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Preparation and characterization of solid lipid nanoparticles containing peptide

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Abstract

Solid lipid nanoparticles (SLN) are an alternative colloidal carrier system for controlled drug delivery. However, only a few have been studied regarding the incorporation of peptides into SLN, due to the hydrophilic peptide not easy to enter the lipophilic matrix of SLN. In the present report, peptide-loaded solid lipid nanoparticles were prepared by a novel solvent diffusion method in an aqueous system. The model peptide gonadorelin was incorporated to study the entrapment efficiency, size, zeta potential (charge) and drug delivery characterization. Gonadorelin and monostearin were dissolved in acetone and ethanol at 50 °C in water bath, the resultant organic solution was poured into an aqueous containing 1% polyvinyl alcohol (PVA) under mechanical agitation. The peptide-loaded solid lipid nanoparticles were quickly produced and separated by centrifugation. The average volume diameter of gonadorelin-loaded SLN is 421.7 nm and the zeta potential of SLN is −21.1 mV dispersed in distilled water. Up to 69.4% of gonadorelin can be incorporated. In vitro release of gonadorelin from SLN is slow. In the test solution of a 0.1N hydrochloric acid for 2 h and then transferred in a pH 6.8 phosphate buffer (simulative gastrointestinal fluid), the drug-release behavior from SLN suspension exhibited a biphasic pattern. After burst drug-release at the first 6 h at a percentage of 24.4% of loaded gonadorelin, a distinctly prolonged release over a monitored period of 12 days was observed and nearly 3.81% of drug was released in each day. In the test solution of a pH 6.8 phosphate buffer (simulative intestinal fluid), the drug-release rate from SLN was similar to that in the simulative gastrointestinal fluid. Further, a novel preparation method in the present research for peptide-loaded SLN was established. These results also demonstrate the principle suitability of SLN as a prolonged release formulation for hydrophilic peptide drugs.

Keywords: Solvent diffusion method in aqueous system; Solid lipid nanoparticles; Gonadorelin; Monostearin; Entrapment efficiency; Prolonged release

1. Introduction

In recent years, many new pharmaceutically active polypeptides have been developed due to the progress of biotechnological techniques and genetic engineering. These new therapeutic biomolecules are usually characterized by large size, short plasma half-life, limited ability to cross cell membranes, and frequent injection of drug over a long therapeutic period are generally required when they are used to treat disease. Therefore, the design of a new dosage form leading to prolonged release should be suitable to overcome such a practical disadvantage.

The depot formulation such as injectable microcapsules composed of biodegradable and biocompatible polymers (i.e., D,L-lactide/glycolide copolymer, PLGA) have already been developed to clinical therapy of analogues of gonadorelin (Rothen-Weinhold

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and Gurny, 1997). Up to now, PLGA and poly(D,L-lactide) (PLA) are the only accepted carrier materials. However, the lower entrapment efficiency of polypeptide on these prolonged release delivery system probably still be existed (Niwa et al., 1994). The stability of drug affected by the local acidic environment after polymer degraded (Castellanos et al., 2003) will be a challenging problem.

Solid lipid nanoparticles (SLN) are an alternative colloidal carrier system for controlled drug delivery (Müller et al., 2000). Compared to other particulate carriers the SLN has more advantages for drug delivery system, such as a good tolerability (Müller et al., 1996a) and biodegradation (Müller et al., 1996b), a high bioavailability by ocular administration (Cavalli et al., 2002), a targeting effect on brain (Yang et al., 1999). In recent years, the study on SLN has markedly increased, especially with the method of high pressure homogenization (Olbrich et al., 2001; Müller and Lucks, 1996).

Solid lipid nanoparticles are a biodegradable particulate drug delivery system (Olbrich and Müller, 1999). Due to the lipophilic material of SLN, only a few investigations have been studied regarding the incorporation of peptides into SLN (Almeida et al., 1997) and few research has been reported on the drug-release from peptide-loaded SLN.

The hot homogenization method with formulating surfactant is the main method for SLN preparation and can easily produce the burst release (Mühlen and Mehnert, 1998). Therefore, the research of prolonged release on SLN is still lacking. The goal of this investigation will be to develop a new preparation method of polypeptide-loaded SLN. It was the work continued from the previous research of our group by a novel solvent diffusion method (Hu et al., 2002). Gonadorelin was used as a polypeptide model drug and the prolonged release behavior of the drug-loaded SLN was observed.

2. Materials and methods

2.1. Materials

Monostearin (Shanghai Chemical Reagent Co., Ltd., China) was used as lipid material of solid lipid nanoparticles. Gonadorelin was kindly donated by Ningbo Hormone Product Co. Ltd. (China). Polyvinyl alcohol (PVA 04-86, Beijing Chemicals Co., Ltd., China) was used as a dispersing agent in water phase. Ethanol, acetone and other chemicals were analytical reagent grade.

2.2. Preparation of solid lipid nanoparticles

The weighed monostearin (400 mg) and the weighed drug (gonadorelin, 4.2 mg) were dissolved completely in a mixture of acetone (6 ml) and ethanol (18 ml) in water bath at 50 °C. The resultant organic solution was poured into 240 ml of an acidic aqueous phase containing 1% PVA (w/v) under mechanical agitate (DC-40, Hangzhou Electrical Engineering Instruments, China) with 400 rpm at different temperature for 5 min. The pH value of the acidic aqueous phase was adjusted to 1.0 by 0.1 M hydrochloric acid. The SLN suspension (original suspension) was quickly produced. The entire dispersed system was then centrifuged (20,000 rpm for 30 min, 3k30, SIGMA Labrorzentrifugen GmbH, Germany) and received the original precipitate. The original precipitate was re-suspended in distilled water (re-suspended suspension, the concentration of lipid was 1.0 mg/ml) by probe-type ultrasonic treatment with 20 times (active every 1 s for a 2 s duration) in ice-bath (400 W, JY92-II, Scientz Biotechnology Co., Ltd., China).

2.3. Measurement of physicochemical properties of solid lipid nanoparticles

The mean diameter of SLN in suspension was determined with Zetasizer (3000HS, Malvern Instruments, UK) after the re-suspended suspension diluted 20 times with distilled water. The zeta potential of SLN in suspension was determined with Zetasizer (3000HS, Malvern Instruments, UK) after diluted 20 times with the original dispersion medium of preparation. For example, the measurement of the zeta potential data of SLN on the condition of the aqueous system with pH 1.0 containing 1% PVA, was operated after the prepared original SLN suspension diluted with the aqueous system with pH 1.0 containing 1% PVA. After the dilution, the pH value of the system is unchanged. The ‘zeta potential of usual aqueous phase (pH 5.7)’ were determined with the re-suspended suspension, diluted with the aqueous system at pH 5.7 containing 1% PVA, at the same solid content as SLN determined sample.

2.4. Determination of gonadorelin

The original suspension was placed in Ultrafree tube with a cutoff of 10,000 Da (Ultrafree, MC Millipore, Bedford, USA) and centrifuged for 5 min at 14,000 × *g* (3K30, SIGMA Labrorzentrifugen GmbH, Germany). The filtrate was determined by HPLC using an Agilent G1310A pump (1100 Series) unit control, an Agilent G1314A Variable Wavelength Detector (1100 Series) set at 230 nm. An Agilent μBondapak^{TM} ODS column (300 mm × 3.9 mm) was used. The mobile phase was consisted of 0.1 M phosphoric acid–methanol–acetonitrile (67/12/26, v/v/v), and the pH value of the 0.1 M phosphoric acid solution was adjusted to 3.0 with triethylamine before use. The flow rate was 1.0 ml/min. The drug entrapment efficiency in the SLN was calculated from Eq. (1).

$$\begin{aligned}&\text{Drug entrapment efficiency}\\&\quad=(\text{weight of drug added in system}\\&\qquad-\text{analysed weight of drug in ultrafiltrates})\\&\qquad\times\frac{100}{\text{weight of drug added in system}}\end{aligned}\qquad(1)$$

2.5. In vitro release kinetics and stability of gonadorelin in SLN

The release rates of gonadorelin were determined in release media consisting of delayed-release test solution 0.1N hydrochloric acid or pH 6.8 phosphate buffer as specified in the USP XXIII (delayed-release (enteric-coated) articles-general drug-release standard, Method A or Method B).

The original precipitate of SLN in preparation was re-suspended in 20 or 30 ml release medium (in 50 ml appropriate glass test-tube) by probe-type ultrasonic treatment with four times (active every 1 s for a 2 s duration) in ice-bath (400 W, JY92-II, Scientz Biotechnology Co., Ltd., China) and then shaken horizontally (Incubator Shaker HZ-8812S, Hualida Laboratory Equipment Company, China) at 37 °C and 60 strokes per minute. The duration of the release test was operated in 22.5 ml of a 0.1N hydrochloric acid for 2 h and then transferred the SLN suspension to a pH 6.8 phosphate buffer (simulative gastrointestinal fluid, addition of 7.5 ml of 0.2 M tribasic sodium phosphate, if necessary, adjust with 2N hydrochloric acid or 2N sodium hydroxide to a pH of 6.8). Another release test was operated in 30 ml of pH 6.8 phosphate buffer (simulative intestinal fluid, prepared by mixing 0.1N hydrochloric acid with 0.2 M tribasic sodium phosphate (3:1), if necessary, adjust with 2N hydrochloric acid or 2N sodium hydroxide to a pH of 6.8).

One milliliter of the suspension was withdrawn from the system at each time interval, and was placed in Ultrafree tube with a cutoff of 10,000 Da (Ultrafree, MC Millipore, Bedford, USA) and centrifuged for 5 min at 14,000 × *g*. At each sampling point in 0.1N hydrochloric acid, an amount of blank release medium equal to the sample volume was added to the test tube. The filtrate was determined using the HPLC method as described above. The free drug (41.8 μg) in each release media was periodically analyzed using the same batch process to evaluate the stability of gonadorelin in gastrointestinal fluid.

3. Results and discussions

3.1. Solvent diffusion method in an aqueous system

The volume mean diameters, zeta potential and the polydispersity indices of SLN prepared with solvent diffusion method in an aqueous system are listed in Table 1. The particles exhibited monodispersed having a volume mean diameter of 421.7 nm with a polydispersity index of 0.235.

Most studies of microparticulate system on slow-release formation of peptide are preferred at the particle sizes of 50–100 μm. There are only a few investigations of incorporation of peptides into nanoparticles (Kawashima et al., 1998; Jain, 2000). It is probably because these technologies are difficult to implement under the conditions needed for peptide stability (Couvreur and Puisieux, 1988).

On our previous research on preparation SLN by solvent diffusion method, the PVA molecular in aqueous phase are adsorbed around the emulsion droplets, which was formed by the diffusion of organic solvent (containing lipid) to aqueous phase, resulting in spontaneous droplet formation in the submicron range. At present research, we used the same method to separate

Table 1
Average diameter, polydispersity index of SLN

Dosage forms	Number average (nm)		Volume average (nm)		Polydispersity
	Mean	Width	Mean	Width	
Drug-free	400.1	99.5	403.2	102.7	0.257
Drug loading	416.5	147.1	421.7	144.7	0.235

the SLN from an acidic dispersed aqueous medium (containing PVA molecular). Under the condition of a usual dispersed aqueous medium with pH 5.7 containing 1% PVA (prepared by 1% PVA in distilled water), coacervation and finally precipitation of lipid did not take place. The physical stability of SLN suspension was excellent. After the nanoparticles suspension was stored at room temperature for 2 weeks, the particle size of drug loaded SLN was 415.3 nm (average volume diameter), and did not change compared to the initial particle size (421.7 nm). There is also no visible aggregation in system during storage. Under the lower pH condition, the zeta potential of system is more nearly zero (as shown in Table 2) and produced aggregation of the SLN, and then the separation of SLN from SLN suspension is easily operated by centrifugation.

3.2. Encapsulation efficiency of drugs

The effect of different temperature of dispersed aqueous phase on drug encapsulation efficiency of SLN is shown in Table 3. The encapsulation efficiencies of SLN depended on the temperature condition of the dispersed aqueous phase and were in the rank order of the low temperature (0 °C), followed by the room temperature (25 °C).

Table 2
The zeta potential of lipid, SLN in the different dispersed aqueous phase

Dispersed aqueous phase	Drug-free SLN		Drug-load SLN	
	Zeta potential (mV)	Width (mV)	Zeta potential (mV)	Width (mv)
Usual aqueous phase (pH 5.7)	−22.8	1.8	−21.1	1.5
Acidic aqueous phase (pH 1.0)	1.8	0.5	0.9	1.2

Peptides may cause special difficulties in their encapsulation, especially because most of them have a significant hydrophobic component of carrier system and thus have a tendency to adsorb onto the surfaces such as glass and plastic. Such adsorption can lead to distinct losses in the amount of peptide available for delivery (Duncan et al., 1995). In addition, the amount of peptide to be incorporated into the drug delivery system is dependent on the physicochemical properties of peptide and the preparation process.

Low temperature condition of the dispersed aqueous phase was performed by in an ice-water bath, and the nanoparticles were prepared in order to improve the encapsulation efficiency of gonadorelin. In the solvent diffusion method in an aqueous system, the diffusion rate of the water miscible organic solvent to aqueous phase was very rapid. The encapsulation efficiency of gonadorelin in SLN was increased to 69.4% as the temperature of the dispersed aqueous phase decreased to 0 °C, which was attributed to the rapid deposit of lipid in droplet form before it could coalesce during stirring and decreased the leakage of gonadorelin into the outer aqueous phase, on the condition of low temperature. It was suggested that the rapid deposition at the interface between the droplets and the aqueous medium would prevent the leakage of peptide

Table 3
Effect of temperature conditions of dispersed aqueous phase on average diameter, drug encapsulation efficiency of solid lipid nanoparticles (w/w)

Temperature conditions (°C) (aqueous phase)	Volume average (nm)	Drug encapsulation efficiency (%)
25	369.5 ± 111.8*	50.4 ± 3.00**
0	421.7 ± 144.7*	69.4 ± 0.31**

Data are represented with mean ± S.D. ($n = 3$).

* $P > 0.1$: significant difference compared with temperature of 25 °C using Student's *t*-test.

** $P < 0.01$: significant difference compared with temperature of 25 °C using Student's *t*-test.

drug, leading to an improvement in the drug encapsulation efficiency, when decreasing temperature of the dispersed aqueous phase. But the volume mean diameters of SLN have no significant difference when the temperature condition of the dispersed aqueous phase is decreased from 25 to 0 °C, which is attributed to the rapid diffusion of organic solvent and little effect of temperature.

3.3. *Release and stability behavior of gonadorelin in SLN in vitro*

The release behavior of gonadorelin in SLN was investigated in the simulative gastrointestinal fluid to simulate the behavior of SLN exposed in the gastrointestinal tract following oral administration. In general, gastric emptying time (GET) values for solid dosage forms such as granules were approximately 2–3 h under feeding and <1 h under fasting, respectively, and the small intestinal transit time was kept 3–4 h. The gonadorelin-loaded SLN prepared with low temperature condition was used as a sample for the release test. The duration of the release test was operated in a 0.1N hydrochloric acid for 2 h and then transferred the SLN suspension to a pH 6.8 phosphate buffer. Considering the possibility of prolonged release of SLN, another release test in a pH 6.8 phosphate buffer was arranged in order to simulate the behavior of SLN exposed in the vivo following hypodermic administration.

The drug-release profiles from the SLN proved linear relationships for zero order release after the burst of drug (Figs. 1 and 2). In the test, drug-release was first carried out in a 0.1N hydrochloric acid for 2 h and then transferred the SLN suspension to a pH 6.8 phosphate buffer, the drug-release behavior from SLN suspension exhibited a biphasic pattern. After burst drug-release at the first 6 h at 24.4% of loaded gonadorelin, a distinctly prolonged release over a monitored period of 12 days was observed and nearly 3.81% of drug was released in each day, calculating from the trendline equation in Fig. 1.

In the test solution of a pH 6.8 phosphate buffer, the drug-release rate from SLN was similar to that in the simulative gastrointestinal fluid, except for the extent of burst release. There is only a half amount of drug (13.9%, compared to that in the simulative gastrointestinal fluid) from SLN was observed at the first 2 h and may be produced by the adsorption of drug on the surface of SLN. After the burst release, a prolonged release was obtained and released 3.97% of drug from the SLN every day over a monitored period of 14 days, calculating from the trendline equation in Fig. 2.

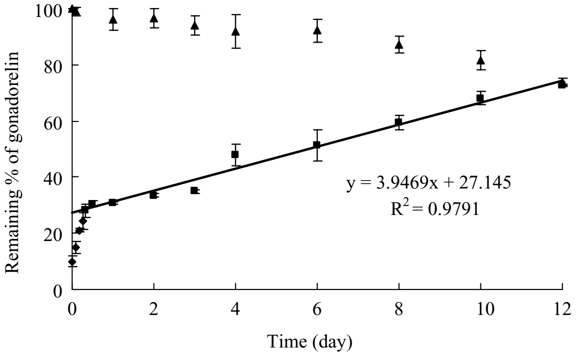

Fig. 1. Release profiles of gonadorelin from SLN and stabilization of gonadorelin during the release test in a 0.1N hydrochloric acid for 2 h and then transferred the SLN suspension to a pH 6.8 phosphate buffer, containing 1.5% glycin as a stabilizer ($n = 3$). Key: (▲) gonadorelin solution; (◆) burst release from SLN; (■) prolonged release from SLN; (—) linearity (prolonged release).

Comparing with the difference of Figs. 1 and 2, we found that the amount of burst drug-release from SLN was much higher in acidic release medium (the simulative gastrointestinal fluid, Fig. 1) than that in neutral release medium (the simulative intestinal fluid, Fig. 2), though the release test operated only 2 h in the acidic release medium. After the burst release at

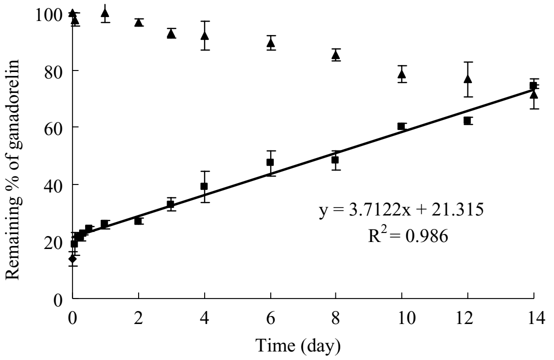

Fig. 2. Release profiles of gonadorelin from SLN and stabilization of gonadorelin during the release test in a pH 6.8 phosphate buffer, containing 1.5% glycin as a stabilizer ($n = 3$). Key: (▲) gonadorelin solution; (◆) burst release from SLN; (■) prolonged release from SLN; (—) linearity (prolonged release).

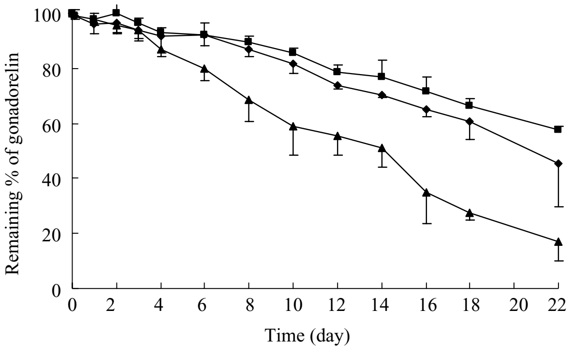

Fig. 3. Stabilization of gonadorelin during the release test in different release media ($n = 3$). Key: (▲) in pH 6.8 phosphate buffer; (■) in pH 6.8 phosphate buffer, containing 1.5% glycin as a stabilizer; (◆) in a 0.1 s hydrochloric acid for 2 h and then transferred the SLN suspension to a pH 6.8 phosphate buffer, containing 1.5% glycin as a stabilizer.

the first 2 h in the acidic release medium, a continuous burst release was observed at the next 4 h in a replaced solution of pH 6.8 phosphate buffer (Fig. 2). As usual, lipid material is easily hydrolyzed especially in acidic condition (Barrault et al., 2002). The difference of drug-release behavior from SLN in different release medium may mean that lipid molecular partially hydrolyzed from the surface of SLN in the acidic medium, and affected the release pattern at the first 2 h in acidic medium and next 4 h in next replaced neutral medium.

The process to produce a prolonged-release formulation for a peptide is difficult and depends on many factors, in particular since inactivation is possible during their incorporation into the release medium. Some environmental factors affected peptide stability (Malgorzata et al., 1996). Gonadorelin and its analogs in aqueous solution is reasonably stable at special pH extent, but labile in acidic and alkaline solution (Powell et al., 1991). In our present test in the simulative intestinal fluid, more than 48.8% of gonadorelin was degraded during incubation for 14 days at 37 °C (Fig. 3). After addition of 1.5% glycin (w/v) in the release medium, the stability of gonadorelin was much improved and the amount of gonadorelin released from SLN was easily determined.

Compared to the usual method of the SLN production with the high pressure homogenization, using a lot of surfactant or drug-solubilizing surfactant, the novel solvent diffusion method in aqueous system is simply operation, needless any special equipment can obtain the sustained release.

4. Conclusion

A novel solvent diffusion method in aqueous system was demonstrated to prepare the peptide loaded solid lipid nanoparticles with the highly gonadorelin encapsulation efficiency after the selection of the optimal temperature condition of dispersed medium. The size distribution of SLN revealed a monodispersed profile (average diameter: 421.7 nm) in distilled water. In vitro release of gonadorelin from SLN in the simulative gastrointestinal fluid, the drug-release behavior from SLN suspension exhibited a biphasic pattern with an initial burst and prolonged release over 12 days, following the zero order release. The drug-release rate from SLN suspension in the simulative intestinal fluid was similar to that in the simulative gastrointestinal fluid, except for the extent of burst release.

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