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Preparation Optimized and Sustained Release Behavior of Nifedipine-γ-Polyglutamic Acid/Chitosan Nanoparticle

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In order to solve the problems caused by the low solubility of nifedipine (NF), such as, low bioavailability and short half-life, a novel drug delivery nanoparticle for sustained release of nifedipine was prepared and its sustained release behavior in systems of different pH were studied. The preparation of this nanoparticle was based on self-assembly of γ-polyglutamic acid (γ-PGA), which was extracted from the fermentation fluid that was produced by *Bacillus subtilis* and chitosan (CS) under normal pressure and temperature. When 2 mL of nifedipine/ethanol solution with a concentration of 0.5 g L^{-1} and 18 mL of γ-PGA solution at pH of 8 and the concentration of 1 g L^{-1} were dropped into 2 mL chitosan solution at pH of 3 and the concentration of 0.4 g L^{-1} to prepare nanoparticle. The particle size (Z-Ave) and polydispersity index (PDI) were 232.2 nm and 0.2795, respectively. The ratio of drug-loading rate and encapsulation rate was 79.72 and 28.44 %, respectively. The accumulative release rate of drug would be well fitted Higuchi equation ($M_t/M_\infty = 0.1601t^{1/2} + 0.0486$, $R^2 = 0.9731$) and Peppas equation ($M_t/M_\infty = 0.2043t^{0.4376}$, $R^2 = 0.9816$). Sustained release performance measurements indicated that there was remarkable sustained release effect of the NF-γ-PGA/CS nanoparticle in circumstance of simulated intestinal fluid, while not so much sustained release effect in simulated gastric fluid. The NF-γ-PGA/CS nanoparticle was an uniformly nano-sized typical sustained release preparation. This nanoparticle could be applied to clinical as sustained release preparation of nifedipine.

Keywords: Nifedipine-γ-polyglutamic acid/chitosan nanoparticle (NF-γ-PGA/CS), Sustained release, Optimization, RSM.

INTRODUCTION

Nifedipine (NF) is the first generation of dihydropyridines category calcium antagonist[1]. It is applied to cure hypertension, stenocardia and coronary disease[2]. Because of its limited solubility in water and evident first-pass effects, it has a low bioavailability and short half-life[3]. To improve it's properties, many approaches have been explored. Forster *et al.*[4] reported a remarkable dissolution rate of nifedipine improved in glass solution, which produced melt extrusion with hydrophilic polymers. Chan *et al.*[5] reported a solid dispersion of nifedipine in poly (ethylene glycol), by which notably enhanced the dissolution of nifepine. Bayrakci *et al.*[6] reported that enhanced nifedipine's dissolution rate by using *o*-phosphorylated calixarenes as drug-solubilizing agents. Park *et al.*[7] developed a nanofibrous sheet-based system to achieve linear release of nifedipine for oral delivery.

Chitosan (CS)[8,9], a biocompatible polysaccharide derived from the N-deacetylation of chitin, is composed of glucosamine and N-acetylglucosamine and it could be decompose by lysozymes *in vivo* and release glycosaminoglycan. γ-Polyglutamic acid (γ-PGA)[10-13] is a poly-amino acid composed of the amino group on the α-carbon and the carboxyl group on the γ-carbon linked by the amide bond. It was extracted from the fermentation fluid which was produced by *Bacillus subtilis*. Chitosan[14-16] and γ-PGA[17-20] were hydrophilic and biodegradable, which already used in field of food and flavor. EI-Ghaffar and Hashem[21] reported a PMMA-γ-CS nanoparticles for α-chymotrypsin immobilization. Akagi *et al.*[22] reported a γ-PGA-Phe nanoparticals which could be multifunctional carriers for pharmaceutical and biomedical. Ashiuchi *et al.*[23] reported an ion-complex nanofiber of γ-PGA and hexadecylpyridinium which shows remarkable water-friendly and antimicrobial. Hajdu *et al.*[24] reported a γ-PGA nano-membrane which could efficient removal of lead ion from aqueous solution.

Sustained release systems were designed for deliver a certain amounts of therapeutic agents to extended duration of time[25,26] or even send the drug to specific target site[27]. The sustained release measure could eliminate the risk of side effects related to oral or parenteral therapies, for instance, decrease

peak serum concentration of cephalexin and prolong the time to peak blood concentration[28]. In prescriptive release medium, sustained release system could release drug relaxed with non-uniform speed. And compare with normal preparation, sustained release system could decrease the frequency of administration and enhance sufferer's compliance to the drug[29]. To avert patients taking the medicine too frequently and drastic fluctuation of blood concentration, oral sustained release tablet of nifedipine was hopeful applied in clinic. After Iler[30] reported that colloid granule with opposite charges were employed to prepare multiple structure *via* alternately adsorption at 1966, self-assembly system has aroused much interest mainly due to the merits of their physicochemical properties. Chitosan[31] is positively charged in acidic or neutral solution, while γ-PGA is negatively charged in alkalescence solution. So chitosan and γ-PGA have potential to form a nanoparticle in temperate condition. Therefore, new types of approach for NF-γ-PGA/CS drug delivery system were highly desirable.

In this study, nine possible effect factors were tested in Plackett-Burman experiment[32] to find out the most remarkable factors. Use level of remarkable factors for Z-Ave and PDI of γ-PGA/CS nanoparticle were optimized by Box-Behnken experiment[33] and influences of additive amount of nifedipine and ethanol were researched *via* single factor experiment. NF-γ-PGA/CS nanoparticle's release behavior in simulated gastric fluid and simulated intestinal fluid were tested and contrasted with Adalat, the commercial tablet. Equations of accumulative drug release rates were fitted.

EXPERIMENTAL

Chitosan (with a relative molecular mass of 150,000 and the degree of deacetylation of 90 %) was purchased from Shanghai Plus Bio-Sci&Tech Co., Ltd, Shanghai, China. γ-Polyglutamic acid was extracted from the fermentation fluid, which was produced by *Bacillus subtilis* with the purity of 95 %. Nifedipine was recrystallized and filtrated from nifedipine tables which were purchased from Nanjing Baijingyu Pharmaceutical Co.,Ltd, Nanjing, China. All other chemicals, including NaOH,HCl and ethanol (99.7 %) were purchased from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China. All chemicals were used as received.

Preparation of NF-γ-PGA/CS nanoparticle: To seek the notable factor for preparation the γ-PGA/CS nanoparticle, single factor experiment and Plackett-Burman experiment was designed. According to the result of the Plackett-Burman experiment (data not show), pH of chitosan, volume of γ-PGA and concentration of γ-PGA were opted to further optimizing experiment *via* three factors three levels Box-Behnken experiment. Best levels of other factors in single factor experiment were chosen to use in Box-Behnken experiment. Levels and experimental factors of Box-Behnken design were showed in Table-1.

γ-PGA was dissolved in distilled water to process γ-PGA solution. Chitosan was dissolved in ethylic acid of 1 % to process chitosan solution with a concentration of 1 g L^{-1}. The O/W emulsion was made by slowly dropping γ-PGA solution into 2 mL of chitosan solution (1 g L^{-1}) at a speed 1 mL h^{-1}, with a stirring rate of 100 rpm, followed by 60 min stirring. By far, the γ-PGA/CS nanoparticle obtained as a dispersion in aqueous solution. Then the solution of γ-PGA/CS nanoparticle was centrifuged in condition of 2000 rpm for 10 min. The precipitation was sent to vacuum freeze drying (Haimen No. 4 light industrial machinery works, Jiangsu, China), in condition of 0.01 MP, -50 °C for 48 h. The optimized levels of the factors were used in the following experiment.

To enhance the solubility of nifedipine, it was dissolved in ethanol to obtain nifedipine solution. Nifedipine solution and γ-PGA solution were added into 2 mL chitosan solution at 0.1 and 1 mL h^{-1} respectively, with a stirring rate of 100 rpm, followed by 1 h stirring. The followed operation was the same as that in preparation of γ-PGA/CS nanoparticle. The optimum level of the addition of nifedipine and ethyl alcohol were tested *via* single factor experiment.

Characterization of NF-γ-PGA/CS nanoparticle: The particle size (Z-Ave) and polydispersity index (PDI) of the nanoparticle were measured employing a Zetasizer model Nano-ZS (Malvern Instruments, United Kingdom) for three times. Average values were calculated. The Z-Ave and PDI measurement was performed by dynamic light scattering. The lower of Z-Ave and PDI, means the nanoparticles were homogeneous and the system more stable. In the analysis, the Z-Ave was as the main index and PDI was as a reference.

Nifedipine solution was scanned by ultraviolet and visible spectrophotometer (TU-1901, Beijing Purkinje General Instrument Co., Ltd, Beijing, China) between wavelength of 200-380 nm to find out the maximum absorption wavelength. Absorbance of a series of nifedipine solution was scanned by ultraviolet and visible spectrophotometer at the maximum absorption wavelength. A certain amount of NF-γ-PGA/CS nanoparticle was dispersed into 10 mL ethanol, then the mixed liquor was disposed by ultrasonic clearer (YS-1012FPM, Shanghai Yandong Ultrasonic Facilities Limited Company, Shanghai, China) at 600 W and 40 KHz for 0.5 h to damage the NF-γ-PGA/CS nanoparticle and let the nifedipine release. The mixed liquor was centrifuged in condition of 3000 rpm for 10 min and the absorbance of the supernatant liquid were measured. Encapsulate rate and drug-loading rate were calculated as follow[34]:

Encapsulate rate (%) = weight of nifedipine in nanoparticle (g)/weight of nifedipine added (g) × 100 %

Drug-loading rate (%) = weight of nifedipine in nanoparticle (g)/weight of nanoparticle (g) × 100 %

The nanoparticle solution of NF-γ-PGA/CS was placed in a dialysis bag (with an interception of 8000-15000). The

TABLE-1
LEVELS AND EXPERIMENTAL FACTORS OF BOX-BEHNKEN DESIGN

Factor	Code	Unit	Level		
			-1	0	1
pH of chitosan	X_1	/	3.0	4.5	6.0
Volume of γ-polyglutamic acid	X_2	mL	5.0	10.0	15.0
Concentration of γ- polyglutamic acid	X_3	g L^{-1}	0.2	0.4	0.6

dialysis bag was placed in a beaker with 100 mL simulate gastric fluid or simulate intestinal fluid in condition of 37 °C with a stirring rate of 100 rpm. 2 mL sample was took every certain time and 2 mL of simulate gastric/intestinal fluid was compensated. The absorbance of the sample at 237 nm[35] were measured. Equations of accumulative drug release rates were fitted. The release behavior in simulate intestinal fluid was contrasted with Adalat powder, which was obtained by smashing the commercial tablet.

RESULTS AND DISCUSSION

Preparation of NF-γ-PGA/CS nanoparticle: The design and response values of Box-Behnken experiment were showed in Table-2. The best was Run 14, its Z-Ave and PDI was 213.5 nm and 0.1425, respectively, when pH of chitosan was 6, volume of γ-PGA was 5 mL and concentration of γ-PGA was 0.4 g L^{-1}.

The second-order empirical model of Z-Ave was regression fitted by Design-Expert 8, the equation was $Y = 285.83-16.69X_1 + 21.31X_2 + 45.75X_3 + 45.50X_1X_2 + 14.63X_1X_3 + 11.38X_2X_3 + 36.58X_1^2-7.17X_2^2-7.29X_3^2$, with $R^2 = 0.9218$ and Adj $R^2 = 0.7811$. It's C.V. was 8.52 %, it means that the difference between model and estimated value was tiny. The quadratic term equation of the regression model could reflect the influence on response of each factor accurately. The second-order empirical model of PDI was regression fitted by Design-Expert 8, the equation was $Y = 0.22-0.019X_1-0.023X_3 + 0.048X_1X_2 + 0.098X_1X_3 + 0.019X_2X_3 + 0.031X_1^2-0.014X_2^2 + 0.050X_3^2$, with $R^2 = 0.8657$ and Adj $R^2 = 0.6867$. It's C.V. was 16.60 %, it means that second-order model could not so well fitted the experiment result. So according to the model of Z-Ave, the optimum level was that pH of chitosan was 3, volume of γ-PGA was 18 mL and concentration of γ-PGA was 0.4 g L^{-1}. Verification tests were did for three times. The Z-Aves were 236.6, 228.7 and 231.4 nm and PDIs were 0.1762, 0.1834 and 0.1789, the average of Z-Ave and PDI was 232.2 and 0.1795, respetively. The result of verification test was worse than Run 14, but better than the others. This might be explained by there was some error in Run 14. All in all, the result of optimum was authentic.

The interaction of two factors reflected on response was showed visualized in the contour and 3D response surface plots on the basis of quadratic term model. In Fig. 1 (a, b), it showed that the influence on response of Z-Ave of pH of chitosan and volume of γ-PGA, they were obviously interacted. And Fig. 1 (c, d) showed that the influence of pH of chitosan and concentration of γ-PGA were obviously interacted. However, Fig. 1 (e, f) showed that the influence of concentration of γ-PGA and volume of γ-PGA, were not obviously interacted, this might because either of them was affected on the addition of γ-PGA in the reaction system.

As expected, the Z-Ave of nanocapsule was strongly dependent on the amount of ethanol added. Fig. 2 showed that with the increasing of ethanol addition, the Z-Aves and PDIs were both first decreased and then increased and the optimum addition of ethanol was 2 mL.

Fig. 3 showed that with the increasing the addition of nifedipine, the Z-Aves were increased while PDIs were first decreased then increased. It is remarkable that after 2 mL of the concentration of nifedipine solution more than 0.5 g L^{-1}, the Z-Ave changed sharply, which was because the encapsulation quantity of nanoparticle was finite. Based on the above results, 0.5 g L^{-1} was choose as the optimum level.

In Fig 4, record 1 was nanoparticle solution and record 2 was dried and redissolved nanoparticle solution, the Z-Ave of NF-γ-PGA/CS nanoparticle was homogeneous. The Z-Ave of nanoparticle was concertrated on 230 nm. But when redissolving the nanoparticle, a few white precipitate could not be dissolved by conventional vibrate. There was a small peak at 5000 nm, that might be some floc, which was formed from γ-PGA and chitosan when freeze drying.

There is a strong absorption peak at 237 nm and a feeble wide absorption peak at 320-360 nm. The result was accord with the literature[35]. It was presented a linear relationship between concentration of nifedipine and absorbance of the test sample from 0.002 to 0.010 g L^{-1}. The standard curve was $y = 58.9x + 0.059$ and R^2, correlation coefficient, was 0.999. It means that the concentration of nifedipine could be obtained by scanning the absorbance.

Encapsulate rate and drug-loading rate assay: According to the foregoing equations, the encapsulate rate and drug-loading rate were calculated as follow.

Encapsulate rate (%) = weight of nifedipine in nanoparticle (g)/weight of nifedipine added (g) × 100 % = 79.72 %

TABLE-2
BOX-BEHNKEN EXPERIMENT DESIGN AND RESPONSE VALUES

Numerical order	Run	X_1	X_2	X_3	Z-Ave/nm	PDI
1	1	-1	-1	0	340.2	0.2895
2	14	1	-1	0	213.5	0.1425
3	6	-1	1	0	326.6	0.2365
4	11	1	1	0	381.8	0.2805
5	2	-1	0	-1	293.5	0.4065
6	15	1	0	-1	233.3	0.1845
7	13	-1	0	1	367.7	0.2220
8	7	1	0	1	366.4	0.3925
9	9	0	-1	-1	238.5	0.3475
10	3	0	1	-1	224.6	0.2685
11	10	0	-1	1	295.5	0.2055
12	4	0	1	1	327.0	0.2045
13	12	0	0	0	271.6	0.2325
14	5	0	0	0	281.8	0.2020
15	8	0	0	0	305.2	0.2255

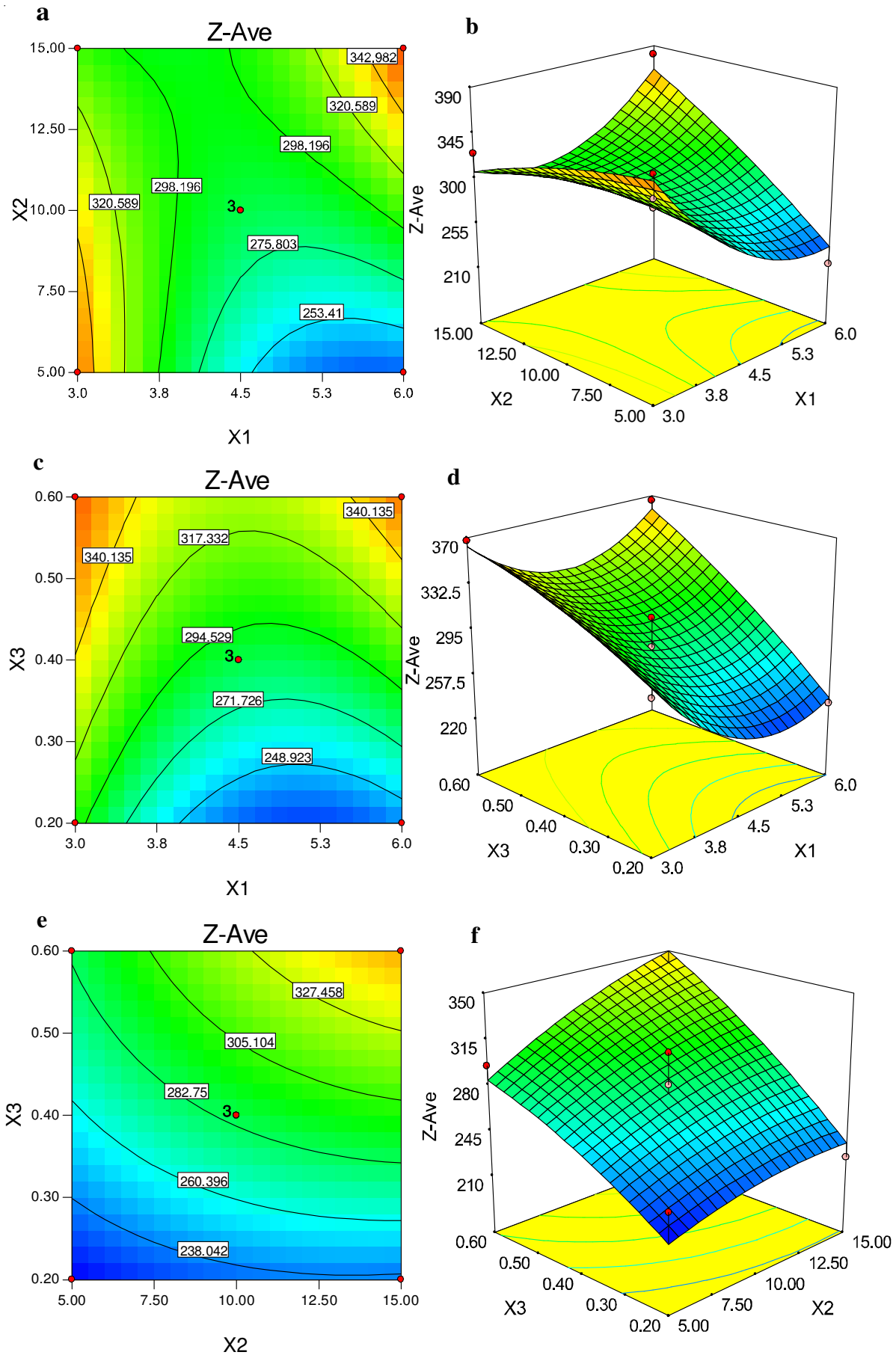

Fig. 1. Contour and 3D response surface plots of two factors with the other factor at central levels; X1, X2, X3 represent pH value of chitosan, Volume of γ-PGA (mL) and mass concentration of γ-PGA (g/L), respectively

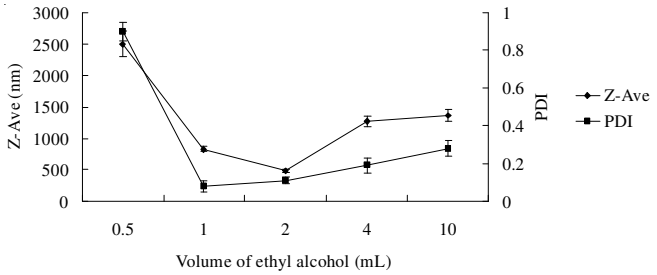

Fig. 2. Effect of ethanol amount on nanoparticle

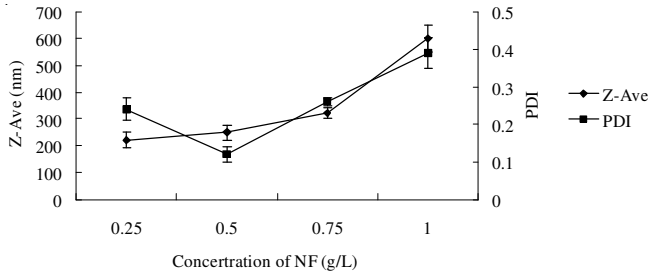

Fig. 3. Effect of the concerrtration of nifedipine on nanoparticle

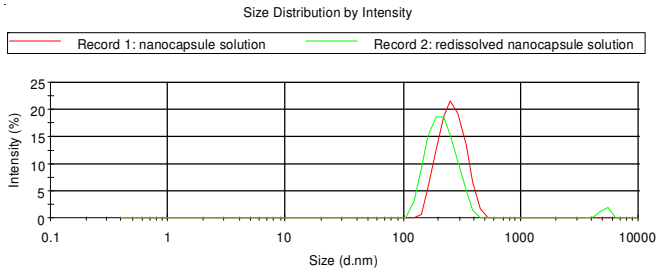

Fig. 4. Size of nanoparticle

Drug-loading rate (%) = weight of nifedipine in nanoparticle (g)/weight of nanoparticle (g) ×100 % = 28.44 %.

After reaction, there was some yellow substance, the nifedipine, attached to the stirrer of magnetic stirring apparatus. These data was acceptable and the encapsulate rate and drug-loading rate were impacted by many factors. The low drug-loading rate could be attributed to the nifedipine's limited solubility in water that caused it could not evenly dispersed in the reaction system.

Release plots of nifedipine: The release plots of nifedipine in different systems were showed in Fig. 5. The NF-γ-PGA/CS nanoparticle has a good sustained release capacity in simulated intestinal juice, which means the NF-γ-PGA/CS nanoparticle prepared by this way has a potential to apply in clinical. But in simulated gastric juice NF-γ-PGA/CS nanoparticle was rapidly released, the accumulative drug release rate rose to 90 % in 9 h. Because of γ-PGA and chitosan were pH-sensitive polymer, the NF-γ-PGA/CS nanoparticle has different existent form and electrical nature in different pH and could not be stable in low pH condition. This NF-γ-PGA/CS nanoparticle could be stable in nature or alkalescence condition, but could not be stable in acidic condition. When the NF-γ-PGA/CS nanoparticle employed to clinical, dosage form and administration route should be considered to give full play to its sustained release capacity.

The release plot of nifedipine in simulated intestinal juice was further tested. In first 3 h, drug released slightly fast,

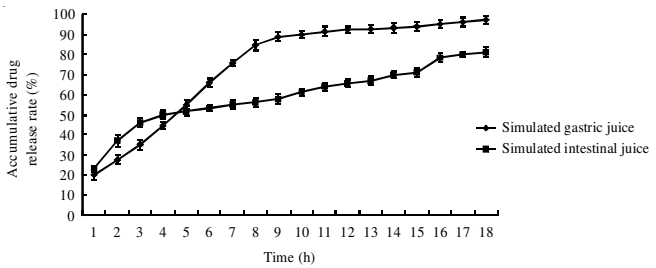

Fig. 5. Release plots of nifedipine in different system

appeared an obvious burst effect and accumulative drug release rates were fitted zero-order kinetic equation, fitted equation was $M_t/M_\infty = 0.0788t + 0.1155$ with $R^2 = 0.9982$. M_t was amount of accumulative drug release at t, M∞ was amount of accumulative drug release in all the period, M_t/M_∞ was accumulative drug release rate, t was release time. At last period of release, drug release rate was decrease obviously. Various models were employed to fit the whole release period and a zero-order kinetic equation was obtained, $M_t/M_\infty = 0.0249t + 0.2728$ with $R^2 = 0.9689$. But the accumulative drug release rate was away from the zero-order kinetic equation at prior period of release, so Level 1 kinetic equation was tried. Level 1 fitted equation was $\ln(1-M_t/M_\infty) = -0.073t-0.0906$ with $R^2 = 0.8994$, degree of fitting was low.

The fitting plots of Higuchi equation was shown in Fig. 6. The Higuchi equation was $M_t/M_\infty = 0.1601t^{1/2} + 0.0486$ with $R^2 = 0.9731$, this model could be account for the release behavior of the NF-γ-PGA/CS nanoparticle. It could be concluded that the releasing mechanism was composite polymer matrix-diffusion[36]. The fitting plots of Peppas equation was illustrated in Fig 7. The Peppas equation was $M_t/M_\infty = 0.2043t^{0.4376}$ with $R^2 = 0.9816$ and n = 0.4376, this model has a high degree of fitting. According to Peppas equation theory, to cylindrical or

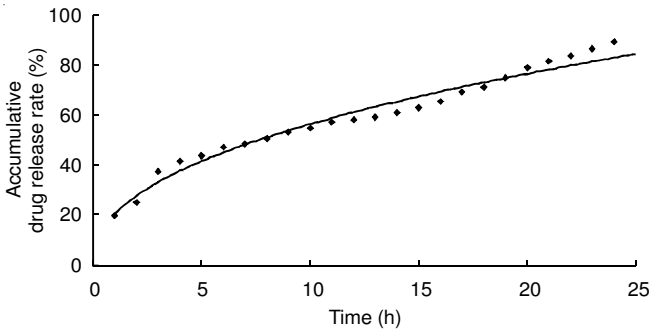

Fig. 6. Fitting plots of Higuchi equation

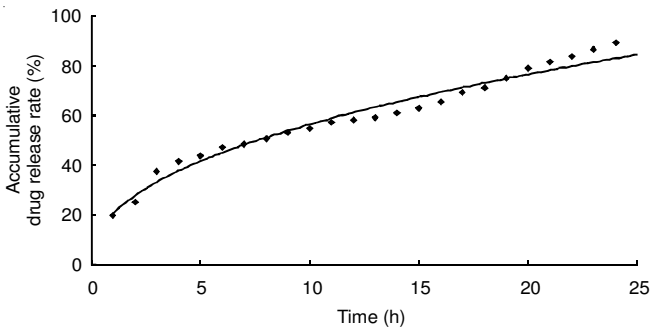

Fig. 7. Fitting plots of Peppas equation

spherical preparation, when $0.43 < n < 0.85$, the releasing mechanism was Fick diffusion and frame erosion[37]. In sum, the releasing mechanism of the NF-γ-PGA/CS nanoparticle was drug diffusion and frame erosion combined action in simulated intestinal juice.

Release plots of the NF-γ-PGA/CS nanoparticle and Adalat tablet: The accumulative drug release rates in simulated intestinal juice of the NF-γ-PGA/CS nanoparticle and nifedipine sustained release table, which trade name was Adalat, for 24 h were shown in Fig. 8. The accumulative drug release rates of Adalat were almost in a line and its zero-order fitting equation was $M_t/M_\infty = 0.032t + 0.1301$ with $R^2 = 0.9902$.

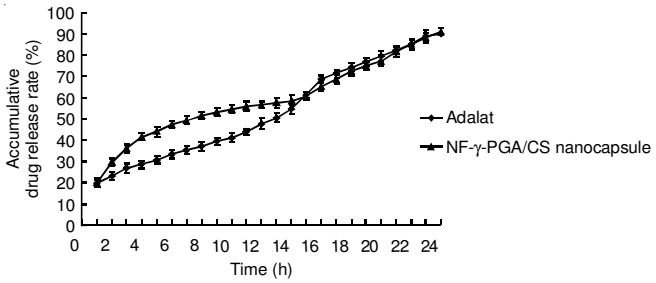

Fig. 8. Release plots of nanoparticle and Adalat

The Adalat tablet was a typical controlled-release preparation, it's release behavior was almost linear. However, the NF-γ-PGA/CS nanoparticle has a obvious burst effect in the first 3 h and could fit with Higuchi equation and Peppas equation, so it was a typical sustained preparation[38]. Contrasted with Adalat tablet, the release rate of the NF-γ-PGA/CS nanoparticle was faster at the prior period, it could be benefit to increase the blood concentration of nifedipine to reach the minimum effective dose. At the last period of release, the accumulative drug release rates of NF-γ-PGA/CS nanoparticle and Adalat tablet were basically the same, it could be benefit to keep the blood concentration of nifedipine stable. This nanoparticle could be applied to clinical as sustained release preparation of nifedipine.

There was also some shortage in this study. Preparing the NF-γ-PGA/CS nanoparticle in this way has an encapsulate rate of 79.72 %. It is not so satisfactory and there is a considerable room of improvement. The dropping speed of γ-PGA and nifedipine solution was 1 and 0.1 g L^{-1}, respectively. This is a low production speed. There must be some flocculent precipitation in the reaction system if dropping faster.

Conclusion

According this study, the uniformly nano-sized NF-γ-PGA/CS nanocapsule was prepered. The NF-γ-PGA/CS nanoparticle was a typical sustained preparation, which could fit Higuchi equation and Peppas equation. This nanoparticle could be applied to clinical as sustained release preparation of nifedipine. The wall materials and the method of preparation could be apply for preparations of other water insoluble drugs.

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