Poly(Lactic-co-glycolic Acid) Microspheres for the Controlled Release of Huperzine A: *In Vitro* and *in Vivo* Studies and the Application in the Treatment of the Impaired Memory of Mice

Dafeng CHU,[a,b] Jingwei TIAN,[b,c] Wanhui LIU,[b,c] Zi LI,[b] and Youxin LI*,[a,b]

[a] *College of Life Science, Jilin University; No. 2699, Qianjin Road, Changchun, 130012, China:* [b] *School of Pharmacy, Yantai University; No. 32, Qingquan Road, Yantai, 264005, China: and* [c] *Research and Development Center, Shandong Luye Pharmaceutical Co., Ltd.; No. 9, Baoyuan Road, Yantai, 264003, China.*

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Huperzine A loaded poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres were prepared by an oil/water (o/w) solvent evaporation technique. With a decrease of the ratio of o/w from 1 : 100 to 1 : 50, the encapsulation efficiency was reduced about 4%. Increasing the PVA concentration from 0.5 to 2% reduced the percentage encapsulation efficiency of huperzine A from 60.7 to 47.4% and the particle size of microspheres from 84.2 to 26.2 μm. The addition of stearic acid improved the encapsulation efficiency, but also accelerated the *in vitro* release of hupezine A from microspheres. After i.m. administration of huperzine A loaded microspheres in mice, huperzine A was sustained released from the PLGA microspheres up to 12 d with a low initial burst. Passive avoidance test of mice showed that the microspheres formulation offered an improved therapeutic efficiency in the treatment of the impaired memory of the mice superior to injection gastric (i.g.) administration of huperzine A suspension at the same dose, whose therapeutic efficiency was similar as that of a 50% reduced dose of the microspheres formulation.

Key words biodegradable polymer; microsphere; huperzine A; pharmacokinetics; pharmacodynamics

Huperzine A, first isolated in 1986 from the Chinese medicinal herb *Huperzia serrata* (THUNB.) TREV., is a reversible, potent, and selective inhibitor of acetylcholinesterase. Compared with other well-known acetylcholinesterase inhibitors for the treatment of Alzheimer's disease, such as physostigmine, galanthamine, tacrine, and even donepezil, huperzine A has showed favorable features such as high potency, long duration of action, low toxicity and better penetration through the blood-brain barrier.[1,2)]

Currently, huperzine A is available as twice daily oral administration (200 to 400 μg/d).[3)] For Alzheimer's patient who suffers memory disorder, it is difficult not to miss scheduled self-medication and therefore, long-term formulations of huperzine A against Alzheimer's disease are attractive for researchers. Several formulation studies have been performed on huperzine A loaded poly(lactic-co-glycolic acid) (PLGA) microspheres using an oil/water (o/w) emulsion solvent evaporation technique.[4—7)] However, the effects of formulation parameters on the properties of huperzine A loaded PLGA microspheres have not been investigated enough.

In this study, preparation, characterization, *in vitro* release, pharmacokinetics and primary pharmacodynamics of huperzine A loaded PLGA microspheres were investigated.

Experimental

Materials Carboxylic group terminated poly(D,L-lactic-co-glycolic acid) (PLGA) RG 502 H (lactide/glycolide ratio, 50/50; Mw, 12000; acid value, 9.7 mg KOH/g) and RG 503 H (lactide/glycolide ratio, 50/50; Mw, 25000; acid value, 4.9 mg KOH/g) were supplied by Boehringer Ingelheim AG (Ingelheim, Germany); Huperzine A was obtained from Joyline & Joysun Pharmaceutical Stock Co. Ltd. (Zhengzhou, China). Polyvinylalcohol (87—89% hydrolyzed; Mw, 13000—23000) and stearic acid was supplied by Sigma-Aldrich (Shanghai) Trading Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade and were obtained from commercial supplies.

Preparation of Huperzine A Microspheres Huperzine A loaded microspheres were prepared using an o/w emulsion solvent evaporation method. Different amounts of drug, polymer and stearic acid were dissolved in dichloromethane. The solution was injected into Polyvinylalcohol (PVA) aqueous solution at 6 °C under vigorous mechanical stirring at 1600 rpm for 3 min. The microspheres were solidified under mild stirring at 150 rpm for 5 h with the temperature increased to 25 °C. The solidified microspheres were filtrated with a 10 μm sieve and washed by distilled water for three times, and then freezing dried (−20 to 25 °C). After sieved with a 154 μm sieve, the microspheres were stored at −20 °C.

Characterization of the Microspheres The particle size of the microspheres was determined using a laser particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, U.K.) with a small volume sample dispersion unit (Hydro SM).

The dried microspheres were fixed with an adhesive sheet on a rigid support and coated with gold. Morphology of microspheres was then studied by scanning electron microscopy (SEM) (JSM-5610LV, JEOL, Tokyo, Japan).

The glass transition temperature (T_g) of the microspheres was analyzed by Differential Scanning Calorimeter (DSC) (822e, Mettler-Toledo Instruments (Shanghai) Co. Ltd., Shanghai, China). Approximately 5 mg samples were heated in sealed aluminum pans with two heating cycles. Investigated temperature ranged from −20 to 100 °C with a heating rate of 10 °C/min.

Determination of Drug Loading Twenty milligrams of the microspheres was dissolved in 1 ml acetone in a 25 ml flask, and then 0.01 M HCl was added up to 25 ml. The precipitated polymer, in which no huperzine A was found, was removed by a 0.45 μm filter and the clear solution was used for analysis, which was carried out using HPLC connected with an UV detector in a mobile phase of the mixture of acetonitrile and 0.2% H_3PO_4 (25 : 75); flow rate, 0.7 ml/min; wavelength, 306 nm; column, ODS C_{18} (250×4.6 mm I.D., 5 μm particle size); injection volume, 20 μl.

The drug loading and encapsulation efficiency were calculated.

$$\text{encapsulation efficiency (\%)} = 100 \times \frac{\text{drug loading determined by HPLC}}{\text{theoretical drug loading}}$$

In Vitro* Release** Five milligrams of microspheres was suspended in 3 ml of 0.01 M phosphate buffered saline (PBS), pH 7.4, containing 0.01% Tween 80, 0.05% sodium azide and 0.04 M sodium chloride in a 5 ml plastic vial (n=3). The suspension was placed in a shaking bath (HZS-H, DongLian electronic Co., Harbin, China) at 40 rpm and 37 °C. Sink conditions were maintained during this study. At preset intervals, the vials were centrifuged at 3000 ***g for 30 min and then 2 ml of the supernatant was drawn and replaced by fresh buffer. The vials were vigorously vibrated before the *in vitro* release was restarted. Huperzine A in supernatant was determined by HPLC

* To whom correspondence should be addressed. e-mail: liyouxin@jlu.edu.cn

method described above.

Pharmacokinetic Study Animal experiments were conducted according to protocols approved by the Animal Care and Use Committee of Shandong Engineering Research Center of Natural Drug. Female and male Kunming mice with body weight 20—22 g were supplied by the Experimental Animal Center of Shandong Luye Pharmaceutical Co., Ltd. (Yantai, China). Huperzine A suspension (5 μg/ml) was prepared by dispersing the huperzine A tablet in sterile water. Huperzine A loaded microspheres suspension (60 μg/ml of huperzine A) was prepared in sterile water containing 1.5% carboxymethylcellulose sodium and 0.9% NaCl. The doses of the administrated huperzine A to mouse were designed according to the following equation: [400 μg (dose of huperzine A per day)/70 kg (body weight)]$_{human}\times$9.01 (the dose conversion factors of weight body between mouse and human).[8]

The present study involved 10 independent groups of 8 mice each and every time point comprised one group. The first group was drug free. Blank blood samples were collected from orbital sinus into heparinized tubes as zero time point by quickly removing the eyeballs. The second group was administrated injection gastricly (i.g.) huperzine A suspension (50 μg/kg) once daily for consecutive 7 d. Blood samples were similarly collected at 2 h after the last drug dosing. Another 8 groups were i.m. administrated huperzine A loaded microspheres suspension at a dose of 600 μg/kg of huperzine A, respectively. Blood samples were also similarly collected at preset time interval after drug administration. Plasma was separated by centrifugation for 10 min at 3000 $\boldsymbol{g}$ and stored at -20 °C until analysis. Huperzine A was analyzed using a LC-MS/MS method, according to the previous report.[9]

Primary Pharmacodynamic Study Utilizing the natural preference of mice for a dark environment, a passive avoidance test (step-through test) was performed.[10—12] The passive avoidance reflex apparatus was provided by Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China), which was separated into light chamber and dark chamber with a connective hole. Testing took place only between 9 and 12 a.m. On the testing day, each mouse was placed in the light compartment. After a habituation period of 3 min, the mouse was removed from the apparatus. Then the electricity switch was turned on and the mouse was placed in the light compartment again. Once the mouse stepped with all four paws into the dark side, the foot-shock (36 V, 55 Hz) was delivered. The mouse could escape from the shock only by stepping back into the safe illuminated compartment. The number of times they entered the dark chamber within 5 min was recorded as the number of errors to valuate their memory capacity.

The present study involved 19 independent groups of 12 mice each. The first and second groups were administrated intraperitoneally saline or an amnesic dose of scopolamine (3 mg/kg) 20 min prior to the test, respectively. The third group was administrated i.g. huperzine A suspension (50 μg/kg) once daily for consecutive 7 d. The mice were tested at 2 h after the last drug administration and 20 min after scopolamine (3 mg/kg) administration. Another 16 groups were divided into two parts of 8 groups each administrated i.m. huperzine A loaded microspheres suspension at a dose of 300 or 600 μg/kg, respectively. The test was conducted at 1, 2, 4, 6, 8, 10, 12, and 14 d postdose and 20 min after scopolamine (3 mg/kg) administration. Comparisons between different groups were done by Student's *t*-test. Data were expressed as mean$\pm$standard deviation (S.D.).

Results and Discussion

Preparation and Characterization of Microspheres Figure 1 showed the spherical and nonporous surfaces of the microspheres. The encapsulation efficiencies of microspheres were depicted in Table 1. Huperzine A is a small molecule and weak base with a solubility of 0.89 mg/ml in purified water and 1.21 mg/ml in 0.5% aqueous PVA (25 °C) and therefore, partitioning of huperzine A into the external aqueous phase lowered huperzine A entrapment efficiency in the case of the o/w method. With the decrease of the ratio of o/w from 1 : 100 to 1 : 50, the encapsulation efficiency was reduced 4.3%. It was probably that a higher concentration of dichloromethane in the external aqueous phase slowed the solidification of microspheres and improved the partition of huperzine A in the aqueous phase. Increasing the PVA concentration from 0.5 to 2% decreased the encapsulation efficiency of huperzine A from 60.7 to 47.4% and the particle size of microshperes from 84.2 to 26.2 μm. Because an increased PVA concentration not only increased the solubility of huperzine A to 1.47 mg/ml in 2% PVA (25 °C), but also improved the dispersibility and stability of the organic droplets in the aqueous continuous phase, resulting in a reduced particle size.

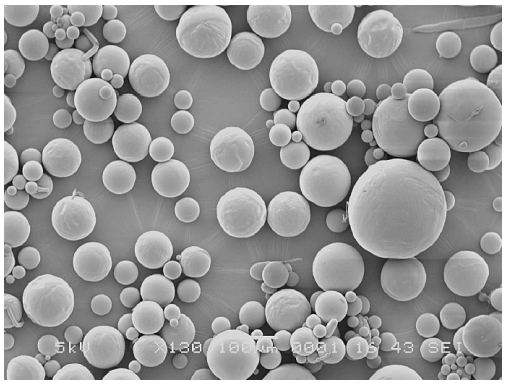

Fig. 1. Surface Morphology of Huperzine A Loaded Microspheres Prepared from PLGA 502 H/503 H (1 : 1) (Batch 7)

It was report previously that carboxylic group of the PLGA polymers influenced the encapsulation efficiency of huperzine A significantly.[5,7] In this study, the similar phenomenon was also found (Table 1). Based on this result, to improve the encapsulation efficiency of huperzine A in microspheres, stearic acid was added together with drug and polymer into dichloromethane. As expected, for 503 H microspheres, when the ratio of stearic acid/drug was increased to 1 : 1 (w/w), the encapsulation efficiency varied from 20.4 to 31.3% (Table 1). When assuming the acidic value was contributed completely by carboxylic terminal group, it can be obtained that the amount of the carboxylic group was 0.0875 mmol per gram polymer in the case of 503 H. Huperzine A with a MW of 242.3 contains a primary amino group. If the improvement of encapsulation efficiency was a result of the interaction between carboxylic group and amino group, theoretically, the most interaction is equal to 2.1% (0.0875$\times$242.3) of huperzine A/503 H (w/w). It indicates that the amount of carboxylic group is too low to get high drug loading, and the addition of stearic acid may improve the encapsulation of huperzine A in the PLGA polymer matrix. In the case of microspheres prepared with 502 H containing double amounts of carboxylic group than 503 H, the theoretical potential maximum drug loading is increased from 2.1 to about 4.2%. Experimental results demonstrated this improvement of more carboxylic terminal group on the drug loading. When the drug in feed varied from 2.5 to 5%, the drug encapsulation efficiency was dropped only 4.1%. But increasing drug in feed continuously from 5 to 12% reduced the encapsulation efficiency about 27% (Table 1). It indicated this improvement is limited by the amount of acidic end group and the superfluous amount of huperzine A than the amount of the acidic end group of PLGA was not encapsulated well in the microspheres and most of them dissolved in the external aqueous PVA solution.

***In Vitro* Release** Figures 2 and 3 showed the *in vitro* releases of huperzine from microspheres prepared from 502 H, 503 H as well as the blends of 502 H and 503 H. The initial

Table 1. Encapsulation Efficiency of Huperzine A Loaded Microspheres[a)]

Batch	Polymer	Drug/polymer in feed % (w/w)	O/w (v/v)	PVA concentration % (w/v)	Actual drug loading % (w/w)	Encapsulation efficiency %
1	502 H	8	1 : 50	0.5	4.23	57.2
2		8	1 : 75	0.5	4.49	60.6
3		2.5	1 : 100	0.5	1.91	78.3
4		5	1 : 100	0.5	3.53	74.2
5		8	1 : 100	0.5	4.56	61.5
6		12	1 : 100	0.5	5.01	46.8
7[b)]	502 H/503 H (1 : 1)	5	1 : 100	0.5	2.89	60.7
8		5	1 : 100	1.0	2.79	58.6
9		5	1 : 100	1.5	2.35	49.3
10		5	1 : 100	2.0	2.26	47.4
11	503 H	5	1 : 100	0.5	0.97	20.4
12[c)]		5	1 : 100	0.5	1.13	23.7
13[d)]		5	1 : 100	0.5	1.49	31.3

a) Polymer/dichloromethane (w/v) was 10% for 503 H and 20% for all other batches. *b*) It was used in pharmacokinetic and pharmacodynamic studies. *c*) Stearic acid/drug (w/w), 1 : 2. *d*) Stearic acid/drug (w/w), 1 : 1.

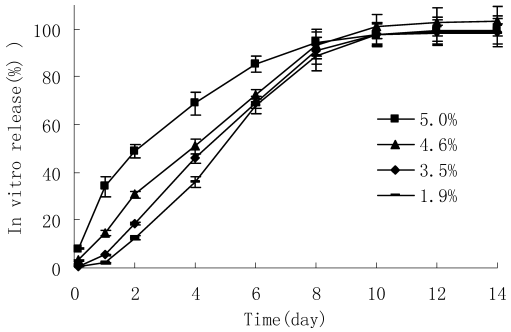

Fig. 2. *In Vitro* Cumulative Releases of Huperzine A Loaded Microspheres Prepared from 502 H with Different Actual Drug Loading of 1.9%, 3.5%, 4.6% and 5.0%, Respectively (Batch 3—6)

Each point represents mean±S.D. (n=3).

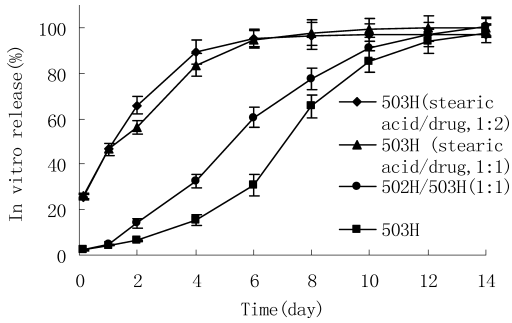

Fig. 3. *In Vitro* Cumulative Releases of Huperzine A Loaded Microspheres Prepared from 502 H/503 H (1 : 1) (Batch 7), 503 H (Batch 11) and 503 H with the Initial Ratio of Steatic Acid/Drug of 2 : 1 (Batch 12) and 1 : 1 (Batch 13)

Each point represents mean±S.D. (n=3).

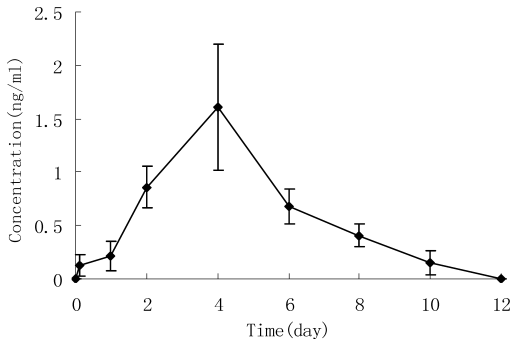

Fig. 4. Plasma Concentration *versus* Time Curve for Huperzine A after i.m. Administration of the Microspheres (Batch 7) Suspension at 600 μg/kg of Huperzine A

Each point represents mean±S.D. (n=8).

bursts were low and different to the significant initial burst in the case of the microspheres prepared from lactide/glycolide ratio of 75 : 25 PLGA with a molecular weight of 20000 and 30000, in which more drugs were distributed on or near the surface of the microspheres, which resulted in the initial burst release.[4)]

A high actual drug loading accelerated *in vitro* release (Fig. 2). The addition of stearic acid also accelerated the *in vitro* release of hupezine A from microspheres significantly (Fig. 3). It may be due to an increased amount of small molecules reduced the T_g of PLGA. Experimental results showed that when stearic acid was added, the T_g of the microspheres was reduced from 42.4 to 40.3 °C (stearic acid/drug, 1 : 2) and 39.4 °C (stearic acid/drug, 1 : 1), respectively. In addition, an increased amount of the hydrophilic carboxylic groups improves the hydrophilicity of microspheres, which leads to an accelerated swelling of the polymeric matrix and diffusion of huperzine A in microspheres.

Pharmacokinetic Study In the case of i.g. administration of huperzine A suspension (50 μg/kg) once daily for consecutive 7 d, the plasma concentrations at 2 h after the last dosing were 1.08±0.34 ng/ml. The mean plasma concentration–time curves of huperzine A after i.m. administration of huperzine A loaded microspheres (Table 1, batch 7) suspension at a dose of 600 μg/kg were shown in Fig. 4. The initial release within 24 h was low, which was similar with the previous studies in dogs.[7)] At the 4th day, the plasma concentration of huperzine A reached the C_{max} of 1.60±0.59 ng/ml, and then slowly fell until the drug was exhausted at the 12th day. A good linear regression relationship was observed between the *in vitro* and *in vivo* releases using the Loo–Riegelman method.[13)]

Primary Pharmacodynamic Study After i.m. administration of scopolamine hydrobromide (3 mg/kg), in the trials on acquisition memory, the memorial impairment of the mice due to scopolamine led to a significant reduction in the sensi-

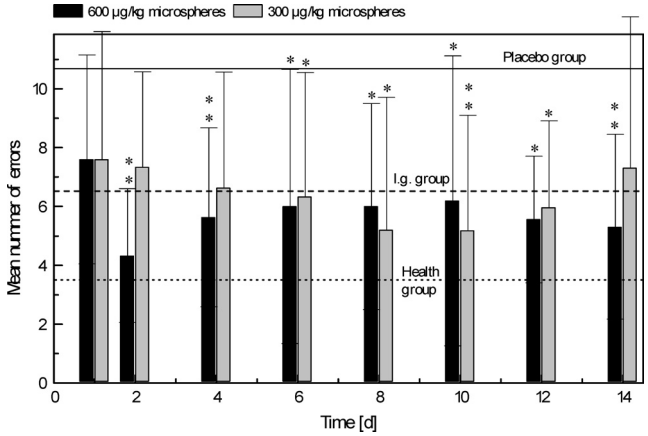

Fig. 5. Passive Avoidance Test of Huperzine A after Oral Administration of the Suspension and i.m. Administration of Microspheres Suspension

Mean number of errors in 5 min. Each point represents mean±S.D. ($n=12$). * and ** expressed $p<0.05$ and $p<0.01$ *vs.* placebo group.

tivity of learning and memory function by the step through avoidance response. In the case of the placebo group in which the mice were treated with scopolamine hydrobromide, the mean number of errors that the mice entered the dark chamber within 5 min was 10.98±5.87. It was significant higher than that of the health group (mean number of errors was 3.55±1.67). This indicated the deficit of the memory of the mice in the placebo group. Figure 5 showed that after i.g. administration of huperzine A suspension, the deficit in the memory of the mice was improved and the mean number of errors was reduced significantly to 6.49±3.69 from 10.98±5.87 ($p<0.05$), which demonstrated the efficiency of huperzine A in the improvement of the impaired memory.

Figure 5 showed that compared with that in the group of i.g. administration of huperzine A suspension, in the microspheres group, where the mice were injected i.m. with the microspheres (Table 1, batch 7) suspension at a dose of 600 μg/kg (50 μg/kg/d, the same dose as the i.g. group), the mean number of errors was reduced to 7.60±3.55 in the first day, which agreed with the low plasma level of huperzine A in the first day after i.m. administration of huperzine A loaded microspheres. But the mean number of errors was reduced to 4.33±2.27 in 2nd day, which was close to that of the health group (3.55±1.67), and then the mean number of errors was kept within 4 to 6 until 14th day. After a single i.m. administration of microspheres, the average mean number of errors within the 14 d study was 5.83±0.92, which was lower than that of the i.g. group with the mean number of errors of 6.49±3.69, This reduction of errors showed a significant improvement of the memorial deficit of the mice treated by scopolamine. After reducing the dose of huperzine A microspheres to half (300 μg/kg or 25 μg/kg/d), the result showed that, though most of the mean number of errors within 5 min was higher than that of the 600 μg/kg microspheres group and the average mean number of errors within the 14 d study was 6.44±0.95, it was comparable to that of the i.g. group. These results indicated that a parenteral sustained delivery formulation of huperzine A had an advantage in the improvement of the therapeutic efficiency over the oral administration. For huperzine A was rapidly absorbed and eliminated *in vivo* after the administration of the immediate formulation of huperzine A, the plasma concentration of huperzine A was fluctuant.[1)] The variation in the plasma concentration might lead to a reduction of the inhibition efficiency of acetylcholinesterase. After i.m. administration of huperzine A loaded microspheres, the continuous inhibition of acetylcholinesterase should maintain acetylcholine at a healthy and stable level far longer than that after administration of the conventional dosage and it will strengthen memory and improve the impaired memory of mice more efficiently than the pulsed inhibition of acetylcholinesterase.

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