dependent on the particle size. A possible uptake in the first cell layers of the cornea, for example, would largely depend on the particle size, as was demonstrated by Calvo et al., who showed that PECL nanoparticles penetrate the first corneal cell layers, while microspheres do not[19].

4.1.2. Hydrocortisone-loaded nanoparticles

Particle sizes obtained vary from 110 to 220 nm. This is remarkably smaller than those measured for the pilocarpine HCl loaded gelatin nanoparticles. This is probably due to a change in the preparation procedure. When the pilocarpine-loaded gelatin particles were prepared, ethanol was added until a faint turbidity was observed, after which cross-linking was started. For the hydrocortisone-loaded gelatin particles, ethanol was added until a clearly visible increase in turbidity was noted, after which 5 ml of water were added to bring the system back to the very beginning of coacervate formation. Probably, the aggregates formed, which were crosslinked, in this case were smaller than the ones obtained when preparing pilocarpine HCl loaded particles, which vary from 310 to 500 nm. Moreover, during hydrocortisone-loaded particle preparation, all volumes used were divided by 2 compared to the production of pilocarpine-loaded spheres. The agitation by the propeller stirrer, however, remained the same, resulting in a more efficient prevention of particle aggregation and consequently smaller particle sizes.

The type of gelatin used did not influence the resulting particle size, as was observed for the pilocarpine HCl loaded particles. The type of cyclodextrin, did not have a significant effect on the particle size either.

4.2. Zeta potential value

4.2.1. Pilocarpine-loaded nanoparticles

Zeta potential measurement results are presented in Table 4. The size and significance of the effects are presented in Table 5.

All zeta potential values of the samples measured are slightly negative. The average zeta potential value for particles prepared at pH 6 equals −6.95 mV, for spheres prepared at pH 4 it amounts to −6.10 mV. Consequently, there is no significant effect of the preparation pH on zeta potential value of the particles obtained.

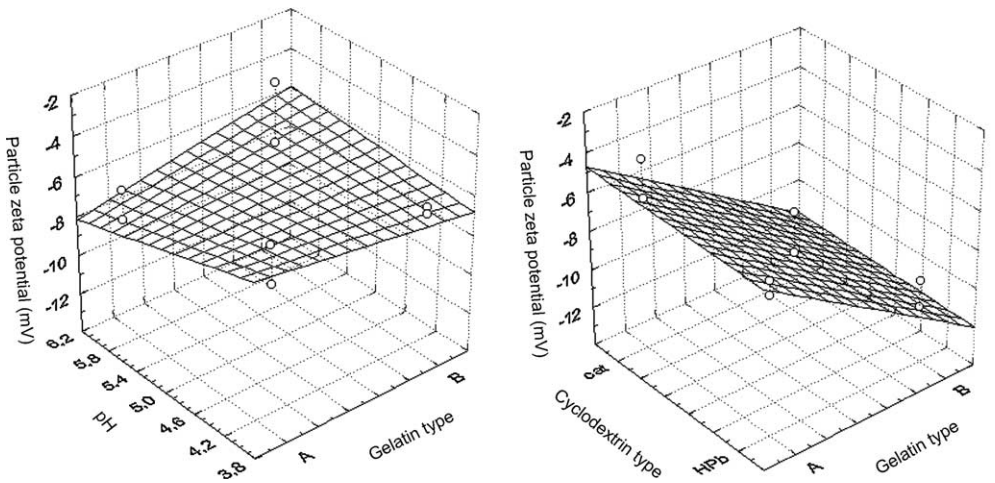
The effect of the gelatin type is equally small. An average zeta potential value of −6.65 mV was found for gelatin type A, and −6.40 mV for gelatin type B.

The electrical charge of gelatin type A and B is positive at a pH level of 4. At a pH level of 6, however, gelatin type A still has a net positive charge, while gelatin type B is negatively charged. Consequently, if the polymer charge would influence the particle zeta potential value, then an interaction between the factor pH and gelatin type can be expected to play a role. As shown in Table 5, there is an interaction effect of 1.15 mV. This is also visible in the graph included in Table 4, as the response surface has a twisted look. Although the interaction effect (1.15 mV) is larger than the effect of the gelatin type (0.25 mV) and the pH (−0.85 mV), it is not significant, due to the large variations in zeta potential value of the replicas. Consequently, one has to conclude that, under the test conditions used, the charge of the gelatin molecule does not significantly affect the particle zeta potential.

Weber et al. performed a study in which they showed that the concentration of the cross-linking agent has an influence on the amount of free amino groups at the surface of gelatin nanoparticles prepared by a desolvation technique. However, when a constant amount of glutaraldehyde was used and gelatin types were compared, they also demonstrated a difference between gelatin type A and B[20]. Consequently, if the amount of free amino groups at the particle surface is different for both gelatin types, one would expect to see a difference in zeta potential value as well. The zeta potential, however, is not only determined by the charges present at the particle surface, but also by the ions present in the dispersion medium. The relatively small values of the zeta potentials found and the lack of effect of the polymer charge can be explained by the presence of salts (sodium metabisulfite and oxidation products) and other ions (H^+, Cl^-, Na^+ and OH^-) in the medium, which can compensate electrical charges present at the particle surface ('shielding'), leading to a diminishing of the zeta potential value.

Table 4
Zeta potential values of pilocarpine HCl and hydrocortisone-loaded gelatin nanoparticles prepared

Pilocarpine HCl-loaded nanoparticles			Hydrocortisone-loaded nanoparticles		
Preparation parameters		Zeta potential value (mV)	Preparation parameters		Zeta potential value (mV)
Gelatin A	1	- 6,6	Gelatin A	1	-6,2
pH = 4	2	- 4,7	Cationic CD	2	-4,2
Gelatin A	1	-8,4	Gelatin A	1	-6,8
pH = 6	2	- 6,9	HP-β-CD	2	-6,1
Gelatin B	1	-6,4	Gelatin B	1	-10,3
pH = 4	2	-6,7	Cationic CD	2	-12,5
Gelatin B	1	-7,8	Gelatin B	1	-11,1
pH = 6	2	-4,7	HP-β-CD	2	-9,7



The results for both replicas prepared (1 and 2) are presented.

Table 5
Calculation of the size and significance of the effects of the preparation factors investigated on the particle zeta potential value of pilocarpine HCl- and hydrocortisone loaded gelatin nanoparticles

	Effect (mV)	SS	d.f.	MS	*F*	*P*
Pilocarpine HCl-loaded nanoparticles						
Gelatin type	0.25	0.1	1	0.1	0.064	0.812
pH	−0.85	1.4	1	1.4	0.742	0.437
Gelatin type × pH	1.15	2.6	1	2.6	1.360	0.308
Error		7.7	4	1.9		
Total SS		12.0	7			
Hydrocortisone-loaded nanoparticles						
Gelatin type	**−5.075**	**51.51**	**1**	**51.511**	**36.500**	**0.004**
Cyclodextrin type	0.125	0.03	1	0.031	0.022	0.889
Gelatin type × cyclodextrin type	−1.125	2.53	1	2.531	1.794	0.252
Error		5.65	4	1.411		
Total SS		59.72	7			

$P = 0.05$, significant effects are printed bold.

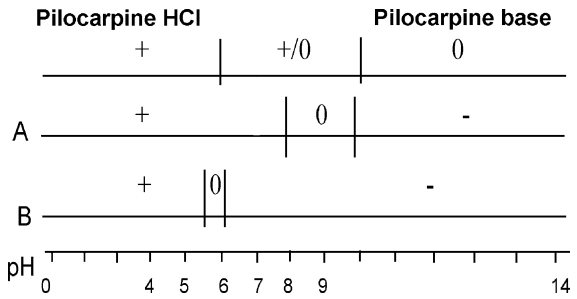


Fig. 1. Electrical charge of pilocarpine HCl and gelatin type A and B in function of the pH value.

4.2.2. Hydrocortisone-loaded nanoparticles

Zeta potential values between −4 and −12 mV were obtained. According to the ANOVA analysis, there is a significant influence of the gelatin type on the zeta potential value. The size of the effect was calculated as −5.1 mV, meaning the average particle zeta potential value decreases with 5 mV when gelatin type B is employed instead of gelatin type A. This can also be observed in the response surface on the graph included in Table 4, which has a clear slope in the direction of the gelatin type. As was shown in Fig. 1, gelatin type A has a higher isoelectric point than gelatin type B, resulting in a different electrical charge of both gelatin types in function of the pH. This difference is reflected in the zeta potential values observed. However, one would expect to observe the same effect when pilocarpine HCl was added as a drug, and this was not the case. This fact was explained by the shielding effect of ions present in the particle dispersion, adsorbing to the particle surface and compensating for charge differences between particles prepared with gelatin type A and B. The shielding effect seems to be less strong for the hydrocortisone-loaded nanoparticles, allowing the differences between the two gelatin types to be expressed as a difference in zeta potential value. The reason for this decreased shielding effect is probably the diminished presence of ions in the hydrocortisone-loaded gelatin particle preparations. No NaOH or HCl was used to adjust the pH to a certain level, as was the case for the pilocarpine-loaded particles. Moreover, the drug itself could play a role. Hydrocortisone carries no electrical charges, while pilocarpine HCl can be considered as a positively charged, protonated, drug molecule accompanied by the negative Cl^- ion.

There was no significant influence of the cyclodextrin type on the particle zeta potential value. One might expect to see an influence of the positively charged cationic cyclodextrin on the zeta potential value, but this was not the case.

4.3. Drug loading

4.3.1. Pilocarpine-loaded nanoparticles

Pilocarpine HCl loadings of the gelatin particles prepared are summarized in Table 6. The size and significance of the effects of the factors investigated is presented in Table 7.

The entrapment of the drug pilocarpine HCl was close to 50% for all samples measured. No differences were observed between types of gelatin nor pH levels, as can be seen in Table 7, and in the graph included in Table 6, which exhibits little slope in either axis. The encapsulation efficiency of around 50% seems to fit in the range of encapsulation efficiencies found in the literature.

The electrical charge of pilocarpine HCl (which has a $pK_1 = 7.15$ and $pK_2 = 12.57$) and gelatin type A and B in function of the pH is presented in Fig. 1. If pilocarpine HCl is considered as a positively charged protonated molecule, with a negative Cl^- counterion, one would expect to find a higher interaction with the gelatin B molecules at a pH of 6, as at this pH, the macromolecule is negatively charged and could electrostatically attract the positively charged pilocarpine molecule. This could result in a higher drug encapsulation for the combination of gelatin B at a pH level of 6. In terms of the experimental design, an interaction effect between gelatin type and pH level should be observed. As shown in Table 7, however, this interaction is not significant, indicating that interactions other than electrostatic ones play a role in the binding of the drug to the particle.

4.3.2. Hydrocortisone-loaded nanoparticles

Drug encapsulation efficiencies varying from 35 to 45% were observed. This is somewhat lower than the values obtained for pilocarpine HCl-loaded nanoparticles, but still in the range of drug loadings found in the literature. It is not clear to which factor the difference between pilocarpine HCl and hydrocortisone loading could be attributed. One could state that the hydrophilicity plays a role, as pilocarpine HCl is more hydrophilic than hydrocortisone. The hydrocortisone–cyclodextrin complex, however, can also be considered as hydrophilic. Other factors involved could be the difference in molecular weight or the difference in initial drug amount added to the preparation between both model drugs.

For the pilocarpine HCl loaded particles, an interaction between the charged drug molecule and the different gelatin polymers at different pH levels was expected to lead to differences in drug loading. This was not the case. When hydrocortisone–cyclodextrin complex was incorporated, no significant effect of the gelatin type was expected, and this is confirmed by the measurement results. The type of cyclodextrin did not alter significantly the drug loading either. The difference in the cyclodextrin used does not lead to a difference in drug incorporation into the particles.

4.4. Drug release

4.4.1. Pilocarpine loaded nanoparticles

All gelatin particle preparations release pilocarpine at a slower rate compared to the reference pilocarpine HCl solution, as shown in Fig. 2. After 3 h, about 50% of the pilocarpine HCl of the reference solution is found in the acceptor compartment, compared to 30% for the gelatin

Table 6
Drug entrapment of pilocarpine HCl and hydrocortisone-loaded gelatin nanoparticles prepared [the results for both replicas prepared (1 and 2) are presented]

Pilocarpine HCl-loaded nanoparticles			Hydrocortisone-loaded nanoparticles		
Preparation parameters		Drug entrapment (%)	Preparation parameters		Drug entrapment (%)
Gelatin A	1	47	Gelatin A	1	35
pH = 4	2	49	Cationic CD	2	41
Gelatin A	1	46	Gelatin A	1	44
pH = 6	2	54	HP-β-CD	2	37
Gelatin B	1	46	Gelatin B	1	45
pH = 4	2	56	Cationic CD	2	42
Gelatin B	1	44	Gelatin B	1	37
pH = 6	2	57	HP-β-CD	2	40

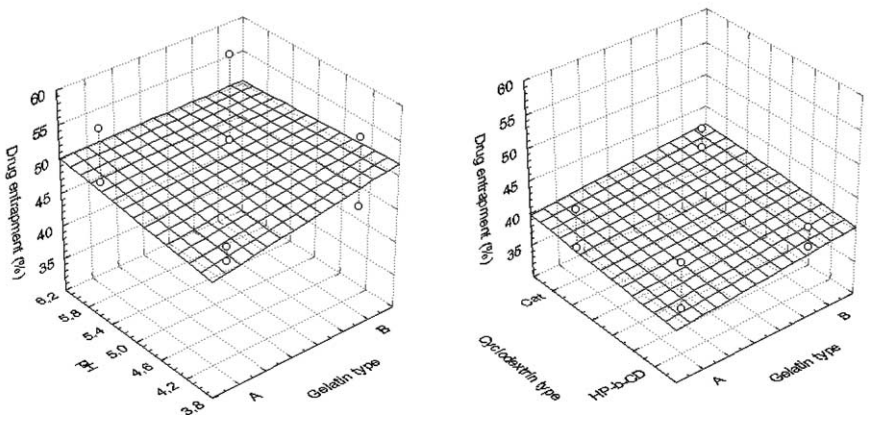


Table 7
Calculation of the size and significance of the effects of the preparation factors investigated on the drug entrapment of pilocarpine HCl and hydrocortisone in gelatin nanoparticles

	Effect (%)	SS	d.f.	MS	*F*	*P*
Pilocarpine HCl loaded nanoparticles						
Gelatin type	1.75	6.1	1	6.1	0.145	0.722
pH	0.75	1.1	1	1.1	0.027	0.878
Gelatin type × pH	−1.25	3.1	1	3.1	0.074	0.790
Error		168.5	4	42.1		
Total SS		178.0	7			
Hydrocortisone loaded nanoparticles						
Gelatin type	1.75	6.1	1	6.12	0.476	0.528
Cyclodextrin type	1.25	3.1	1	3.12	0.243	0.648
Gelatin type × cyclodextrin type	3.75	28.1	1	28.12	2.184	0.213
Error		51.5	4	12.88		
Total SS		88.9	7			

$P = 0.05$, significant effects are printed bold.

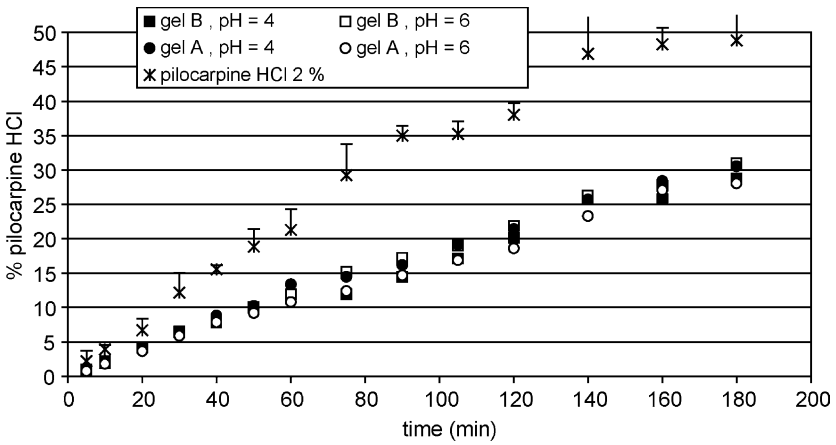


Fig. 2. Pilocarpine HCl release from gelatin nanoparticles prepared with gelatin type A or B at a pH level of either 4 or 6 (for the sake of clarity, only the error bars (S.D.) for the pilocarpine HCl solution are shown).

nanoparticles. There is little difference between the release patterns of the various particle preparations, as shown in Fig. 3.

Considering the standard deviations on the results, no significant differences in the drug release rate between the different series is observed. The pH during particle preparation has a significant effect on particle size. This effect, however, is not observed in the drug release studies. Probably the difference in size between the gelatin particles prepared at a pH level of 4 and 6 was not large enough to result in a significantly different pilocarpine release rate. The use of different types of gelatin, the other factor in the experimental design, does not influence drug release either.

In order to study the drug release mechanism, the power law expression was used, which is given in Eq. (2) [21].

$$\frac{M_t}{M_\infty} = kt^n \tag{2}$$

In this equation M_t/M_∞ is the fractional release of the drug, k is a constant incorporating structural and geometric characteristics of the controlled release device, and n is the release exponent, indicating the mechanism of drug release.

This equation is a simplification of a more general equation, describing the Fickian diffusion of a water-soluble drug from a plane sheet polymeric device. This simplification can be used in cases where $M_t/M_\infty < 0.6$, meaning less than 60% of the drug is released.

The value of n in the power law is an expression for the drug release mechanism. In Fig. 4 a graph is presented in which the release data of pilocarpine HCl from the gelatin particles are plotted as log M_t/M_∞ as a function of log t. The $n$value was calculated as the slope of the straight lines fitted using the least squares method. The value of n for each type of preparation is presented in Table 8.

The value of n is close to 1, indicating a release approaching zero-order kinetics, implying that the drug is released at a constant rate.

Gelatin undergoes a type I degradation, meaning that hydrolytically unstable crosslinks are broken resulting in the formation of water-soluble polymer fragments [22]. In the case of pilocarpine HCl loaded gelatin nanoparticles, however, the release of the drug may be controlled by factors other than the breakdown of the polymer network. Considering the fact that the release of the drug is rather

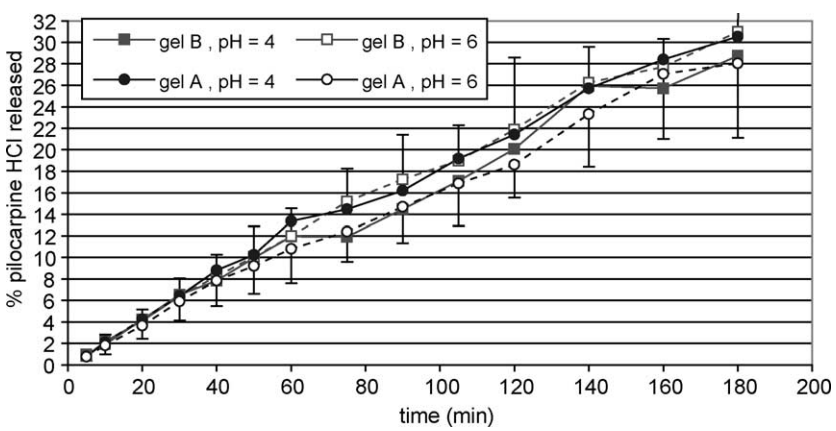


Fig. 3. Drug diffusion test results from gelatin nanoparticle prepared with gelatin type A or B at a pH level of either 4 or 6 (only the standard deviations for particles prepared with gelatin type B at pH 6 and those prepared with gelatin type A at pH 6 are shown).

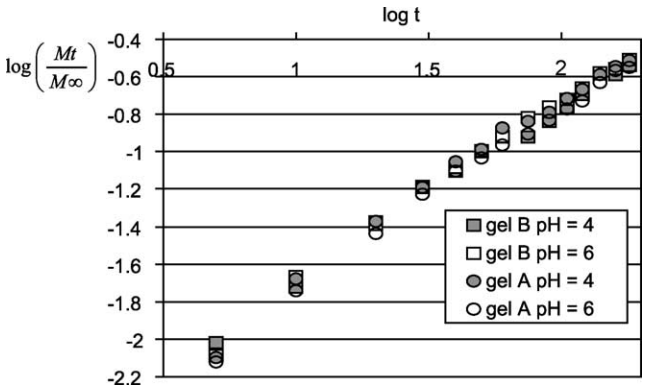


Fig. 4. log M_t/M_∞ plotted as a function of log $t$for pilocarpine release from various gelatin nanoparticle preparations.

Table 8
Kinetic exponent of drug release n of various gelatin particle preparations evaluated

	n	R^2
Pilocarpine HCl loaded nanoparticles		
Gelatin A, pH 4	0.9802	0.9903
Gelatin A, pH 6	0.9799	0.9937
Gelatin B, pH 4	0.9340	0.9923
Gelatin B, pH 6	0.9801	0.9937
Hydrocortisone loaded nanoparticles		
Cat CD, gelatin type B	0.9311	0.9825
Cat CD, gelatin type A	0.9029	0.9506
HP-β-CD, gelatin type A	0.9633	0.9365
HP-β-CD, gelatin type B	0.8662	0.9777

fast, and that pilocarpine is a relatively small molecule (with a molecular weight of 208 g/mol), one could assume that the drug is released by diffusion out of the particle matrix. A possible explanation for the zero order drug release could be as follows. In the donor compartment of the diffusion cell there is a balance between the pilocarpine present in the water phase and the drug present in the nanoparticles. Each time a part of the pilocarpine in solution diffuses to the acceptor compartment, a part of the drug encapsulated diffuses out of the particles, ensuring a constant concentration of drug in the donor phase. This could explain the zero order release pattern, where a constant amount of pilocarpine is transferred from the donor to the acceptor as a function of time.

4.4.2. Hydrocortisone-loaded nanoparticles

A graphical representation of the amount of hydrocortisone found in the acceptor compartment of the diffusion cell as a function of time is given in Fig. 5. After 5 h, almost 60% of the hydrocortisone is found in the acceptor compartment for the reference solutions of hydrocortisone complexed with HP-β-CD and Cat-CD. The particle preparations show a slower release, up to 30–40 % after 5 h. The preparations where HP-β-CD was used to complex the hydrocortisone show the slowest release, less than 30% after 300 min. When the cationic cyclodextrin was employed, over 30% of the drug was found in the acceptor compartment for the particles prepared with gelatin type B and more than 40% in the case of gelatin type A nanoparticles. Although the standard deviations of the measurements are quite high,

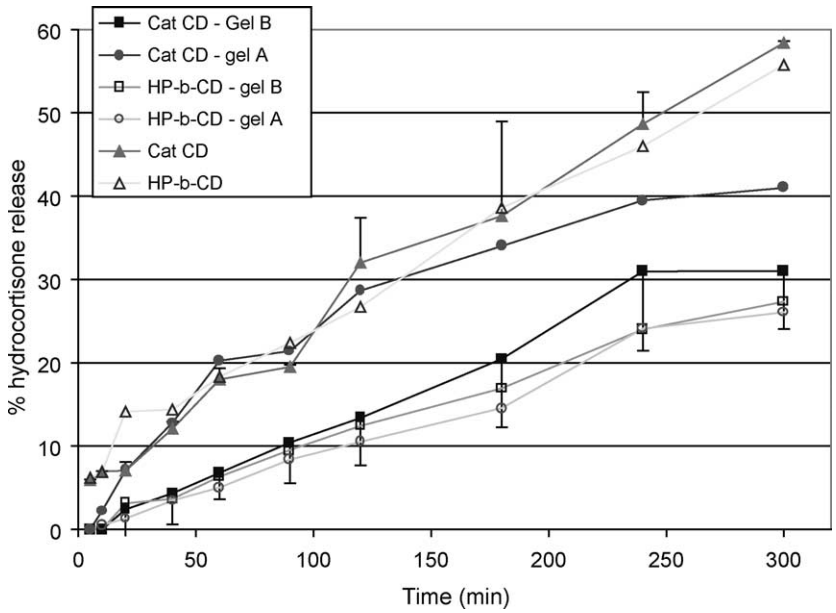


Fig. 5. Hydrocortisone release from gelatin nanoparticles (for the sake of clarity, only the error bars (S.D.) for the hydrocortisone–Cat CD complex and the same complex encapsulated in gelatin type B nanoparticles are shown).

there does seem to be an influence of the cylcodextrin type on the drug release. The effect is probably not due to the cyclodextrin alone, because then one would expect to see a difference in release between the two reference solutions as well, which is not the case. Probably, there is an interaction effect between the gelatin and the cyclodextrin, altering the release of the drug.

The drug release mechanism was also studied using the power law. The values of the kinetic exponents calculated are summarized in Table 8.

A value of 1 for n indicates a zero order release, while a value between 0.5 and 1 suggests an anomalous, non-Fickian release. The values observed for the hydrocortisone-loaded nanoparticles are close to 1, but not as close as the values found for pilocarpine HCl loaded nanoparticles. We can assume that the hydrocortisone release mechanism is anomalous, but close to zero order.

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