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Preparation and in vitro release studies of thymosin-loaded PLA microspheres

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Abstract: To obtain a kind of convenient oral dosage form of protein, which can be fully absorbed and is efficient and safe, the thymosin-loaded PLA(polylactic acid) microspheres are prepared by the emulsification-solvent evaporation method and the orthogonal design is used to optimize the technology of preparation. The form of the medicament microspheres of thymosin are proved by differential thermal analysis (DTA). The drug content is determined by the Lowry method, and the package ratio of medicament microspheres of thymosin and drug release in vitro are calculated. The results show that the average diameter and encapsulation efficiency of the product prepared according to the optimized formulation are 13.8 μm and 80.7%, respectively. The in vitro release behavior within 12 h can be described by the Higuchi equation with $T_{1/2}$ = 295 min. There are no significant changes in size distribution and residual drug contents after being stored at 25 ℃ and 40 ℃ for 90 d, respectively. Due to the fact that its thymosin content and package ratio meet the requirement, and its releasing half life is long, the thymosin-loaded PLA microsphere has a favorable application future.

Key words: thymosin; polylactic acid; microsphere; in vitro release

Thymosin, originally purified from the thymus of the calf or pig, is regarded as a main sequestering peptide which stimulates lymphocyte growth. Thymosin is found to be involved in multiple biological processes as an immune-potentiating agent. Clinical study provides a rationale for the use of thymosin in reconstituting immunocompetence with primary or secondary immunodeficiency diseases, pre-existing immune deficits, the development of autoimmune diseases, resistance to tumor induction by malignant cells or oncogenic viruses are also addressed[1]. At present, the dosage forms of thymosin products used in clinics refer to injection or orally administrated capsules or tablets[2]. With the aim to improve the bioavailability of thymosin through oral administration, thymosin-loaded microspheres with good dispersal are used as drug carriers for controlled delivery to enhance the absorption of polypeptides via the gastrointestinal tract[3]. And, the degradation of thymosin-loaded microspheres by gastrointestinal tract enzymes may be slowed down[4]. In order to obtain convenient oral dosage forms of protein, which can be fully absorbed and are efficient and safe, thymosin-loaded PLA microspheres are prepared by the emulsification solvent evaporation method in the present study. The effects of process parameters are investigated and the behavior of drug release is evaluated in vitro.

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1 Instruments and Reagents

Instruments: Hitachi X-650 scanning electron microscopy, 722 grating spectrometer (Third Analytical Instrument Factory in Shanghai).

Reagents: thymosin (Modern Medical Center, Southeast University, Lot # 030530), PLA (molecular weight is 60 000, Shanghai Institute of Organic Chemistry), bovine serum albumin (National Institute for the Control of Pharmaceutical and Biological Products, 15.6 mg/sticks), gelatin (Shanghai Yan' an manufacturers), Folin reagents.

2 Experiment and Results

2.1 Preparation of PLA microspheres

PLA was dissolved in dichloromethane, and then the mixed emulsifier (tween 80 and span 80, 5∶1) was added into the solution. By adding 1% gelatin solution (containing 1% thymosin), the mixture was stirred to obtain an emulsion. The resultant W/O emulsion was sonicated for 10 min at 10 ℃ and then added to the 5% mixed emulsifier gelatin solution by stirring at 950 r/min. The resultant W/O/W emulsion was sonicated for 5 min and stirred slowly at 45 ℃ in a water bath for 5 h. The PLA microspheres were separated, washed by isopropanol and dried at 60 ℃ for 24 h.

2.2 Optimization of process parameters

2.2.1 Orthogonal design

Orthogonal experiments were designed to investigate the effects of five process parameters: PLA concentration (A), water-oil volume ratio (B), stirring speed (C), the concentration of the mixed emulsifier (D) and the concentration of gelatin in the external water phase (E). The test was taken at four levels (see Tab. 1), according to $L_{16}(4^5)$.

Tab. 1 Experimental factors and levels

Level	Factor				
	A/%	B	C/(r·min^{-1})	D/%	E/%
1	2	1∶3	300	0.5	0.1
2	5	1∶6	600	0.8	0.3
3	8	1∶10	800	1.0	0.5
4	10	1∶12	1 000	1.5	1.0

2.2.2 Evaluation parameters

Directly observed by optical microscopy, the average particle size of microspheres s_1, the uniformity of size distribution s_2 and the degree of adhesion between the microspheres s_3 was evaluated and the score of S ($S = s_1 + s_2 - s_3$) were calculated. The higher the score of S is, the better the qualities of the microspheres are.

2.2.3 Process optimization

Poor analysis shows that the effects of five process parameters on the size of the microspheres and the degree of adhesion between the microspheres is $A > C > D > B > E$. So, process optimization for $A_2B_3C_4D_2E_3$ results in: a PLA concentration of 5%, a solution volume ratio of 1% gelatin and PLA solutions of 1∶10, a stirring speed of 1 000 r/min, a mixed emulsifier concentration of 0.8%, and a concentration of gelatin in the external water phase at 0.5%.

2.3 Characterization of thymosin-loaded PLA microspheres

2.3.1 Thermal analysis

Differential thermal analysis was used to demonstrate the formation of PLA microspheres. Data were normally collected between 40 ℃ and 400 ℃ at a scanning nominal rate of 10 ℃/min. Fig. 1 shows the DTA curve of the thymosin-loaded PLA microspheres.

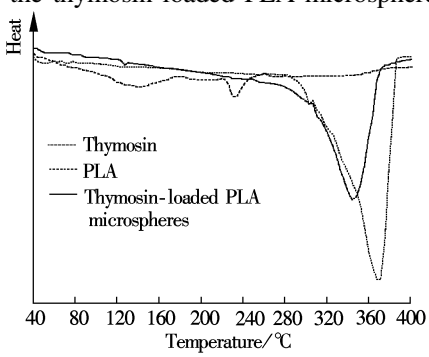

Fig. 1 DTA curve

The DTA curve of the thymosin showed an endothermic peak at 140.1 and 229.9 ℃. Another endothermic peak at 362.2 ℃ belonged to the PLA, and only an endothermic peak at 336.5 ℃ belonged to thymosin-loaded PLA microspheres. The result demonstrates that thymosin-loaded microspheres have been formed.

2.3.2 Structure and morphology of PLA microspheres

The morphologies of the thymosin-loaded PLA microspheres were observed by scanning electron microscopes (SEM). SEM images show that the microspheres with size distributions of 4 to 24 μm and an average diameter of 13.8 μm are round and uniform (see Fig. 2).

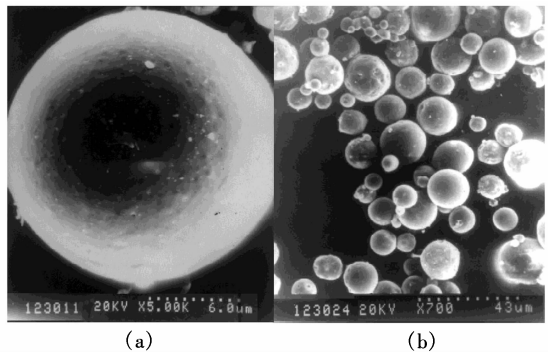

Fig. 2 Microspheres by scanning electron microscope. (a) 5000×; (b) 700×

2.4 Drug loading and embedding rate

The content of thymosin in microspheres was determined according to *China Biological Products* (2000 version) assay (Lowry Folin phenol reagent).

2.4.1 Standard curve

12.8 mg bovine serum albumin was dissolved in 0.03 mol/L phosphate-buffered saline (PBS, pH 7.8) to form a 0.3 mg/mL standard protein solution. Add two solutions to six tubes, as shown in Tab. 2. The blending solutions were heated at 55 ℃ in a water bath for 5 min, and a cooling bath for 10 min, then the absorbance at 650 nm (A) was measured. By linear regression of A to the standard protein concentration (C), a standard curve equation $A = 0.941C + 0.024$, with $r = 0.9996$ and linear range of 30 to 270 μg/mL was obtained.

Tab. 2 Standard curve solution mL

Number	Bovine serum albumin solution	Phosphate-buffered saline solution
0	0	1
1	0.1	0.9
2	0.3	0.7
3	0.5	0.5
4	0.7	0.3
5	0.9	0.1

2.4.2 Drug loading and embedding rate

Abrasive microspheres (equivalent to 125 mg) prepared by optimizing the process were dissolved in 15 mL PBS with centrifuging (2 000 r/min) for 5 min. The concentration of protein samples was calculated from the standard curve equation[5]. Drug loading and encapsulation efficiency of microspheres were determined according to *Chinese Pharmacopoeia*[6]. The results reveal that the average drug loading of microspheres is 0.2 mg/mg while the average encapsulation rate is 80.7%.

2.5 In vitro release studies

According to *Chinese Pharmacopoeia*[6], the thymosin-loaded microspheres were placed in a release medium with a pH 6.8 PBS solution. The test was performed at (37 ± 0.5)℃, 3 000 r/min. 150 mg (appropriately 30 mg thymosin) microspheres and thymosin powder 30 mg were placed in dialysis membranes. Regular samples were taken after having been placed in the dissolution cup for 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 h and 2, 4, 6, 8, 10, 12, 15, 20 min, respectively. The content of thymosin was determined by the Lowry method, and the cumulative release rate was calculated from the standard curve equation Q (see Fig. 3).

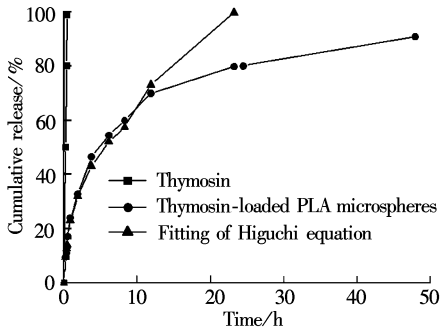

Fig. 3 Profile of microcapsule's release in vitro at 37 ℃ (n = 6)

The results demonstrate that $T_{1/2}$ of the thymosin powder and microspheres were about 7 and 295 min, respectively. The in vitro release behavior of the thymosin-loaded PLA microspheres (former 12 h) acted in accord with the Higuchi equation $Q = 20.35\ T_{1/2} + 2.47$ (r = 0.996 0). The release curve obviously shows that the microsphere control release and burst release phenomenon is more serious initially. For example, the cumulative release in the first 2 h is nearly 30%.

2.6 Stability[7]

An appropriate amount of thymosin-loaded PLA microspheres was kept at 25 ℃ and 40 ℃ for 90 d. Changes in appearance and color were observed and the content of thymosin was determined. The results indicate that thymosin-loaded PLA microspheres placed at 25 ℃ for 90 d showed fewer changes in morphology and size distributions while slight adhesions were observed under 40 ℃. The microspheres showed good stability due to the fact that the remaining doses were 100.0% and 98.1%, respectively.

3 Conclusion

Hydrophilic and lipophilic balance values and the amount of emulsion can be adjusted with the use of mixed emulsifiers in the preparation of primary emulsions. A more lasting film is formed to prevent polymerization of emulsion droplets effectively, and the stability of the emulsion is improved. Improper stirring during the preparation of resultant emulsions leads to a combination of internal and external water phases. Sufficient emulsification time facilitates the formation of microspheres with small particle sizes and smooth surfaces.

The average particle diameter of thymosin-loaded PLA microspheres prepared by the emulsification-solvent evaporation method is tiny, and its thymosin content and package ratio meet the requirements[8]. And its releasing half-life is long, which is forty-three times that of the thymosin. The biology consistent characteristics and haemal consistent characteristics of thymosin-loaded PLA microspheres are favorable and thymosin-loaded PLA microspheres have a favorable application future. The study of the quality and analysis of thymosin-loaded PLA microspheres will be developed in our future work.

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胸腺肽聚乳酸微球的制备和体外释药研究

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摘要:为了寻找适于多肽分子吸收,免疫学上“有效”、“安全”,且服用方便的口服制剂,用 W/O/W 型乳化挥发法制备胸腺肽聚乳酸微球,正交实验方法优化了制备工艺. 通过差热分析证明载药微球已较好形成,Lowry 法测定药物的含量,计算微球的载药量、包封率及体外释药量. 结果表明,所得微球平均粒径为 13.8 μm,平均包封率为 80.7%,前 12 h 的体外释药符合 Higuchi 方程,$T_{1/2}$ = 295 min,在 25 ℃和 40 ℃分别放置 90 d,微球的粒径分布和剩余药量无显著变化. 微球的载药量和包封率符合要求,释药半衰期长,具有良好的应用前景.

关键词:胸腺肽;聚乳酸;微球;体外释药
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