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A novel surface modified nitrendipine nanocrystals with enhancement of bioavailability and stability

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ABSTRACT

In this study, chitosan, a cationic polymer with positive charge, was introduced to modify the nanocrystals of nitrendipine with negative charge. The nanocrystals were prepared *via* precipitation–high pressure homogenization method. Then the nanocrystals were dispersed into chitosan solution, and the free chitosan was removed by centrifugation to obtain the chitosan modified nanocrystals, which remained the same particle size. However, the zeta-potential changed to positive after modification.

The physical stability of the chitosan modified nanocrystals was remarkably improved under ambient conditions. During the *in vitro* dissolution test, the modified nanocrystals showed a certain degree of slow-release property. In the *in vivo* study, the C_{max} of nitrendipine remained the same, however, the T_{max} delayed from 0.75 h to 1.5 h with the chitosan modified nanocrystals. The surface modification by chitosan improved the bioavailability compared with the initial nanocrystals, which had demonstrated significant improvement of bioavailability compared to the traditional coarse powder form. Based on the experimental results, modification of the nanocrystals with certain polymer was supposed to be a good method to control the *in vitro* and *in vivo* behaviors of the nanocrystals, which could further increase the bioavailability of the water insoluble drug.

1. Introduction

Drug nanocrystals are pure solid drug particles with crystalline character and a particle size in the nanometer range (Junghanns and Müller, 2008). In recent years, drug nanocrystals have been the subject of much interest due to their novel physical properties, which mainly depend on crystal size (Date, 2004; Gao et al., 2008b). It has been reported that the dissolution rate and saturation solubility of some water insoluble drugs can be increased by reduction of the particle size, hence, the bioavailability is improved (Patravale et al., 2004; Rabinow, 2004). However, it is difficult to modify the *in vitro* release and *in vivo* profiles of drug nanocrystals in the carrier free drug delivery system.

Chitosan is a cationic polysaccharide, that consists of 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose *via* a β1–4 linkage (Dash et al., 2011). It is abundant in nature and is considered to be one of the most promising biopolymers for drug delivery purposes because of its interesting properties: biocompatibility, biodegradability and mucosal adhesiveness (Kean and Thanou, 2010; Kumar, 2000).

Nitrendipine is a calcium channel blocker which is practically insoluble (about 1.9–2.1 μg/ml) in water and has poor oral bioavailability (Wang et al., 2006). In this study, nitrendipine nanocrystals were prepared *via* precipitation–high pressure homogenization method. Then the chitosan modified nitrendipine nanocrystals were developed using the charge interaction between the negatively charged nanocrystals and the positively charged chitosan. The physical properties of the nanocrystals were characterized by X-ray diffraction (XRD) and differential scanning calorimetry (DSC). *In vitro* and *in vivo* tests were carried out to compare the dissolution rate and oral bioavailability of the nanocrystals before and after the modification by chitosan.

2. Materials and methods

2.1. Materials

Nitrendipine (99% purity) was purchased from Nanjing Pharmaceutical Factory (China). Polyvinyl alcohol (PVA, 05-88) was generously supplied by Shin-Etsu Chemical Ind. Co. Ltd (Japan). Chitosan (degree of deacetylation was >90% and molecular weights were 30, 100 and 500 kDa) were purchased from Golden-Shell Biochemical Co., Ltd (China). Acetone (analytical grade) was bought from Ruijingte Chemical Agent Company (Tianjin, China). Cyclohexane, isopropyl alcohol, methanol and acetonitrile

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(chromatographic grade) were purchased from Concord Chemical Agent Company (China). Sodium lauryl sulfate (SLS) was purchased from Tianjin Bodi Chemical Holding Co., Ltd (China).

2.2. Preparation of nitrendipine nanocrystals

Nitrendipine nanocrystals were prepared using a tandem precipitation–high pressure homogenization process. Briefly, nitrendipine was dissolved in acetone (100 mg/ml, w/v), then, 0.5 ml of the solution was transferred to 25 ml of PVA solution (1 mg/ml) at 10 °C. The mixture was homogenized using a high pressure, piston-gap homogenizer (ATS AH100D) for 20 cycles at 1000 bar. The obtained samples were centrifuged at 10,000 × *g* for 45 min at 4 °C to collect the initial nanocrystals.

2.3. Preparation of chitosan modified nitrendipine nanocrystals

The initial nanocrystals (50 mg) were dispersed into 25 ml of chitosan solutions (0.2%, w/w) and vortexed for 5 min. After the adsorption of the chitosan onto the nanocrystals, the samples were then centrifuged at 10,000 × *g* for 45 min at 4 °C to remove the free chitosan.

2.4. Particle size and zeta-potential

Laser diffraction (LD, Coulter_LS 230, Beckmann-Coulter Electronics, Krefeld, Germany) with polarization intensity differential scattering (PIDS) was applied to investigate the particle size. The LD data obtained were evaluated using the volume distribution as the particle size and the Span values. The particle size gives the average particle diameter, while the Span value is a statistical parameter used to evaluate the particle size distribution and calculated using the following equation:

$$\text{Span} = \frac{D_{90} - D_{10}}{D_{50}} \quad (1)$$

where D_{10}, D_{50} and D_{90} represent the particle diameters at 10%, 50% and 90% of the volume distribution.

The lower the Span value, the narrower is the particle size distribution.

The zeta potential was measured using laser Doppler microelectrophoresis (Zetasizer nano ZS, Malvern instruments Ltd., UK) with the nanocrystals diluted in water according to the manufacturer's manual.

2.5. Morphology of nitrendipine crystals

The particle morphology of nitrendipine coarse powder and the nanocrystals were examined using scanning electron microscopy (SEM, Hitachi S-4800, Japan) and transmission electron microscopy (TEM, H-600, Hitachi, Japan), respectively. The coarse powder was coated with a thin layer of gold and viewing by SEM. The nanocrystals were stained with 2% (w/v) phosphotungstic acid and placed on copper grids coated with a thin film of poly(vinyl formal) for viewing by TEM.

2.6. Differential scanning calorimetry (DSC) analysis

DSC analysis was performed using a thermal analyzer (TA-60WS, Shimadzu, Japan) in a dry nitrogen atmosphere. Al_2O_3 was used as a reference, and the heating curves were recorded at a scan rate of 10 °C/min from 30 °C to 350 °C.

2.7. X-ray diffraction (XRD) measurements

The samples were analyzed using a X-ray diffractometer (D/MAX 2400, Rigaku, Japan) with Cu Kα radiation at a wavelength of 1.542 Å, generated at 30 mA and 40 kV. Samples were analyzed in the 2-theta range from 5° to 50° using a step size of 0.02°.

2.8. In vitro dissolution

In vitro dissolution studies were carried out in a RCZ-6B drug dissolution apparatus (Huanghai Medicament Test Factory, Shanghai, China) using the paddle method in the Chinese Pharmacopoeia 2010. Distilled water containing 0.1 M hydrochloric acid and 0.1% SLS (w/v) was used as dissolution medium. The temperature was maintained at 37 ± 0.5 °C, and the rotation speed of the paddle was 100 rpm. A commercial nitrendipine tablet (Tianjin pacific Pharmaceutical Co. Ltd) was used as the reference preparation. Accurately weighed samples containing the equivalent of 5 mg nitrendipine were added to the dissolution medium (900 ml). Samples of approximately 5 ml were taken from the dissolution medium at predetermined time points, and passed through a 0.1 μm syringe filter (Shanghai Huan'ao Trading Company, China). The filtrates were determined by HPLC (Hitachi L7110 pump equipped with an L7420 UV–VIS detector, Japan). The analytical column was a Diamonsil™ C_{18} column (5 μm, 200 mm × 4.6 mm) and the mobile phase was composed of methanol and water (80:20, v/v). The flow rate was 1.0 ml/min, and the UV-detector was set at 236 nm. All dissolution experiments were performed in triplicate, and all sample analysis was carried out in triplicate.

2.9. In vivo bioavailability in rats

Male Wistar rats were supplied by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China), and the animal experiment protocol was evaluated and approved by the Animal Ethics Committee, Shenyang Pharmaceutical University. Twenty male Wistar rats weighing 200 ± 20 g were fasted overnight with free access to water. The animals were then divided into four groups, with five rats in each group. The initial nanocrystals and chitosan modified nanocrystals were orally administered at a dose of 25 mg/kg, and the coarse powder was orally administered at a dose of 200 mg/kg. The commercial tablet (Tianjin pacific Pharmaceutical Co. Ltd) was orally administered as the reference preparation at a dose of 200 mg/kg. All the formulations were orally administered after well dispersed in distilled water.

Blood samples (0.3 ml) were collected by retro-orbital puncture at predetermined time points, and the plasma was collected by centrifugation at 10,000 rpm for 5 min. Nimodipine was employed as the internal standard. Plasma samples (100 μl) were vortexed and extracted with 0.5 ml mixed organic solvent (cyclohexane:isopropyl alcohol = 50:2, v/v) on a vortex mixer (XW-80A, Shanghai, China) for 5 min. After centrifugation at 10,000 rpm for 5 min, the organic layer was transferred to another clean tube and evaporated under a stream of nitrogen gas. All the samples were then stored at −20 °C until analysis.

Nitrendipine was determined by HPLC (Hitachi L7110 pump equipped with an L7420 UV–VIS detector, Japan). The separation was carried out on a Diamonsil™ C_{18} column (5 μm, 200 mm × 4.6 mm) and the mobile phase was composed of a mixture of methanol, acetonitrile and water (50:25:25, v/v/v). The flow rate was 1.2 ml/min, and the UV-detector was set at 236 nm. Each sample was re-dissolved in 30 μl mobile phase, and 20 μl was subjected to HPLC analysis.

Standard pharmacokinetic (PK) parameters (mean ± S.D.) of nitrendipine were derived from plasma concentration *versus* time profiles using a non-compartmental model with WinNonlin®

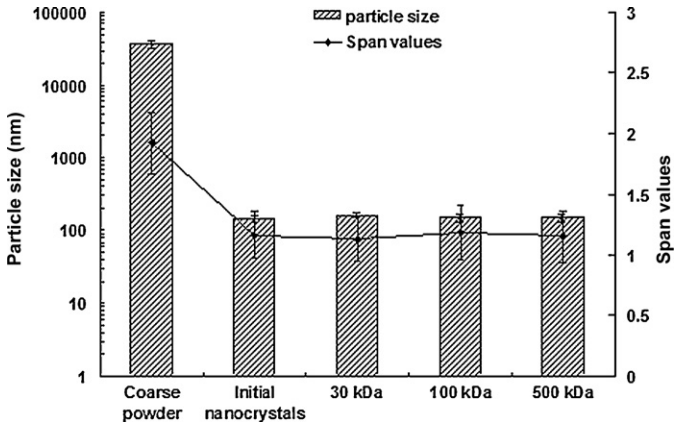

Fig. 1. Effect of molecular weight of chitosan on the particle size of nitrendipine nanocrystals (means ± S.D., $n = 3$).

Professional Version 3.1 software (Pharsight Corp., Mountain View, CA). The PK parameters that were calculated included the maximum peak concentration of the drug in plasma (C_{max}), the time to reach this maximum concentration (T_{max}), the elimination rate constant (Lambda_z) and the area under the curve ($AUC_{0\rightarrow 24}$). The relative bioavailability (Fr) was compared with the commercial tablet. All results were presented as mean ± S.D. values, and Student's *t*-tests and ANOVA were performed to determine the significance of any differences.

3. Results and discussion

3.1. Effects of chitosan on the particle size and zeta-potential

Fig. 1 shows the effect of molecular weight of chitosan on the particle size of nitrendipine nanocrystals. According to Fig. 1, the mean particle size of nitrendipine coarse powder was 36.6 μm, and the initial nanocrystals were prepared successfully with an average particle size of 175 nm. The higher Span value of the coarse powder suggested that nitrendipine crystals were irregular in particle size, and the crystals were more uniform in the sample of initial nanocrystals according to the lower Span value. The particle size increased a little after modification by chitosan, but no significant difference was demonstrated. The molecular weight of chitosan showed no significant influence on the particle size and size distribution of the nanocrystals due to the similar particle size and Span value of the nanocrystals that modified by different molecular weight of chitosan. The steric stabilization effect is supposed to increase with the increasing thickness of the adsorbed layer, which increases with increasing molecular weight of the polymer (Chibowski and Wis'niewska, 2002; Nsib et al., 2006). Accordingly, chitosan with an average molecular weight of 500 kDa was chosen for further study.

Figs. 2 and 3 show the effect of concentration of chitosan on the particle size and zeta-potential of nitrendipine nanocrystals. The concentration of chitosan showed no significant influence on the particle size, however, the zeta-potential increased with the increasing concentration of chitosan. No significant difference on the Span value of the samples was detected, which suggested the similar particle size distribution of the samples. According to Fig. 3, the positive surface charge confirmed the deposition of the positively charged chitosan on the surface of the negatively charged nanocrystals. No significant difference was demonstrated in the zeta-potential between the two groups of 0.2% chitosan and 0.3% chitosan, which indicated the saturated adsorption of chitosan in the 0.2% chitosan solution. Claesson and Ninham investigated the adsorption of chitosan using the surface force technique, and the

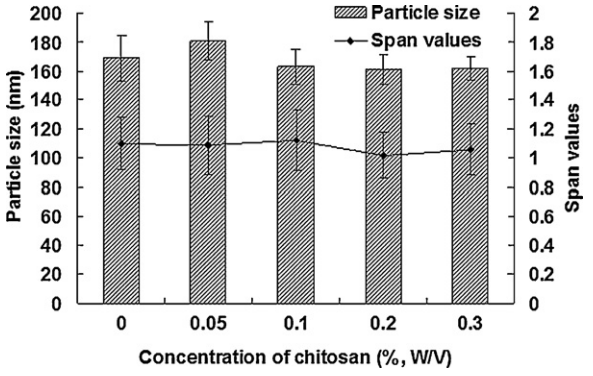

Fig. 2. Effect of concentration of chitosan on the particle size of nitrendipine nanocrystals (means ± S.D., $n = 3$).

results indicated that chitosan could form an irreversibly adsorbed layer onto the surface of negatively charged mica (Claesson and Ninham, 1992). The interaction between chitosan and the surface of the negatively charged nanocrystals was similar with that between chitosan and the surface of the negatively charged mica. Accordingly, an irreversible chitosan layer was supposed to form on the surface of the negatively charged nanocrystals.

3.2. Morphology of nitrendipine crystals

The morphology of the coarse powder (a), the initial nanocrystals (b) and the chitosan modified nanocrystals (c) are shown in Fig. 4. The coarse powder was irregular in shape with a broad particle size distribution. The initial nanocrystals were flaky in shape with more uniform particle size, and the modification by chitosan did not change the morphology and particle size of the nanocrystals.

3.3. X-ray diffraction (XRD) measurements

Fig. 5 shows the X-ray diffraction patterns of the coarse powder (a), the initial nanocrystals (b), the chitosan modified nanocrystals (c) and chitosan (d). The XRD pattern of the coarse powder showed characteristic high-energy diffraction peaks at 2θ values between 8° and 30°, indicating the crystalline structure of nitrendipine. No characteristic intense peak was found in the XRD pattern of chitosan, indicating an amorphous state. The initial nanocrystals presented a similar XRD pattern to that of the coarse powder, indicating that the initial crystalline state had been maintained, however, the degree of crystallinity decreased. The surface area of the drug increase significantly due to the decrease of the particle size to the nanometer range (Pouton, 2006). The molecules on the surface are more weakly bound and less constrained in their

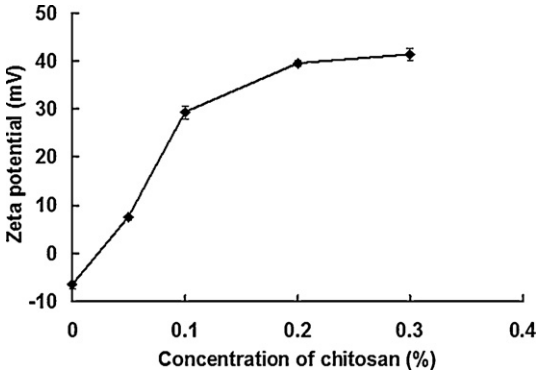

Fig. 3. Effect of concentration of chitosan on the zeta potential of nitrendipine nanocrystals (means ± S.D., $n = 3$).

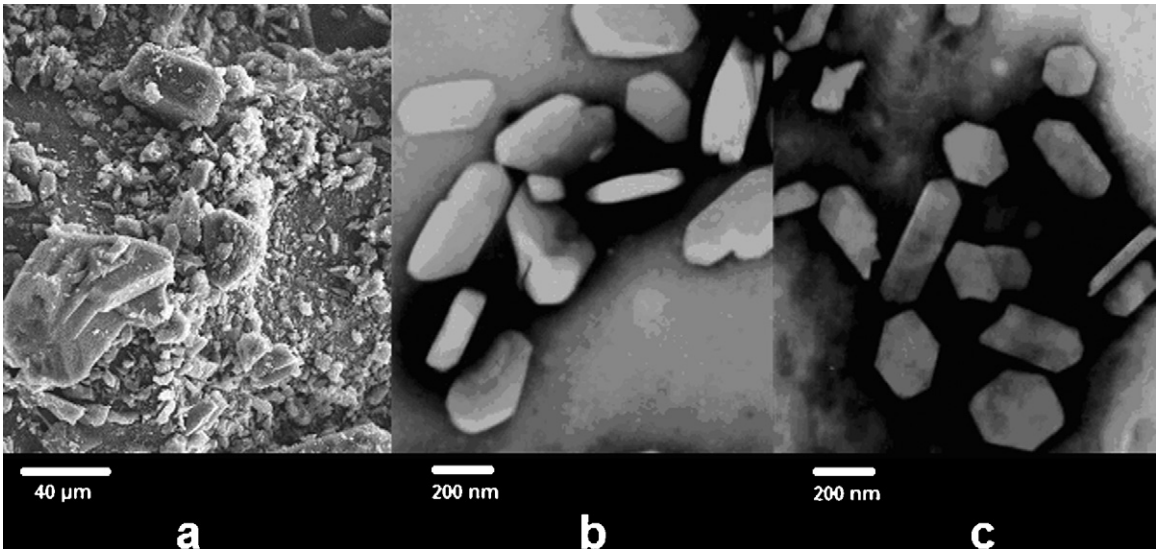

Fig. 4. The morphology of the coarse powder (a), the initial nanocrystals (b) and the chitosan modified nanocrystals (c).

thermal motion than molecules in the body of crystals, which is supposed to be responsible for the decrease of the degree of crystallinity (Schmidt et al., 1998). The characteristic intense peak of nitrendipine at 2θ value about 10° increased significantly after modification by chitosan. The interaction between the nanocrystals and chitosan was supposed to be responsible for the change in the XRD pattern.

3.4. Differential scanning calorimetry (DSC) analysis

Fig. 6 shows the DSC results of chitosan (a), the chitosan modified nanocrystals (b), the initial nanocrystals (c) and the coarse powder (d). The coarse powder exhibited a sharp endothermic peak at 160.25 °C indicating the melting point. No endothermic peak was detected in the DSC curve of chitosan, indicating the amorphous state. In the thermograms of the initial nanocrystals and the chitosan modified nanocrystals, the endothermic peaks at 156.78 °C and 155.60 °C, ascribed to the melting of nitrendipine, indicated that nitrendipine was present in crystalline form. However, the endothermic peak of nitrendipine drifted about 4 °C and 5 °C to the left, perhaps due to size reduction of the crystals (Lai et al., 1996; Schmidt et al., 1998).

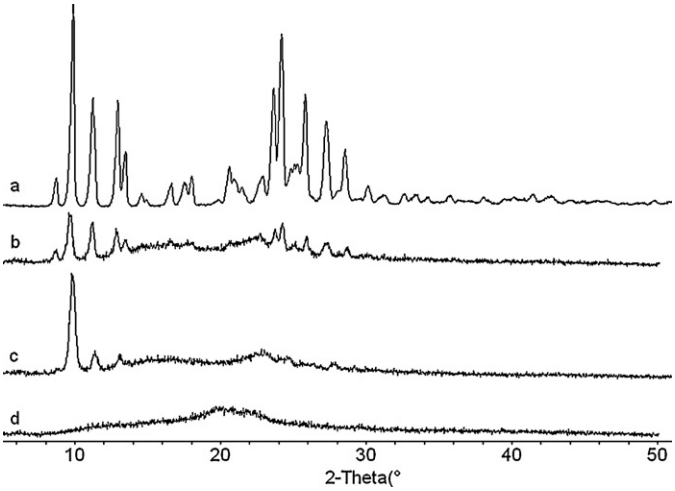

Fig. 5. X-ray diffraction patterns of the coarse powder (a), the initial nanocrystals (b), the chitosan modified nanocrystals (c) and chitosan (d).

3.5. Particle size stability

The unique nano-scale structure of nanocrystals provides significant increases in the surface area to volume ratio which results in the stability problems, such as sedimentation, agglomeration and crystal growth (Wu et al., 2011). The particle size stability of the nanocrystals is critical, because it ensures the maintenance of the special properties that depend on crystals size (Gao et al., 2008a; Van Eerdenbrugh et al., 2008). To evaluate the influence of chitosan on the particle size stability, the freeze dried samples of the initial nanocrystals and the chitosan modified nanocrystals were placed in sealed vials under 30 °C for 24 days. According to Fig. 7, the particle size of the initial nanocrystals increased significantly under ambient conditions, however, the particle size of the chitosan modified nanocrystals remained constant. Electrostatic repulsion and steric stabilization are two main mechanisms that can be used to resolve the stability problems. For the initial nanocrystals, the zeta-potential is only −6.58 mV which is far below the minimum value to keep the particle size constant. However, the zeta-potential of the chitosan modified nanocrystals is about +40 mV, which could provide greater electrostatic repulsion to prevent growing of the particle size. Furthermore, the chitosan deposited on the surface of the nanocrystals could provide steric stabilization effect that further increased the particle size stability of the nanocrystals. The

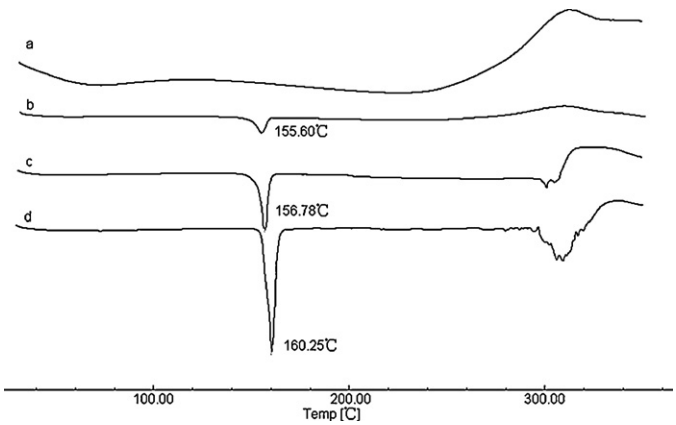

Fig. 6. Differential scanning calorimetry curves of chitosan (a), the chitosan modified nanocrystals (b), the initial nanocrystals (c) and the coarse powder (d).

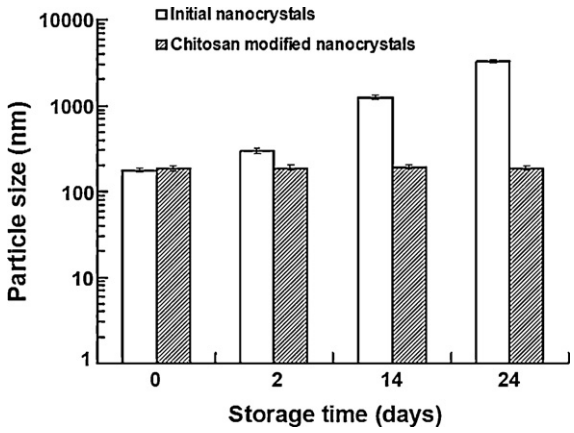

Fig. 7. Particle size stability of the initial nanocrystals and the chitosan modified nanocrystals (means ± S.D., $n = 3$).

result could be explained by the adsorption of the chitosan layer, that increased the electrostatic repulsion effect and the steric repulsion effect, hence, improved the stability of the nanocrystals (Sun et al., 2010).

3.6. In vitro dissolution

The *in vitro* dissolution profiles are shown in Fig. 8. The nanocrystals significantly improved the dissolution rate of nitrendipine. Only about 7% of the drug dissolved from the coarse powder during the 60 min study, however, about 80% and 20% of the drug dissolved from the initial nanocrystals and the chitosan modified nanocrystals within 1 min, respectively. It cost 3 min and 45 min for the drug to dissolve completely from the initial nanocrystals and the chitosan modified nanocrystals. The commercial tablet, a solid dispersion of nitrendipine, represented a significant improvement in the dissolution rate compared with the coarse powder. About 20% of the drug dissolved from the commercial tablet in 5 min, however, only about 60% of the drug dissolved from the commercial tablet during the 60 min study. Both the dissolution rate of the coarse powder and the commercial tablet were very slow, which indicated that the dissolution rate was the rate-limiting step for absorption of nitrendipine from the coarse powder and the commercial tablet. According to the Noyes–Whitney equation, the dissolution rate of a drug crystal is proportional to the surface area available for dissolution and the diffusion layer thickness (Kesisoglou et al., 2007). A reduction in the particle size will significantly increase the surface area available for dissolution and decrease the diffusion layer thickness, thus increasing the dissolution rate (Hintz and Johnson, 1989). In addition, an increase in the saturation solubility by the reduction of particle size is also expected, which will lead to a further increase in the dissolution rate (Junghanns and Müller, 2008). The chitosan modified nanocrystals presented a certain degree of slow-release character compared with the initial nanocrystals. The poor wetting ability of the chitosan on the surface of the nanocrystals are supposed to result in the slow-release of the drug by affecting the rate of penetration of the dissolution fluid at the surface and formation of a gel-like barrier (Säkkinen et al., 2002). The results indicate that transformation of the drug powder into nanocrystals is a useful method to increase the dissolution rate of a water insoluble drug, and the surface modification is a good method to control the *in vitro* dissolution behavior of the nanocrystals.

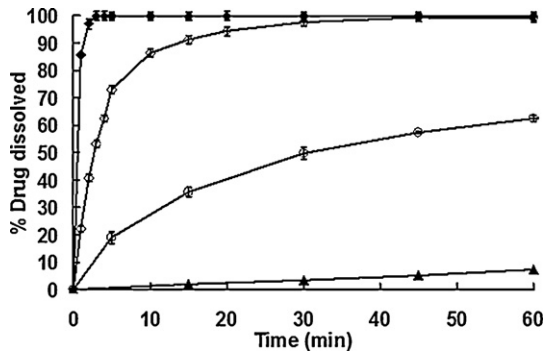

Fig. 8. Percentage of dissolved nitrendipine from the coarse powder (▲), the initial nanocrystals (♦), the chitosan modified nanocrystals (◊) and the commercial tablet (○) (means ± S.D., $n = 3$).

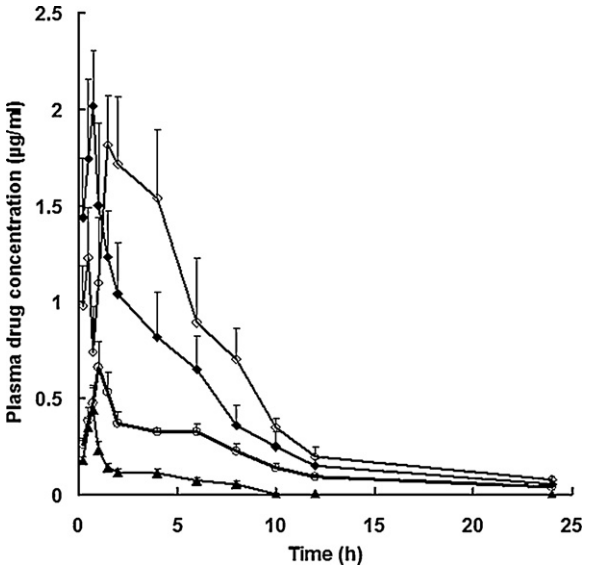

Fig. 9. Average plasma drug concentration *versus* time profiles after oral administration of the coarse powder (▲), the initial nanocrystals (♦), the chitosan modified nanocrystals (◊) and the commercial tablet (○) (means ± S.D., $n = 4$–5).

3.7. In vivo bioavailability in rats

The plasma drug concentration *versus* time profiles and pharmacokinetic parameters of nitrendipine are presented in Fig. 9 and Table 1, respectively. The plasma drug concentration profile of the initial nanocrystals represents significant improvement in drug absorption compared with the coarse powder and the commercial tablet ($p < 0.01$), and the chitosan modified nanocrystals further increased the bioavailability compared with the initial nanocrystals ($p < 0.05$). The C_{max} of the chitosan modified nanocrystals was similar to that of the initial nanocrystals, and both of them were significantly increased compared with the coarse powder and the commercial tablet ($p < 0.01$). However, the T_{max} of the chitosan modified nanocrystals was delayed significantly from 0.75 h to 1.5 h compared with the initial nanocrystals and the coarse powder ($p < 0.01$). The T_{max} of the commercial tablet was 1.0 h, which is prolonged due to the slow-release of drug from the commercial tablet. The elimination rate constant (Lambda_z) of nitrendipine showed no statistically significant difference among the four formulations. The relative bioavailability of the coarse powder, the initial nanocrystals and the chitosan modified nanocrystals were 23.60%, 1804.38% and 2524.57%, respectively, compared with the commercial tablet. Nitrendipine is a biopharmaceutical classification system Class II substance (poorly soluble and highly permeable), which means dissolution is the rate-limiting step for absorption (Choi et al., 2003). According to the results of the *in vitro* dissolution study, the nanocrystals significantly improved the dissolution rate

Table 1
Pharmacokinetic parameters of different nitrendipine formulations in rats (n = 4–5, means ± S.D.)..

Formulations	Coarse powder	Initial nanocrystals	Chitosan modified nanocrystals	Commercial tablet
C_{max} (μg/ml)	0.44 ± 0.11	2.01 ± 0.29[a]	2.00 ± 0.19[a]	0.62 ± 0.11
T_{max} (h)	0.75 ± 0.00[a]	0.75 ± 0.00[a]	1.63 ± 0.25[a,b]	1.00 ± 0.00
Lambda_z	0.14 ± 0.03	0.12 ± 0.05	0.14 ± 0.03	0.10 ± 0.03
AUC_{0-24} (μg h/ml)	0.97 ± 0.04[a]	9.27 ± 1.91[a]	12.97 ± 1.96[a,c]	4.11 ± 0.40
Fr	23.60%	1804.38%	2524.57%	–

[a] Statistically significant compared with the commercial tablet ($p < 0.01$).
[b] Statistically significant compared with the initial nanocrystals ($p < 0.01$).
[c] Statistically significant compared with the initial nanocrystals ($p < 0.05$).

of nitrendipine. The increased oral bioavailability of the nanocrystals could be explained by the significantly improved dissolution rate, which could retain the drug in the soluble form during the gastrointestinal dilution and permeation processes. Chitosan is an absorption enhancer ascribed to the opening of the tight junctions of epithelial cell barriers (Thanou et al., 2001). And the deposition of the positively charged chitosan on the surface of the nanocrystals further increase the interaction of the nanocrystals with the negatively charged membrane of intestinal cells (Behrens et al., 2002). Consequently, chitosan presence on the surface was supposed to increase the transportation of nitrendipine into the circulation. However, the existence of the chitosan layer on the surface of the nanocrystals decreased the dissolution rate of the drug, which delayed the T_{max} of nitrendipine in rats. The *in vivo* results lead to the conclusions that transformation of the coarse powder into nanocrystals is an effective approach to increase the oral bioavailability of nitrendipine, and the surface modification is a potential approach to obtain a slow-release character and further increase the bioavailability.

4. Conclusions

We have demonstrated the effective molecular interaction between the negatively charged nitrendipine nanocrystals and the positively charged chitosan. Chitosan shields the nanocrystals as a consequence of the electrostatic interactions leading to an increase in the particle size stability. Furthermore, the chitosan modified nanocrystals showed a certain degree of slow-release character, and the bioavailability of nitrendipine was further increased.

Acknowledgements

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