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Factorial design, physicochemical characterisation and activity of ciprofloxacin-PLGA nanoparticles

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Abstract

Poly(lactide-co-glycolide) nanoparticles incorporating ciprofloxacin HCl were prepared by means of a W/O/W emulsification solvent evaporation method. The stabiliser selected was poly(vinylalcohol). A 2^4 full factorial design based on four independent variables was used to plan the experiments and the variable parameters were the number of homogenisation cycles, addition of boric acid to the inner water phase containing the drug, ciprofloxacin concentration in the inner water phase and oil:outer water phase ratio. The effects of these parameters on the particle size, zeta potential, drug loading efficiency and drug release were investigated. Also the effect of gamma irradiation on the particle size and drug release was evaluated and DSC and XRD analyses of the compounds and the nanoparticles were performed. The activity on two series of microorganisms, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, was examined.

Keywords: PLGA; Nanoparticles; W/O/W emulsification solvent evaporation method; Ciprofloxacin; Experimental design

1. Introduction

Micro- and nanoparticles are frequently made of poly(lactic acid) (PLA) or its copolymer with glycolic acid (PLGA), due to the biocompatibility and biodegradability of these materials (Anderson and Shive, 1997). Other frequently applied synthetic polymers are poly(ε-caprolactone) (PECL), poly(alkylcyanoacrylates) (PACA) and Eudragit® (Sintzel et al., 1996; Pignatello et al., 2002; Vauthier et al., 2003). Also natural polymers like gelatine, albumin or chitosan can be employed (Vandervoort and Ludwig, 1999; De Campos et al., 2001; Merodio et al., 2002).

The use of these polymer particles is widespread and among other administration routes, their application can be ocular (Couvreur et al., 1995; Le Bourlais et al., 1998). Ophthalmic use of a dispersed system of nanoparticles overcomes problems of rapid elimination after instillation and low bioavailability of topical eye drops, due to the defence mechanisms of the eye, including reflex blinking, lachrymation and an increased tear flow, and the barrier function and low pain threshold of the cornea. Biodegradable nanoparticles have the advantage of a controlled release of the drug incorporated to obtain the required tear levels and therapeutic effects and the possibility of mucoadhesion

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to prolong the residence time of the particles in the precorneal area or of endocytosis through the cornea epithelium, depending on the particle size and size distribution (Zimmer et al., 1991; Calvo et al., 1996; zur Mühlen et al., 1998; Takeuchi et al., 2001; Romero-Cano and Vincent, 2002). The nanoparticles' physicochemical characteristics and therefore also the release kinetics can be controlled by modifying the preparation method or additives (Gabor et al., 1999).

PLGA was chosen in this research, because some other polymers employed show disadvantages for ocular application. PACA nanoparticles appeared not to be very well tolerated by the ocular mucosae, causing cell lysis (Vauthier et al., 2003). In contrast with the relative rapid biodegradation of PACA and PLGA, PECL is a slowly biodegradable carrier material and for albumin the release rate is difficult to control (Sintzel et al., 1996).

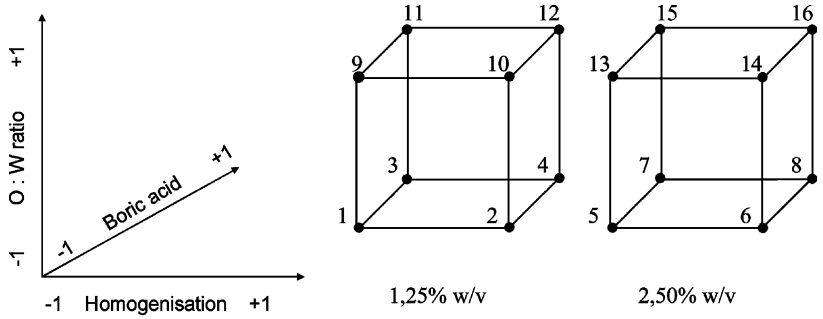
Ciprofloxacin, a frequently used antimicrobial agent in ophthalmology, was selected as drug for incorporation in the nanoparticles, because it has proven to be a powerful topical antibiotic for use as a single agent in the treatment of bacterial conjunctivitis and keratitis caused by *Staphylococcus aureus* and numerous gram-negative bacteria and because the frequency of spontaneous resistance to ciprofloxacin is very low (Limberg and Buggé, 1994). It has been reported that fluoroquinolones possess an in vitro efficacy against gram-negative as well as gram-positive ocular pathogens, superior to other antibiotics tested (Jensen and Felix, 1998; Egger et al., 2001).

In present research, we attempt to optimise the formulation of PLGA nanoparticles, containing ciprofloxacin, by trying to determine which factors influence the physicochemical properties of the nanoparticles formed. Among these properties are particle size, which controls the residence time of the particles at the eye surface and is an important parameter considering endocytosis and drug release kinetics, particle zeta potential, which influences physical stability and possible mucoadhesion, drug entrapment efficiency, which should be maximised and drug release kinetics, which should be optimised for the purpose. The factors examined were the number of homogenisation cycles, addition of boric acid to the inner water phase containing the drug, ciprofloxacin concentration in the inner water phase and oil:outer water phase ratio. A 2^4 full factorial design was used to plan and perform the experiments. This methodology allows the determination of the influence of the different factors on the nanoparticles' properties, requiring a minimum of experiments. The effect of gamma sterilisation of the particles on drug release and particle size was also investigated, considering sterility as a requirement of the pharmacopoeia for ophthalmological dosage forms. XRD and DSC measurements were conducted before sterilisation to determine the possible interactions between drug and PLGA polymer and to determine in which crystalline state ciprofloxacin HCl was present in the particles. Microbiological tests were performed before and after sterilisation to evaluate the activity of the nanoparticles against strains of *Pseudomonas aeruginosa* and *S. aureus*, two of the most common ocular pathogens causing bacterial infection of the human cornea (Armstrong, 2000; Callegan et al., 2000; Moreau et al., 2002). The activity of the nanoparticles was compared to a ciprofloxacin solution and blank nanoparticles without ciprofloxacin.

2. Materials and methods

2.1. Materials

The poly(lactic-co-glycolic acid) or PLGA polymer chosen was Resomer® RG 503 (Boehringer Ingelheim, Ingelheim am Rhein, Germany) with a molecular weight of 40,000 and a D,L-lactide:glycolide 52:48 molar ratio. Poly(vinylalcohol) or PVA MW 30,000–70,000 was purchased from Sigma Chemicals Co. (St. Louis, USA). Ciprofloxacin HCl was supplied by Roig (Barcelona, Spain). Dichloromethane was obtained from Sigma-Aldrich (Steinheim, Germany) and acetonitrile (HPLC grade) from Acros Organics (New Jersey, USA). Filtered (Porafil 0.20 Membranfilter, Düren, Germany) purified Milli Q water (Millipore, Mollsheim, France) was used throughout the experiments. Salts for the preparation of Simulated Lachrymal Fluid (SLF) were obtained from Merck (Leuven, Belgium) (KCl and NaCl), Merck (Darmstadt, Germany) ($NaHCO_3$) and Sigma Chemicals Co. (St. Louis, USA) ($CaCl_2$ and $MgCl_2$). SLF is an electrolyte solution composed of 1.7893 g/l KCl; 6.3118 g/l NaCl; 2.1842 g/l $NaHCO_3$; 0.0670 g/l $CaCl_2{\cdot}2H_2O$; 0.1572 g/l $MgCl_2{\cdot}6H_2O$, adjusted with



Homogenisation		Boric acid in W_1		Conc. cipro. HCl		O:W ratio	
1 cycle	3 cycles	without	with	1,25% w/v	2,50% w/v	1 : 5	2 : 5
-1	1	-1	1	-1	1	-1	1

Fig. 1. Cubes representing the different types of nanoparticles prepared, sample numbers from 1 to 16, axes standing for upper and lower level of three factors, both cubes representing the fourth factor. The values for the upper and lower level of the variables used in the experimental design are given in the table.

0.1N HCl to a pH of 7.4 ± 0.1 (Van Haeringen, 1981).

2.2. Factorial design of experiments: 2^4 full factorial design with one replica

Four different factors and their influence on the nanoparticles' properties were evaluated using a 2^4 full factorial design, composed of four variables which are set at two levels each. The four factors investigated were the number of homogenisation cycles, addition of boric acid to the inner water phase containing the drug, ciprofloxacin concentration in the inner water phase and oil:outer water phase ratio. The different preparations were made in duplicate in order to estimate the experimental error. The design required a total of 2^4 experiments with one replica each or in total 32 preparations. For each factor, the lower and higher value of the lower and upper level can be represented by a 1 or a −1 sign, which is shown in Fig. 1. To obtain a clearer view, the preparations of the factorial design can also be represented on cubes, where the different axes represent the different variables. Because there are more factors than axes on a 3D-graph, the different samples can be represented on the corners of two cubes.

A factorial design is frequently employed for the planning of a research because it provides the maximum of information, requiring the least experiments (Erden and Celebi, 1996; Vandervoort and Ludwig, 2002). To perform the statistical analysis of the data, the Statistica® software (Statsoft, Tulsa, USA) was used.

2.3. Preparation of ciprofloxacin-loaded PLGA nanoparticles

2.3.1. Preparation of ciprofloxacin solution

Solutions of two different concentrations of ciprofloxacin HCl (1.25 and 2.50% w/v) were prepared by dissolving the drug, with or without 1.65% w/v of boric acid, in the inner water phase W_1. The solutions were sonicated for 30 s at 20 W (amplitude 50%) and then for 45 s at 35 W (amplitude 70%) (Branson 450-D, 102-C with microtip, Branson, Danbury, USA).

2.3.2. W/O/W emulsification solvent evaporation method

The nanoparticles were prepared by W/O/W emulsification solvent evaporation followed by high-pressure homogenisation (Vandervoort and Ludwig, 2002). Two millilitres of an aqueous ciprofloxacin solution were emulsified by means of sonication for 1 min at 20 W (amplitude 50%) (Branson 450-D, 102-C with microtip, Branson) in an organic phase which consisted of 500 mg of PLGA dissolved in 5 or 10 ml of

dichloromethane. The resulting W/O emulsion was dispersed in 25 ml of the first outer water phase, a 1% w/v PVA stabiliser solution, and sonicated for 30 s at 15 W (amplitude 40%) to obtain a multiple W/O/W emulsion, which was homogenised employing a Microfluidizer M-110L (Microfluidics, Newton, USA) at a pressure of 50 bar for one or three cycles. The emulsion was then diluted in the second outer phase, W_3, consisting of 120 ml 0.3% w/v of PVA in water in order to minimise coalescence of the emulsion and aggregation of the particles formed. The organic solvent was allowed to evaporate during 4 h at room temperature under agitation (700 rpm) with a magnetic stirrer (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Münich, Germany). Consequently the polymer, insoluble in the water phase, precipitated as solid particles. The resulting nanosuspension was subsequently cooled down to −18 °C and freeze-dried (Leybold-Heraeus D8B, GT-2A, Germany). Each sample was prepared in duplicate.

2.4. Nanoparticle size and zeta potential analysis

The mean particle size Z_{ave} of the nanoparticles was determined by Photon Correlation Spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). A portion of the freshly prepared suspension was diluted hundred times with filtered purified water before measuring. The Z_{ave} of each sample was determined four times and the average value was used for calculations in the factorial design and plotting of response surfaces. Afterwards the mean particle size of two replicas was calculated.

The zeta potential of the nanoparticles was determined using Electrophoretic Light Scattering (ELS). Five milligrams of freeze-dried nanoparticles were suspended in 10 ml of Simulated Lachrymal Fluid. This dispersion was injected in the capillary of the Zetasizer 3000 and determined 20 times. Afterwards, the average value was used for calculations in the factorial design and plotting of response surfaces. The mean zeta potential values for two replicas were also calculated.

2.5. Drug loading determination

Twenty milligrams of freeze-dried nanoparticles, accurately weighed, were dispersed in 10.0 ml of purified water and gently sonicated for 10 min (Julabo USR3, Julabo, Seelbach, Germany). The samples were centrifuged at 3000 rpm for 3 h (Cetra-MP4 centrifuge, International Equipment Company, Miami, USA) and the ciprofloxacin HCl concentration in the supernatant was determined by a validated HPLC method, like described by Barichello et al. (1999) for other drugs with comparable water solubility to ciprofloxacin. The HPLC system consisted of a Gilson 321 pump (Gilson, Villiers-le-Bel, France), a UV-Vis 152 detector (Gilson, Villiers-le-Bel, France), a μ Bondapack™ C_{18} 125 Å 10 μm column (Waters, Milford, USA) and an HP 3395 integrator (Hewlett-Packard Company, Palo Alto, USA). The mobile phase and flow rate used corresponded to the monograph in the European Pharmacopoeia (1997). Ciprofloxacin HCl was detected at 278 nm and the concentration in the supernatant was calculated using a calibration curve.

The entrapment efficiency or EE of the samples was determined using the following equation: EE (%) = (actual drug loading/theoretical drug loading) × 100%.

2.6. In vitro release tests

The in vitro release experiments were carried out using diffusion cells, whereby a dialysis membrane with a MWCO of 12,000–14,000 Da (Medicell International, London, UK) separated the donor compartment, consisting of 50.00 mg of nanoparticles, dispersed in 5.0 ml 5.07% w/v mannitol solution in water, and the acceptor compartment, filled with 18.0 ml of Simulated Lachrymal Fluid. The acceptor compartments were stirred magnetically at 200 rpm (Variomag Electronicrührer Poly 15, H + P Labortechnik GmbH, Münich, Germany). At suitable time intervals samples of 1.0 ml were withdrawn from the acceptor compartment and replaced by the same volume of SLF-solution. The drug content of the samples was determined by the above described HPLC method.

2.7. Gamma irradiation

Some of the samples were gamma-irradiated by using Co^{60} as irradiation source (Gammir I-Sulzer irradiation unicell, IBA-Mediris, Fleurus, Belgium) and received a dose of 25 kGy, considered as adequate for the purpose of sterilising pharmaceutical products

when the bioburden is not known, according to the European Pharmacopoeia.

2.8. Differential scanning calorimetry (DSC) analysis

DSC experiments were carried out to determine the possible interactions between drug and PLGA polymer and to determine in which crystalline state ciprofloxacin HCl was present in the particles. Five to ten milligrams of the PVA, PLGA or ciprofloxacin powders or of the lyophilised samples were put in aluminium pans (TA Instruments, Brussels, Belgium) and were hermetically sealed. The heating rate was 5 °C/min, nitrogen served as purge gas and the system was cooled by means of liquid N_2. The DSC-7 calorimeter (Perkin-Elmer, Norwalk, USA) was calibrated for temperature using octadecane and indium, and for enthalpy using indium, sealed in aluminium pans with a sealed empty pan as a reference.

2.9. X-ray diffraction (XRD) analysis

X-ray powder diffractometry analyses were performed to find out whether ciprofloxacin HCl was present in the nanoparticles in a crystalline or an amorphous state and to confirm the results obtained with DSC. A Philips PW diffractometer (beam 173 nm) was used and samples were exposed to monochromatic Cu Kα radiation (0.45 kV × 20 mA, $\lambda = 1.5406$ Å) obtained by Ni filtration and a system of D/R/S slides of 1°, 0.2 mm and 1°, respectively. The diffraction pattern was determined in the area $4^\circ < 2\theta < 65^\circ$, using a stepwise method (0.2°/s).

2.10. Determination of the antibacterial activity of ciprofloxacin-loaded nanoparticles

The antimicrobial effectiveness of the nanoparticles was assessed in comparison with the free drug (ciprofloxacin aqueous solution) and blank nanoparticles without ciprofloxacin by measuring the minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) on *P. aeruginosa* (ATCC 9027) and *S. aureus* (ATCC 6538). The microorganisms chosen are two of the most common ocular pathogens causing bacterial infection of the human cornea (Armstrong, 2000; Callegan et al., 2000; Moreau et al., 2002).

In this way, the activity of the nanoparticles can be compared with that of a ciprofloxacin solution with the same drug concentration and changes in MIC and MBC, related to the dosage form or sterilisation process can be detected. It was also checked whether blank nanoparticles, and thus the PLGA polymer or its acid degradation products, exhibited any antibiotic effect, which could interfere with the determination of the activity of the drug. Since the potency of the released drug to kill microorganisms can be proven, this microbiological assay is supplementary to the in vitro release tests with diffusion cells, which consisted of a chemical analysis of the drug contents via HPLC.

The MIC values (μg/ml), being the lowest concentrations of antibiotic inhibiting visible growth after 24 h of incubation at 37 °C, were determined in Tryptone Soy Broth (E & O Laboratories, Bonnybridge, Scotland) in 24 Well Cell Culture Clusters (Corning Incorporated, Corning, USA) after serial two-fold dilutions of the drug solution and nanoparticles' dispersions (Page-Clisson et al., 1998). The dilutions were prepared on day 1, inoculation with microorganisms occurred on day 1, 2, 3 or 4, and the wells were inspected for turbidity 24 h after inoculation. The MBC values (μg/ml) were determined by subculturing the negative dilutions on TSA plates without antibiotic and inspecting for formation of colonies after 24 h of incubation at 37 °C. The MBC technique establishes the lowest level of a bactericidal agent which will kill at least 99.9% of the organisms in a standard inoculum, whereby growth of organisms which were inhibited but not killed can be detected.

A positive control for growth consisted of *P. aeruginosa* or *S. aureus* in broth and a negative control for sterility consisted of uninoculated broth.

3. Results and discussion

3.1. Physical characterisation

3.1.1. Size before sterilisation

The particle size is an important parameter, as the biopharmaceutical properties of a nanoparticles formulation can be influenced by its physicochemical properties. For example, smaller particles possess a larger surface area, which in turn can lead to a faster release of the drug incorporated. In this way, the size

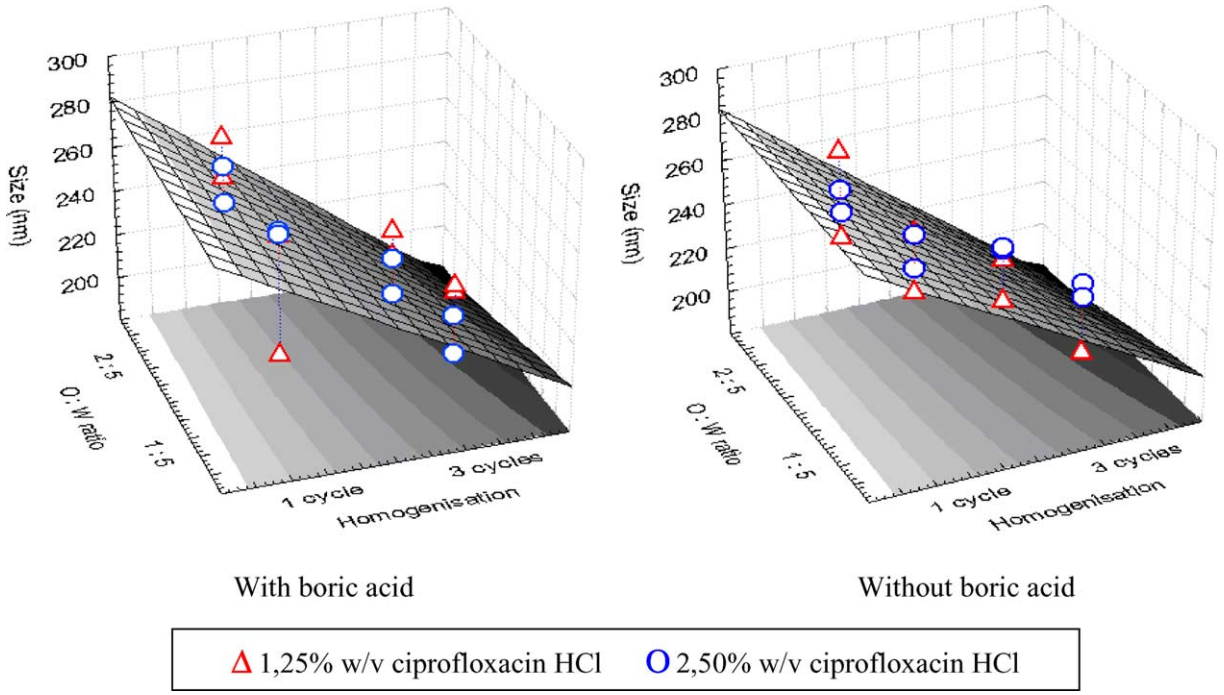


Fig. 2. Graphical representation of the particle size of PLGA nanoparticles in a 3D surface plot.

and release properties of the nanoparticles are interrelated (Yoncheva et al., 2003). The size can also play an important role in endocytosis possibilities of nanoparticles (Zimmer et al., 1991; Calvo et al., 1996).

The sizes of the samples and replicas are graphically represented in Fig. 2. The effect of homogenisation on the nanoparticles' size is visible in the plane of the surface plot that slopes downwards towards more homogenisation cycles, which shows that homogenisation has a marked influence. The effects of the other factors were negligible. The effect of the different factors of the design on the size of the nanoparticles is summarised in Table 3. Particle sizes ranging from 180 to 275 nm were measured. For the particles which were homogenised for three cycles instead of one, a significant reduction in particle size was observed. This was due to the fact that during a larger number of homogenisation cycles, smaller emulsion droplets were formed, resulting in smaller nanoparticles. In Table 3, the negative effect of homogenisation on particle size is shown, which means that in this case, the mean particle size of 234.68 nm was reduced with 46.02 nm when the particles were homogenised for two more cycles. Particle size can thus be controlled by adapting the number of homogenisation cycles. This is comparable to the influence of stirring rate on the particle size, like reported by O'Donnell and McGinity (1997). It was found that the other variable factors did not have a significant ($P < 0.05$) influence on the size of the nanoparticles, although the same authors reported a decrease in the mean diameter of microparticles, when the volume of solvent increased, like in our case from an *O*:*W* ratio 1:5 to 2:5, due to a change in viscosity. However, their preparation method consisted of O/W emulsification, without an inner water phase. The unchanged size as a function of ciprofloxacin HCl content and aqueous to organic phase ratio is in agreement with the work of Chorny et al. (2002).

3.1.2. Size after sterilisation

Some of the samples were gamma-irradiated, in order to determine the influence of sterilisation on particle size and on drug release, which will be discussed later. The size of the investigated samples increased slightly, meaning that an aggregation of the nanoparticles occurred. A *t*-test for independent

Table 1
Mean size ($n = 4$) and standard deviation of the nanoparticles before and after gamma sterilisation

Sample number	Mean size (nm) before gamma	S.D.	Mean size (nm) after gamma	S.D.	*P*-value
5	255.8	4.57	295.1	2.56	**5.51E−06**
15	246.3	26.91	286.2	7.76	**0.03**
Replica 5	270.4	1.65	308.9	1.96	**9.03E−08**
Replica 15	262.7	10.83	281.4	1.70	**0.01**

Statistically significant *P*-values ($P < 0.05$) are printed in bold, sample numbers correspond to those given in Fig. 1.

samples has been conducted using the Statistica® software (Statsoft, Tulsa, USA). The results of this analysis are presented in Table 1. From this table, one can conclude that there was a significant increase in particle size after gamma sterilisation. This is in agreement with the results of Montanari et al. (2001), who reported a change in particle size distribution after gamma-irradiation of clonazepam-loaded PLGA microspheres.

3.1.3. Zeta potential measurement

The zeta potential is also an important physicochemical characteristic of the nanoparticles. The charge of the particles can influence the stability of the formulation: extremely positive or negative zeta potential values cause larger repulsive forces. An eventual interaction with the mucus layer at the eye surface occurs especially with positively charged particles. The zeta potential of the nanoparticles was examined in Simulated Lachrymal Fluid, which possessed a similar electrolyte composition compared to physiological tear fluid. The results from the zeta potential measurements are given in Table 2. The size of the effects of the different parameters on the zeta potential is shown in Table 3. The values of the zeta potential of all particles were approximately the same in SLF and varied between individual extreme values of −3.8 and −10.7 mV. The relative high salt concentration of SLF caused the equalising of the zeta potential values, due to the fact that ions of the medium compressed the electrical double layer which surrounds the dispersed particles, thereby making their surface charges similar. Only a small difference in zeta potential was obtained between the different kinds of nanoparticles prepared. The zeta potential slightly diminished when the emulsion was homogenised for three cycles instead of one, but nor this effect neither the other factors' effects were significant ($P < 0.05$). An influence of the concentration of the cationic drug ciprofloxacin HCl on the zeta potential could be expected when the drug would be present at the particle surface. Since such an effect was not observed, almost the entire portion of the drug was probably incorporated inside the nanoparticles. Also the number of homogenisation cycles did not influence the particle's charge, although one could expect this to happen, since the nanoparticles' size decreased, and the total free surface increased in this way. Homogenisation could thus have affected the surface charge, but this was not the case.

3.2. Drug loading of the nanoparticles

Drug loading levels of the PLGA nanoparticles, expressed as entrapment efficiency percentages and absolute drug loadings, are graphically presented in

Table 2
Mean zeta potential values of the various nanoparticles preparations ($n = 2$)

	Zeta potential of PLGA nanoparticles (mV)			
	Without boric acid		With boric acid	
Homogenisation	One cycle	Three cycles	One cycle	Three cycles
1,25% cipro (*O*:*W* 1:5)	−5.2	−6.5	−5.5	−6.5
1,25% cipro (*O*:*W* 2:5)	−5.6	−6.5	−5.6	−6.3
2,50% cipro (*O*:*W* 1:5)	−6.2	−6.0	−5.6	−5.9
2,50% cipro (*O*:*W* 2:5)	−5.1	−6.7	−5.4	−7.9

Table 3
Effects of the different independent factors on the properties of the nanoparticles investigated

Parameter	Mean value	Homogenisation	Boric acid	Conc. drug	*O*:*W* ratio
Size	234.68 nm	**−46.02**	4.31	3.48	−5.13
Zeta	−6.01 mV	−1.03	−0.12	−0.16	−0.23
EE	61.72%	**18.15**	0.87	−8.52	−8.03
Drug loading	18.94 μg/mg	**6.06**	−0.67	**9.92**	−3.65
Release 6.5 h	12.97%	**−10.93**	**5.91**	2.92	**7.16**
Release 24 h	26.05%	**−18.49**	4.80	7.86	**12.14**

Statistically significant effects ($P < 0.05$) are printed in bold.

Fig. 3. In Table 3, an overview of the effects of the different variable parameters on the drug encapsulation is given. Entrapment efficiencies between 28 and 87% and absolute drug loading levels between 6 and 37.5 μg drug/mg nanoparticles were measured. The only parameter having an effect on the drug entrapment was the number of homogenisation cycles. The effect was positive, which means that higher entrapment efficiencies were determined for nanoparticles which had undergone three homogenisation cycles. The mean entrapment efficiency increased from almost 62% towards 80% when increasing the number of homogenisation cycles. The higher homogenisation intensity resulted in a reduction in size of the emulsion droplets and an increased surface area. The resulting accelerated dichloromethane removal led to increased encapsulation efficiency (Castellanos et al., 2001). Boric acid was added to the inner water phase because, according to Sun et al. (2002), the partition coefficient of ciprofloxacin in an *n*-octanol/buffer system reached its maximum value around the p*I*, being 7.46, and reached its minimum values above pH 10 or below pH 5. Therefore, a boric acid solution with a pH of 4.8 was chosen due to its widespread use in ocular formulations. In this acidic environment, ciprofloxacin is better soluble than in the outer water phase, an aqueous PVA-solution, with a pH value of about 7. The tendency of the drug to diffuse from the inner towards the outer water phase during preparation of the nanoparticles would then decrease. In this way, the entrapment efficiency would increase. However, this expected effect was not observed, like previously reported by Chorny et al. (2002). Changing the *O*:*W* ratio did not have a significant ($P < 0.05$) effect on the drug entrapment in the nanoparticles. Even though the entrapment efficiencies did not change significantly when the drug concentration in the inner water phase was increased from 1.25 to 2.5% w/v, the mean absolute drug loading increased significantly from 19 to 29 μg drug/mg nanoparticles. The explanation is that the same percentage of the drug was encapsulated employing either of both ciprofloxacin concentrations, so the absolute amount of drug in micrograms per milligram nanoparticles increased when the concentration of ciprofloxacin in the inner water phase increased. In Table 3, which shows the effects of the different factors, both homogenisation and drug concentration have significant ($P < 0.05$) effects on absolute drug loading. The mean drug loading of almost 19 μg/mg nanoparticles was increased with 6 and 10 μg/mg, respectively.

3.3. Release of ciprofloxacin HCl

3.3.1. Release before sterilisation

A summary of the effects of the different parameters on the average release values after 6.5 and 24 h is presented in Table 3. These time intervals were chosen because the effects caused by the variation of the different parameters were clearer after longer time periods.

The results showed that addition of boric acid had a slightly positive influence on the drug release, but only after 6.5 h. This means that when boric acid was present in the inner water phase, the drug was released faster out of the nanoparticles. This can be due to the fact that, like reported in the review of O'Donnell and McGinity (1997), buffers or salts added to the internal aqueous phase promoted an influx of water from the external phase due to the difference in osmotic pressure, which resulted in more porous particles and caused a faster drug release. Boric acid can also influence the hydrolysis rate of PLGA, although Tsuji and Nakahara (2002) proved similarity in hydrolysis

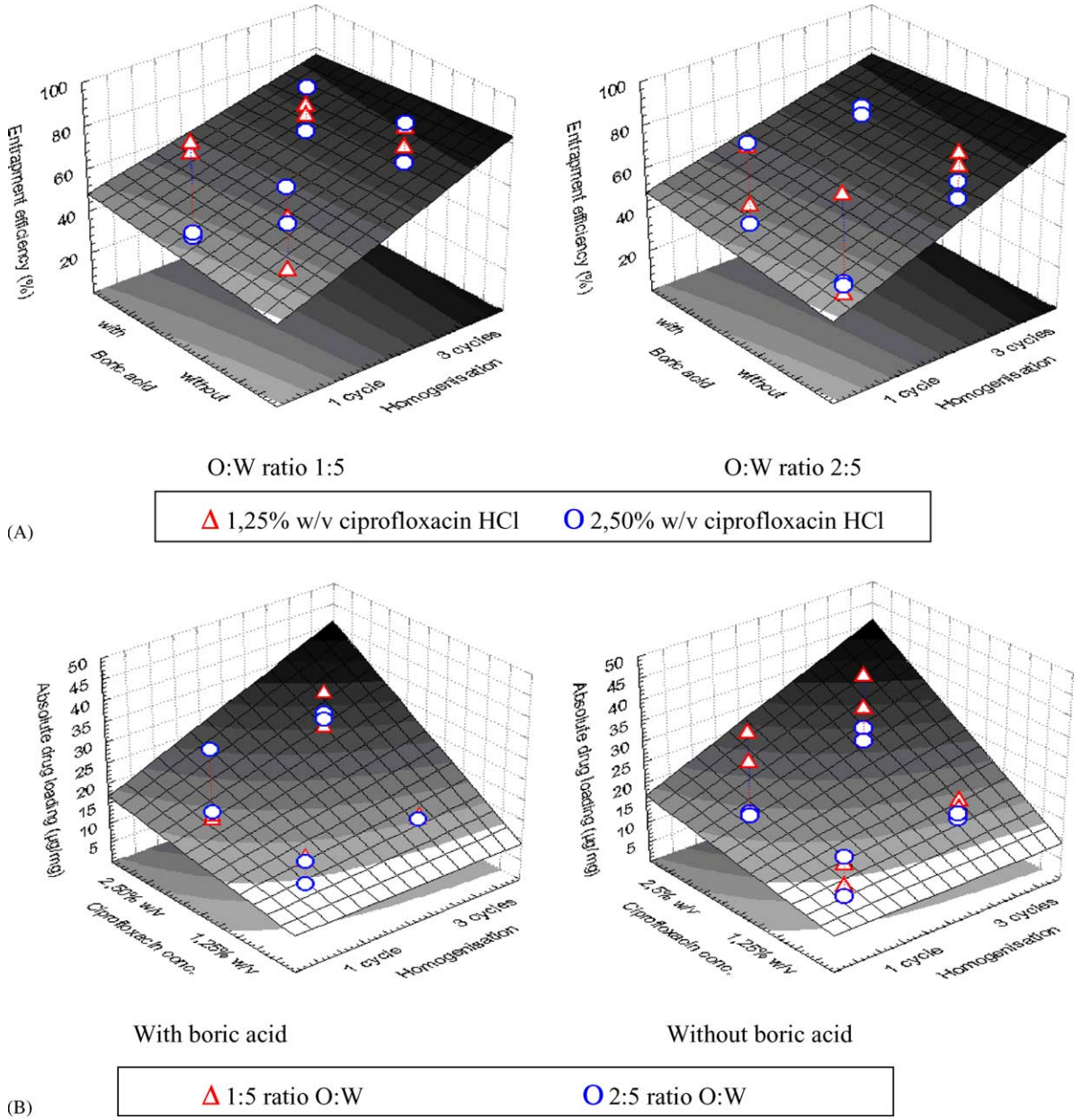


Fig. 3. Three dimensional surface plots of entrapment efficiencies (A) and absolute drug loading amounts (B) of the nanoparticles prepared.

rate constant values (k) between poly(L-lactide) films at pH 2 and pH 7.4.

Homogenisation had a negative influence on the drug release; this retardation can be explained by the fact that during the homogenisation process, emulsion droplets are smaller when three cycles are applied instead of one cycle. One would expect that drug release would be faster, unless the resulting nanoparticles are not only smaller but the polymer would also be more tightly packed. Ueda and Kreuter (1997) have

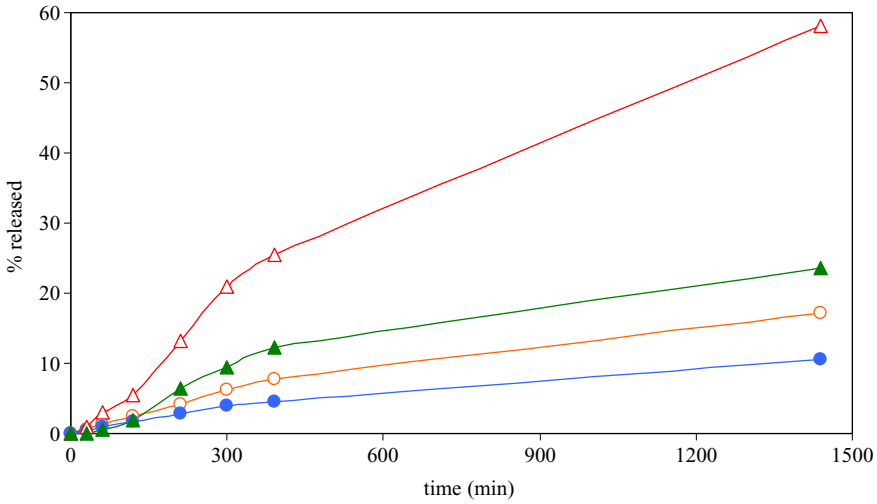


Fig. 4. Drug release profiles of nanoparticles with high ciprofloxacin HCl concentration and no boric acid in the inner water phase. Open symbols: one homogenisation cycle; filled symbols: three homogenisation cycles; circles: ratio *O*:*W* 1:5; triangles: ratio *O*:*W* 2:5.

reported that beside the surface area of nanoparticles, their dense matrix structure could also influence the drug release process. The opposite result would however also be possible, since smaller particles possess a larger surface area. The effect of homogenisation on drug release was thus contradictory, since an increase of the number of homogenisation cycles did not only have an effect on the size of the nanoparticles, but also on the drug encapsulation efficiency, which had in turn an effect on the release of ciprofloxacine. In this way, it is possible that the effect of homogenisation on drug release was not direct, but instead influenced by the other physicochemical parameters.

When changing the *O*:*W* ratio from 1:5 to 2:5, a faster release of ciprofloxacine out of the nanoparticles was observed, like already pointed out by Chorny et al. (2002). More dichloromethane was present in the formulation; evaporation thus took a longer time and hardening of the particles was slower. In this way, the nanoparticles possessed a more porous structure, resulting in an increase of the diffusion rate compared to particles with a denser structure.

Like previously reported by Hyon (2000), the concentration of drug in the inner water phase had no effect on drug release percentages.

An illustration of the resulting profiles of the in vitro release experiments is presented in Fig. 4. The drug release from particles with an *O*:*W* ratio 2:5 which have undergone one homogenisation cycle is the highest, the drug release from nanoparticles with an *O*:*W* ratio 1:5 which have undergone three homogenisation cycles is the slowest. Particles with *O*:*W* ratio 2:5 submitted to three cycles or *O*:*W* ratio 1:5 submitted to one homogenisation cycle show an intermediate release rate. The drug release out of the particles can thus be controlled by varying the number of homogenisation cycles or the *O*:*W* ratio.

3.3.2. Release after sterilisation

From the comparison of the drug release profiles before and after sterilisation (Fig. 5) can be concluded that, except for one sample, drug release after gamma sterilisation of the nanoparticles is faster. According to Nugroho et al. (2001), chain scission takes place within the polymer during irradiation. The free radicals, which are hence formed, can recombine and crosslink or can stay separated, which causes a decrease in the polymer's MW, a faster polymer degradation and thus a faster drug release. This assumption is in agreement with the results from Montanari et al. (2001), who evaluated the effects of gamma irradiation on the stability of clonazepam-loaded PLGA microspheres. These authors found that the release rate increased slightly after irradiation.

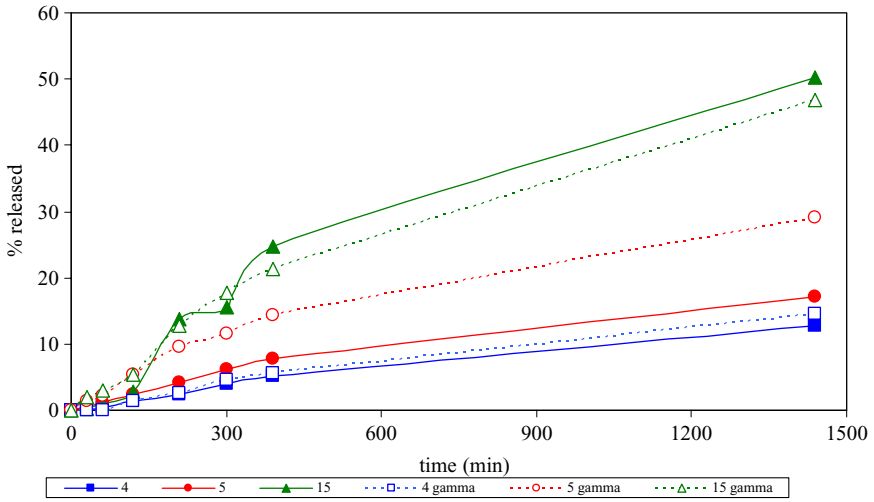


Fig. 5. Mean drug release ($n = 2$) of samples before and after gamma-irradiation. Filled symbols, full line: before; open symbols, dotted line: after gamma-irradiation. Sample numbers correspond to Fig. 1.

3.4. Differential scanning calorimetry (DSC) and X-ray diffraction (XRD)

To determine whether the ciprofloxacin HCl was incorporated in the nanoparticles in its crystalline or amorphous form, an XRD- and a DSC-study were conducted.

The graphs depicted in Fig. 6 show the XRD patterns of PVA, PLGA and ciprofloxacin powder and of the nanoparticles. It appeared that PVA and PLGA were amorphous polymers and that the ciprofloxacin powder was crystalline. The graph of the nanoparticles clearly demonstrates that they consisted of a mixture of PLGA and PVA, since the graphs of these two compounds can be superimposed to form the graph of the nanoparticles. Also the conclusion can be drawn that the drug is present in the nanoparticles in an amorphous state, since no crystalline diffraction pattern is distinguishable.

DSC is a thermal analytical technique which provides information about the physical properties of products, for example about the crystalline or amorphous nature of the samples. Quantitative information about exothermic, endothermic and heat capacity changes in function of temperature or time is provided (Clas et al., 1999). DSC can also demonstrate a possible interaction between different compounds of a mixture (Fathy et al., 2002). The DSC curves of PVA, PLGA and ciprofloxacin HCl are depicted in Fig. 7. It was demonstrated that PVA was an amorphous polymer with a T_g of 29 °C. PLGA was also amorphous and the T_g of this polymer amounted 44 °C. The DSC curve showed also a large relaxation endotherm. The curve of the drug showed two endothermic peaks. The one at 156 °C represents the dehydration peak; the one at 330 °C corresponds to the endothermic melting peak. The melting of the drug was followed by thermal decomposition.

DSC curves of the nanoparticles are presented in Fig. 8. In Fig. 8A, a curve is shown of a sample in which two separated T_g's at 27 and 39 °C for PVA and PLGA are visible. Ciprofloxacin was present as an amorphous compound. The T_g of the amorphous ciprofloxacin could not be measured, since the melt of the drug decomposed. In Fig. 8B, one single T_g between that of the two polymer components is visible at 36.6 °C. DSC analyses did not show interactions between the drug ciprofloxacin and the polymers PLGA and PVA. The absence of plasticization effects suggested the absence of interactions between the polymer and the drug. The interaction between PVA and PLGA was rather small. Since in some of the samples the glass transition of the polymers was not changed, the drug was present as a separate amorphous phase in the nanoparticles, like recently reported by Passerini and Craig (2002). The authors

described MTDSC, SEM and XRD analyses on ciclosporin A-loaded PLGA microspheres. Contrary to the ciprofloxacin in the nanoparticles of present study the ciclosporin starting material was already present in a semi-crystalline form, during the preparation process however, the drug was transformed to an amorphous state.

3.5. Antibacterial activity of the ciprofloxacin-loaded nanoparticles in vivo

The antibacterial effectiveness of ciprofloxacin HCl loaded into nanoparticles was assessed in comparison with free drug in aqueous solution and blank nanoparticles using a microbiological method. An

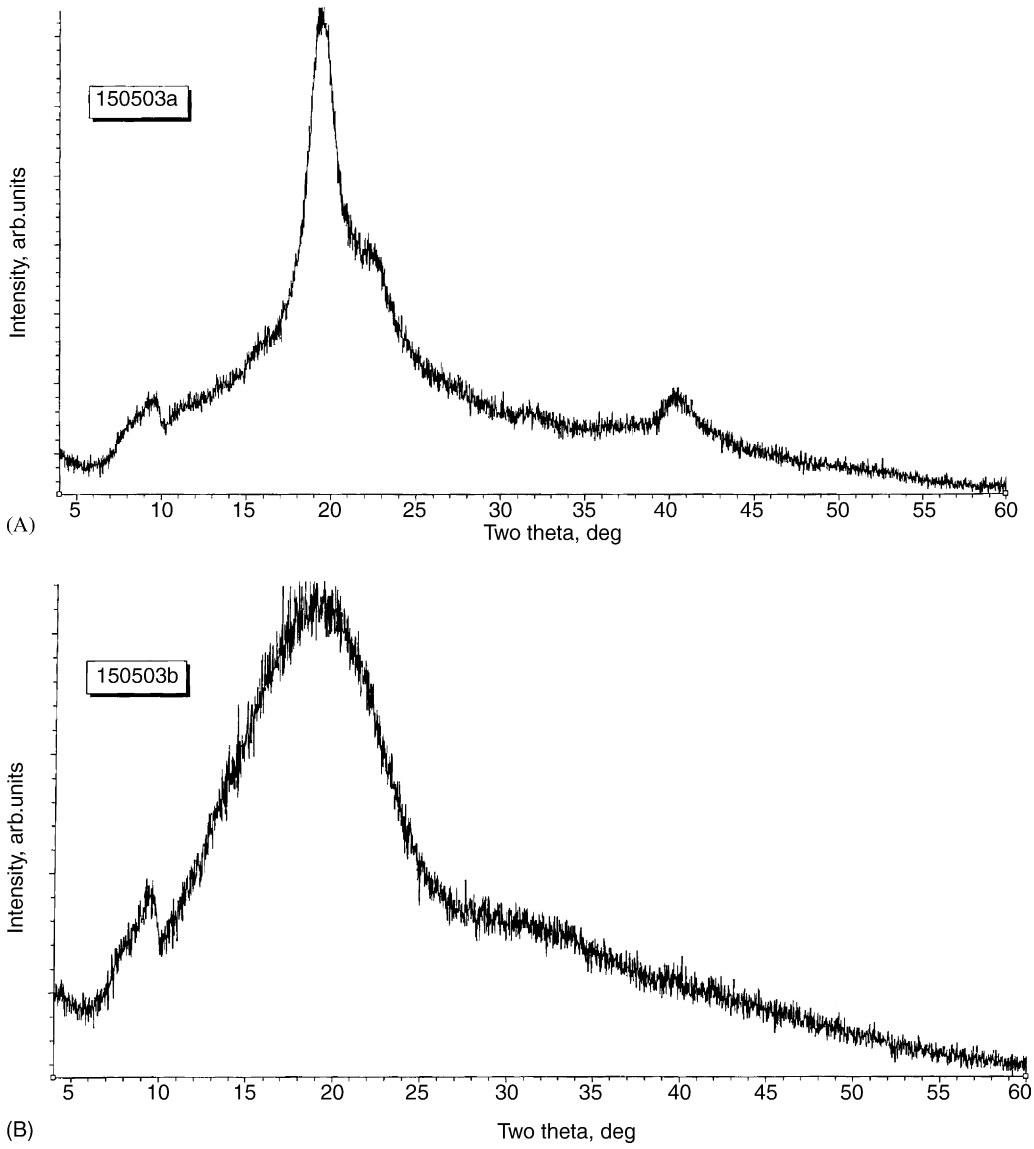


Fig. 6. Powder X-ray diffraction patterns of PVA (A), PLGA (B), ciprofloxacin HCl (C) and of the nanoparticles (D).

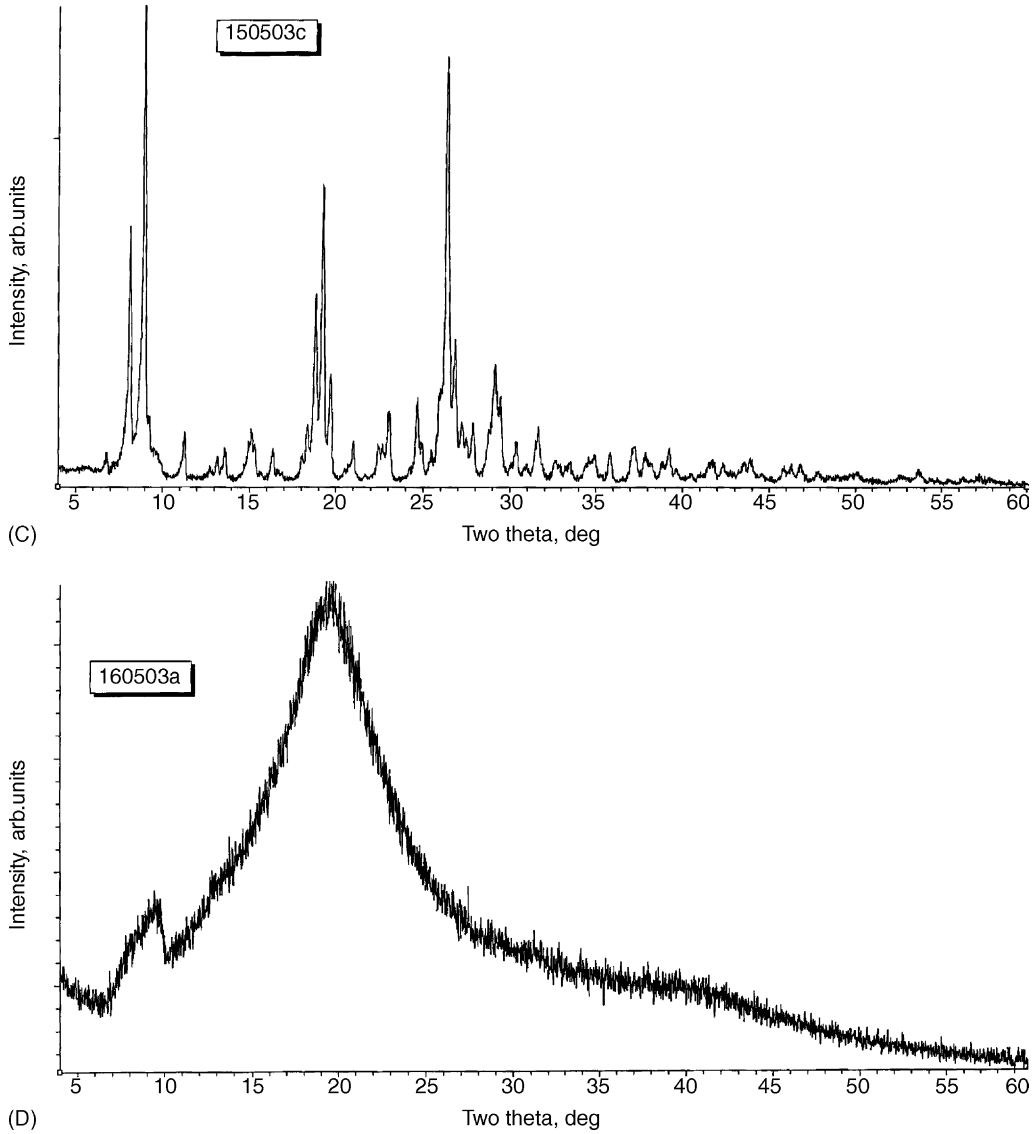


Fig. 6. (*Continued*).

overview of the results is presented in Table 4. The MIC and MBC values were followed during 4 days, but did not change as a function of time neither of the solution nor of the nanoparticles. The MIC values lay between 0.146 and 0.293 μg/ml for *P. aeruginosa* and 0.586 and 1.17 μg/ml for *S. aureus*, whatever the form used. For *P. aeruginosa*, the MBC value of the ciprofloxacin solution varied between 0.293 and 0.586 μg/ml and between 0.293 and 1.17 μg/ml for the nanoparticles. For *S. aureus*, MBC values lay between 0.586 and 1.17 μg/ml. So, only the MBC value for *P. aeruginosa* depended on the dosage form used, the MIC for *P. aeruginosa* and the MIC and MBC values for *S. aureus* were comparable between the solution and the nanoparticles. The blank nanoparticles did not show any antimicrobial activity, though the acid environment after degradation and release of lactic and glycolic acid could have inhibited bacterial growth. Thus, the microbiological assay demonstrated that the incorporation of ciprofloxacin HCl into

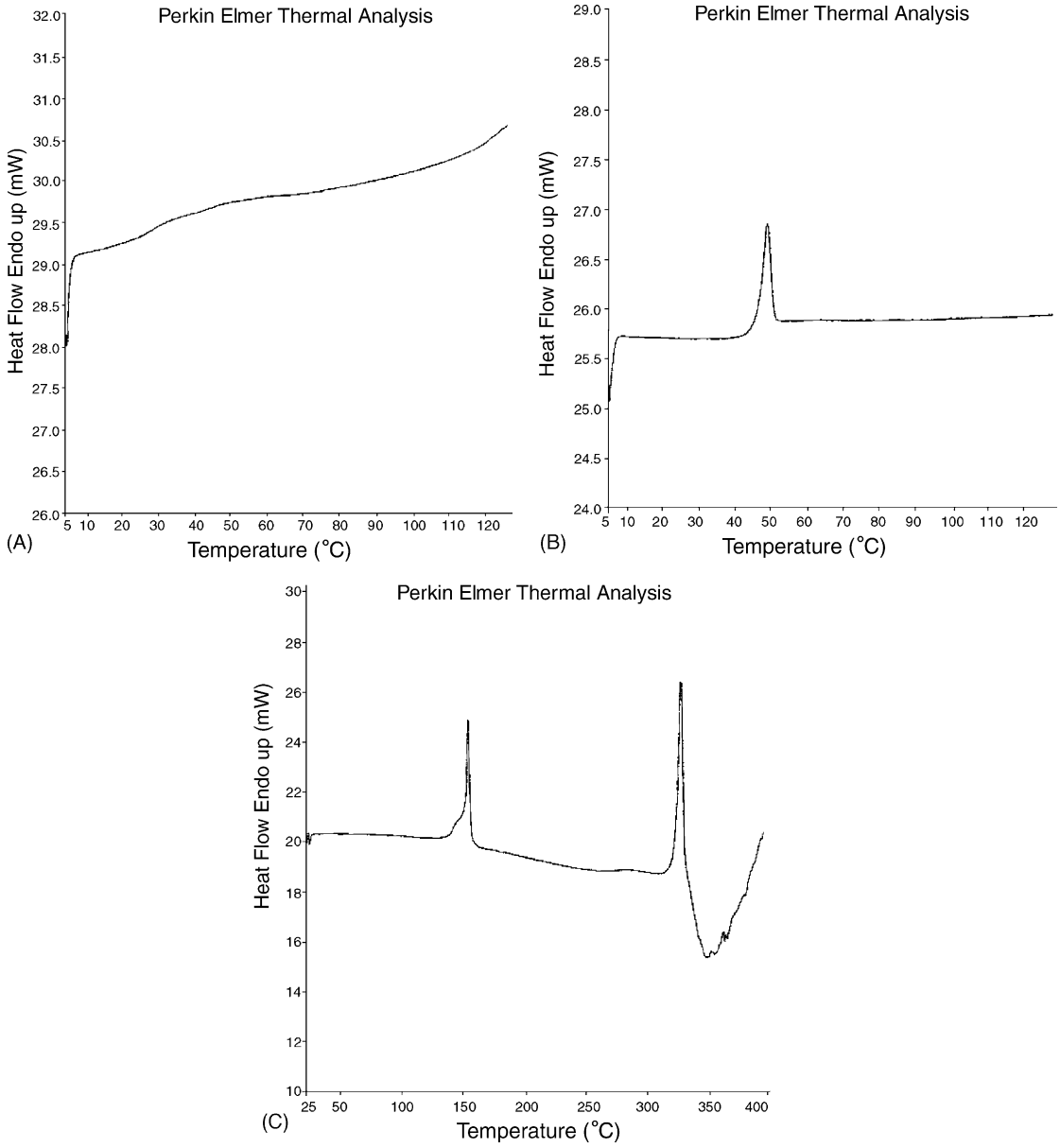


Fig. 7. DSC curves of PVA (A), PLGA (B) and ciprofloxacin HCl (C).

PLGA nanoparticles did not change either its MIC or its MBC for *S. aureus*. For *P. aeruginosa*, MIC values remained unchanged, though the MBC value increased when nanoparticles were applied instead of the aqueous solution. Gamma irradiation did not have any influence on the MIC and MBC values of the nanoparticles, at least for the microorganisms examined here. One can conclude that although the drug has not been released for 100% after 24 h (Table 5), the concentration released is large enough to kill microorganisms to the same extent as the aqueous solution.

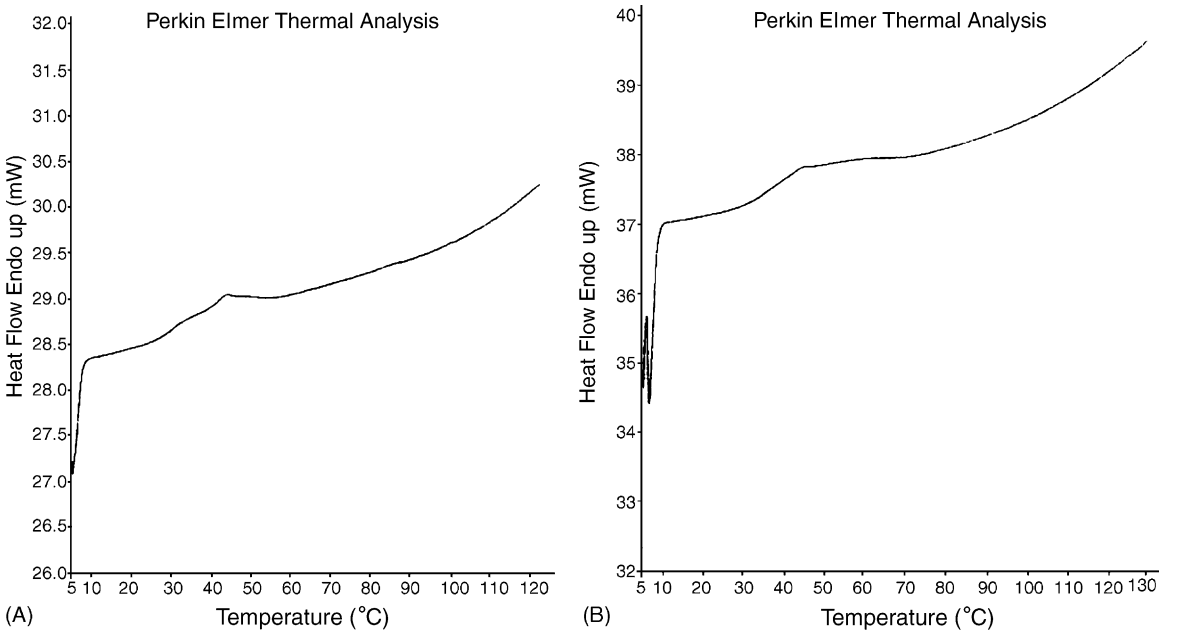


Fig. 8. DSC curves of the nanoparticles.

Table 4
MIC and MBC values in μg/ml of the antimicrobial agents on *Pseudomonas aeruginosa* (Ps. aer.) and *Staphylococus aureus* (St. aur.)

Bacteria	Cipro aq. sol.		NPs		NPs γ-ster.	
	MIC	MBC	MIC	MBC	MIC	MBC
Ps. aer.	0.146–0.293	0.293–0.586	0.293	0.293–1.17	0.146–0.293	0.293–1.17
St. aur.	0.586–1.17	0.586–1.17	0.586–1.17	0.586–1.17	0.586–1.17	0.586–1.17

Cipro aq. sol.: ciprofloxacine HCl aqueous solution; NPs: nanoparticles; NPs γ-ster.: gamma-sterilised nanoparticles.

Table 5
Mean drug release after 24 h, determinations in duplo

	Drug release of PLGA nanoparticles after 24 h (%)			
	Without boric acid		With boric acid	
Homogenisation	One cycle	Three cycles	One cycle	Three cycles
1,25% cipro (*O*:*W* 1:5)	31.25	11.25	12.76	12.73
1,25% cipro (*O*:*W* 2:5)	20.94	15.43	49.13	23.50
2,50% cipro (*O*:*W* 1:5)	17.19	10.66	42.91	21.16
2,50% cipro (*O*:*W* 2:5)	58.04	24.54	50.20	15.22

4. Conclusions

Poly(lactide-co-glycolide) nanoparticles incorporating ciprofloxacin HCl were prepared by the W/O/W emulsification solvent evaporation method, followed by high pressure homogenisation. After testing the effects of different preparation factors on the nanoparticles' physicochemical properties, the following conclusions can be drawn. Homogenisation decreased the particle size and release rate of ciprofloxacin, but increased the entrapment efficiency. Addition of boric acid to the inner water phase increased drug release rates, but only after 6.5 h. In terms of formulation, this parameter has not been very

useful. The *O*:*W* ratio influenced the drug release rate in a way that drug release was faster when the volume of the organic phase was larger. An increase in concentration of ciprofloxacin seemed to have no influence on the properties investigated, although the absolute drug loading increased when the concentration of the drug in the preparation increased. None of the factors studied was able to give rise to a change in zeta potential values, when measured in Simulated Lachrymal Fluid. The drug release rate was faster after gamma sterilisation, and particle size increased slightly. The optimal parameters for this ocular drug delivery system would be: three homogenisation cycles, the lowest *O*:*W* ratio used, the highest drug concentration and no boric acid in the inner water phase. A combination of these parameters gives rise to a small, reproducible particle size with narrow size distribution and a high entrapment efficiency. In vivo studies should be performed and tear levels measured to know which release kinetics are optimal to treat ocular infections.

DSC and XRD experiments revealed that the drug was present in the nanoparticles in an amorphous state and that no interactions between drug and polymer were measurable. Microbiological activity tests proved that, although not 100% of the drug incorporated was released from the nanoparticles after 24 h, the activity against *P. aeruginosa* and *S. aureus* of the nanoparticles and aqueous solution was comparable.

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