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Preparation and *In Vivo* Evaluation of Huperzine A-Loaded PLGA Microspheres

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Huperzine A-loaded microspheres composed of poly(D,L-lactide-co-glycolide) were prepared by an O/W emulsion solvent evaporation method. The characterization of the microspheres such as drug loading, size, shape and release profile was described. The *in vitro* release in the initial 7 days was nearly linear with 10% released per day. Thereafter drug release rate became slow gradually and about 90% drug released at day 21. The *in vitro* release rate determined by dialysis bag method had a good correlation with the *in vivo* release rate. Huperzine A aqueous solution was intramuscularly injected (i.m.) at 0.4 mg/kg and microspheres were intramuscularly injected at 8.4 mg eq huperzine A/kg in rats. The maxium plasma concentration (C_{max}) after i.m. microspheres was only 32% of that after i.m. solution. Drug in plasma could be detectd until day 14 and about 5% of administered dose was residued at the injection site at day 14. The relative bioavailability of huperzine A microspheres over a period of 14 days was 94.7%. Inhibition of acyecholinesterase activity (AchE) in rat's cortex, hippocampus and striatum could sustain for about 14 days. In conclusion, huperzine A-loaded microspheres possessed a prolonged and complete drug release with significant inhibition of AchE for 2 weeks in rats.

Key words: Huperzine A, Poly(lactide-co-glycolide) microsphere, *In vitro* release, *In vivo* release, Acetylcholinesterase

INTRODUCTION

There has been much interest in using microspheres made from poly (D,L-lactic-co-glycolic acid) (PLGA) or poly(D,L-lactic) (PLA) for the sustained release of peptide and other active drugs. It can prolong the duration of drug effect significantly and improve the compliance of patients. There is also no need to be embedded by surgical operation and to be removed after the drug released completely. The total dose and some adverse reactions may be reduced because it can sustain the steady plasma concentration for a long time. When using the depot formulation, the effective dose of the leuprorelin was reduced to 1/4 and 1/8 of that needed for repeated parenteral administration of a plain solution due to the promotion of down-regulation by continuous hits on the receptors resulting from sustained peptide levels in the target organ (Okada, 1997).

Alzheimer's disease (AD) is a slowly progressive neuropsychiatric illness, principally characterized by short term memory deficits. The patients with AD suffered from marked reduction of cholinergic neuronal function resulting in a deficiency in acetylcholine (Ach) concentration in the brain and these reduction are associated with impairments in memory (Becker and Giacobini., 1988). Thus cholinergic enhancement strategies have been at the forefront of efforts to pharmacologically palliate the cognitive symptoms. Huperzine A, an alkaloid extracted from Chinese herb Huperzia serrata (Thunb) Trev, is a potent and selective acetylcholinesterase inhibitor with a rapid absorption and penetration into the brains (Tang,1996). It is mainly used for the treatment of Alzheimer's disease patients. At present huperzine A is administered 2~3 times a day by oral. In order to improve the compliance of patients and relieve its side effects, huperzine A biodegradable PLGA microsphere was developed (Fu *et al.*, 2005). The main objective of the present article was to study pharmacokinetics and pharmacodynamics of huperzine A-loaded

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microspheres using rats model. The *in vitro / in vivo* drug release rate was determined and its relationship was investigated in this paper. The pharmacological response was assessed by detecting acetylcholinesterase (AChE) in rat's cortex, hippocampus and striatum.

MATERIALS AND METHODS

Materials

Poly(D,L-lactide–co-glycolide) (PLGA) (W_η 15000, lactide /glycolide ratio, 75/25) was purchased from Chengdu institute of organic chemistry, Chinese Academy of Sciences. Huperzine A was obtained from Beijing institute of pharmacology and toxicology. Polyvinyl alcohol (PVA-124), dichloromethane (DCM), ethanol was obtained from Beijing chemical reagents company. All other materials or solvent were of reagent or analytical grade.

Microspheres preparation

O/W emulsion solvent evaporation method was applied to fabricate huperzine A-PLGA microspheres. About 400 mg PLGA and 20mg huperzine A were added to 1 mL of a mixture of DCM:ethanol (4:1). After completely dissolved, it was poured into 3.5% PVA-124 aqueous solution and then the mixture was emulsified by using a propeller stirrer (SXJQ-1, Zhengzhou, China) at 600 rpm for 10 min at 25°C. Then stirring at 300 rpm was continued for 4 h to evaporate the organic solvent. The harden microspheres were filtered, rinsed with distilled water and dried under vacuum.

Determination of huperzine A content in the microspheres

Huperzine A was extracted with 25 mL of a mixture of DCM and ethanol (1:1) from the microspheres. The solution was directly measured at 313nm by using a UV/VIS spectrophotometer (UV-160, SHIMADAZU, Japan). The polymers did not interfere with absorbance of the drug at the specified wavelength. The encapsulation efficiency is expressed as the ratio of detected and added drug amount. Each measurement was performed triplicate.

Particle size and span determination

Microspheres were mounted on a slide glass and inspected under an optical microscope (IMT, Olympus, Tokyo, Japan) connected to a video camera (ICD-740, Olympus). The video signals were displayed on a computer and 500 particles were measured for each batch of the microspheres. D_{10}, D_{50} and D_{90} indicated respectively the value that 10%, 50% and 90% of the detected particle's diameter was smaller than.

$$\text{Span} = \{D_{90} - /D_{10}\} / D_{50}$$

Microscopic observations

The microspheres were mounted onto metal stubs using a double-sided adhesive tape. After vacuum-coated with a layer of gold, the surface of huperzine A microsphere was observed by scanning electron microscopy (Hitachi S-450, Japan).

In vitro release assays

About 25 mg of microspheres were weighted and added to dialysis bag with cut off molecular weight 1 kDa, 1 mL phosphate buffered saline (PBS, 0.01M, pH 7.4) was then added. The dialysis bag containing microsphere suspension was kept in a beaker flasks filling 50 mL phosphate buffered saline (PBS, 0.01M, pH 7.4) containing 0.02% sodium azide. Incubation was conducted at 37°C by shaken at a rate of 72 rpm. At predetermined intervals, 5 mL of medium was drawn out and the same volume of fresh medium was replenished. Huperzine A concentration was determined in triplicate at 307 nm by spectrophotometer. Each measurement was performed triplicate.

Animal experiment

The experiments were carried out in Sprague-Dawley rats (225~275 g), the rats were purchased from Beijing institute of Pharmacology and Toxicology. The animals had free food and water before and during the experimental time. Huperzine A aqueous solution was administered intramuscularly into the hind leg of rats at a dose of 0.4 mg/kg. Blood samples were withdrawn from the eye socket at 3, 6, 10, 15, 30, 60, 120, 180, 240 and 360 min after i.m. (n=4).

Microspheres eq 8.4 mg huperzine A/kg were administered intramuscularly into the hind leg of rats after dispersed in 1 mL of saline containing 0.5% carboxymethyl cellulose and 0.1% Tween 80 (six rats per group, per time periods). Rats were decapitated at 1, 2, 3, 5, 7, 10, 14 and 16d after injection, the blood was collected and the brains were dissected on ice into frontal cortex, hippocampus and striatum. The microspheres were collected carefully from the injection site.

In vivo release assays

Microspheres collected from the injection site were homogenized in 10 mL pH 7.4 phosphate buffered saline (PBS, 0.01M, pH 7.4). 1 mL of homogenates was mixed with 50 μL internal standard (40.4 μg/mL *N*-methyl huperine A solution) and 100 μL of pH 10.83 Na_2CO_3-$NaHCO_3$ buffer solution. The resulting mixture was extracted with 4 mL of ethyl acetate on a vortex for 2 min and centrifugated at 3000 rpm for 10 min. The organic layer was taken and dried under nitrogen. The residue were diluted with HPLC mobile phase and determined by HPLC method according to the method described in our previous report (Fu *et*

al., 2004). The average relative recovery of huperzine A was about 103.64%. The intra and inter-assay coefficients of variation were 6.24 and 2.56%, respectively.

in vivo release percentage = (the weight of drug in microspheres at different sample points)/(the weight of drug in mircrospheres injected)×100%

Determination of drug plasma concentration

Blood samples were centrifuged at 3500 rpm for 15 min, 0.5 or 1 mL plasma were mixed with 50 μL internal standard (0.4 μg/mL *N*-methylhuperine A solution) and 100 μL of pH 10.83 Na_2CO_3-$NaHCO_3$ buffer solution. The resulting mixture was extracted with 5 mL of chloroform on a vortex for 2 min and centrifugated at 3000 rpm for 10 min. The organic layer was taken and dried under nitrogen. The residue were diluted with HPLC mobile phase and determined by HPLC (Fu *et al.*, 2004). The low detection limit was 2.5 ng/mL. The relative recovery of huperzine A was 95.54%, the intra and inter-assay coefficients of variation were 10.91 and 11.88% ,respectively.

AChE assay

Frontal cortex, hippocampus and striatum were weighted and were homogenized in 10 mM pH 7.2 phosphate buffered saline (PBS, pH 7.2) according to the ratio of 1:8,1:8 and 1:19 (w/v) respectively. 10 μL, 10 μL and 8 μL homogenates were applied to determine the AchE activity respectively according to the microcolorimetric method (Li *et al.*, 1986). AchE inhibition percentage was expressed as following: AchE inhibition percentage = (AchE of saline control group – AchE of microspheres group)/AchE of saline control group × 100%

DATA analysis

3P97 pharmacokinetic program was used to analyse plasma concentration-time data. The maximum plasma concentration (C_{max}) and the time of its occurrence (T_{max}) were actual detected value. The area under the plasma concentration-time curve (AUC) and mean residence time (MRT) was caculated by statistical moment theory. The area under the average plasma concentration-time curve from 0 to last sampling time (AUC_t) was calculated using the linear trapezoidal rule and was extrapolated to infinity (AUC_{inf}).The elimination rate constant (Kel) was estimated from the slope of the terminal phase of the drug plasma concentration. The relative bioavailability of i.m. huperzine A microspheres (F) was caculated by the following equation:

$$F = \frac{AUC(microsphere)/Dose(microsphere)}{AUC(solution)/Dose(solution)} \times 100\% .$$

Significant differences between the observed data were assessed by Student's t-test.

RESULTS AND DISCUSSION

Morphology, size, drug loading and *in vitro* release of huperzine A-loaded microspheres

As shown in Fig. 1, the microspheres were spherical in shape with a smooth surface. The mean diameter of five batches microspheres was 63.08 ± 3.29 μm, span was 0.73 ± 0.08 (*n*=5). The encapsulation efficiency and drug loading was 78.08 ± 3.43 and 3.80 ± 0.17% (n=5), respectively.

Fig. 2 showed the in vitro release profiles of five batches huperzine A-loaded microspheres. The *in vitro* release behavior had a good reproduction. In the initial 7 days release was nearly linear with 10% released per day. Thereafter a gradual slow release was observed and about 90% drug released at day 21.

In vivo release rate

In vivo release profiles of huperzine A microspheres could be described as biphase. Drug released from

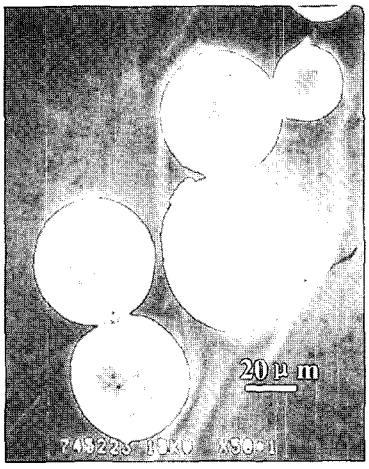

Fig. 1. Scanning electron microscope picture of Huperzine A microspheres

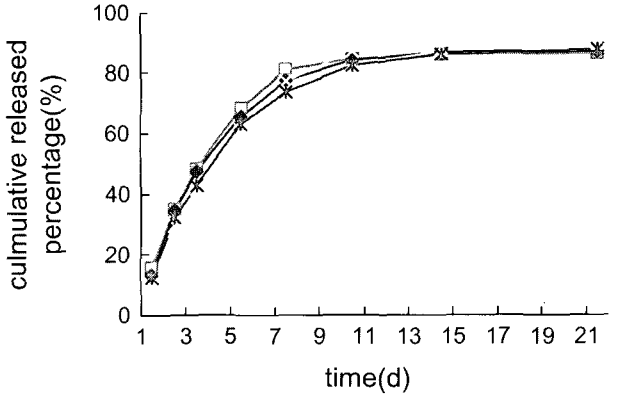

Fig. 2. *In vitro* release profiles of huperzineA from 5 batches of PLGA microspheres

microspheres at a rate of 17% per day in the initial 3 days, followed by a nearly linear release with 5% released per day from day 3 to day 14 (Fig. 3). About 95.38% of dose released from microspheres during 14 days. The *in vitro* release rate determined by dialysis bag method had a good correlation with the *in vivo* release rate (Fig. 4). Dialysis bags simulated better *in vivo* conditions and could avoid microspheres losing during sampling compared to immediate release method. It was also reported by others that a good *in vitro/in vivo* correlation could be obtained by this method (Diaz *et al.*, 1999; Woo *et al.*, 2001).

Pharmacokinetic studies of huperzine A-solution and microspheres

Plasma concentration–time profiles of huperzine A after i.m. administration of huperzine A solution and huperzine A microspheres was shown in Fig. 5 and Fig. 6. The pharmacokinetics parameters was shown in Table I. As shown in Fig. 6, drug plasma concentration maintained at a higher level (more than 30 ng/mL) in the initial 3 days after i.m. injection huperzine A-loaded microspheres, C_{max} of 51.5 ± 4.7 ng/mL emerged at the second day. This was correspondent with drug initial burst release from microspheres. C_{max} per dose after i.m. microspheres was only 32% of that after i.m. solution although 30% drug had been released from microsphers at day 2. This might be owed to that drug released steadily and slowly from microspheres at injection site during this period. Drug concentration decreased markedly after 3 days and keeped at a lower level of 6.3 ng/mL~19.2 ng/mL during day 5 to day 14. Drug plasma was below to detect limitation at day 16. The relative bioavailability of huperzine A microspheres from 0 to 14d was 94.70%, suggesting that the encapsulated huperzine A had almost been absorbed in rats over the period of 14 days.

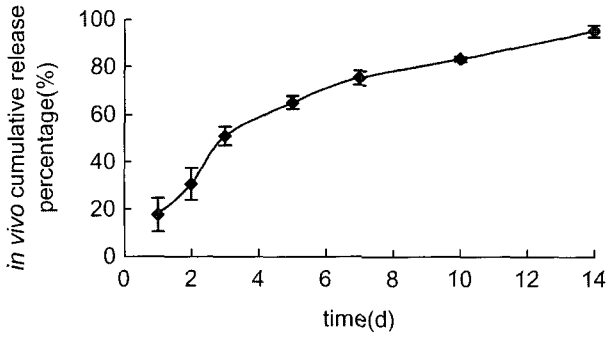

Fig. 3. *In vivo* cumulative release profiles of huperzine A-loaded PLGA microspheres at rat's injection site (n=6)

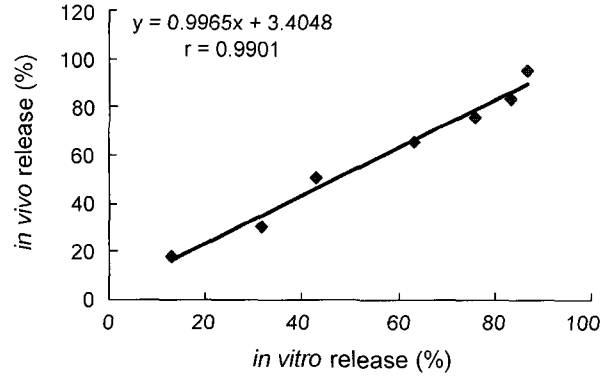

Fig. 4. *In vivo-in vitro* correlation of huperzine A-loaded PLGA microspheres

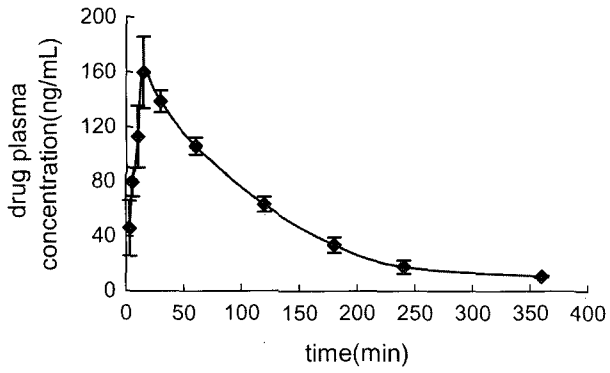

Fig. 5. Drug plasma concentration versus time profile after intramuscularly injection of huperzine A solution to rats (Mean ± S.D., n=4)

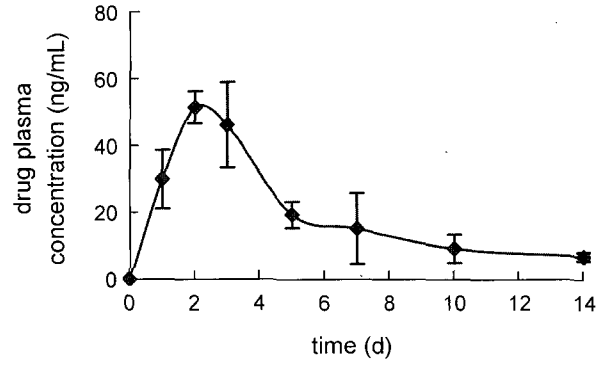

Fig. 6. Drug plasma concentration versus time profile after *intramuscularly injection of huperzine A microsphere to rats* (Mean±S.D., n=6)

Table I. Pharmacokinetic parameters following intramuscularly injection of Huperzine A solution solution and microspheres to rats

parameters	huperzine A solution (0.4 mg/kg)	huperzine A microspheres (8.4 mg eq huperzine A/kg)
T_{max}	11.51 ± 3.41[a]	48.0[b]
C_{max} (ng/mL)	159.66 ± 26.16[c]	51.47 ± 4.70[d]
AUC_t (ng·h/mL)	297.12 ± 56.43[e]	6548.81[f]
AUC_{inf} (ng·h/mL)	329.32 ± 66.29	7080.24
MRT	2.11 ± 0.57[a]	187.44[b]

[a]unit min, [b]unit h, [c]Mean ± S.D., n = 4, [d]Mean ± S.D., n = 6, [e]AUC_{6h}, [f]AUC_{336h}

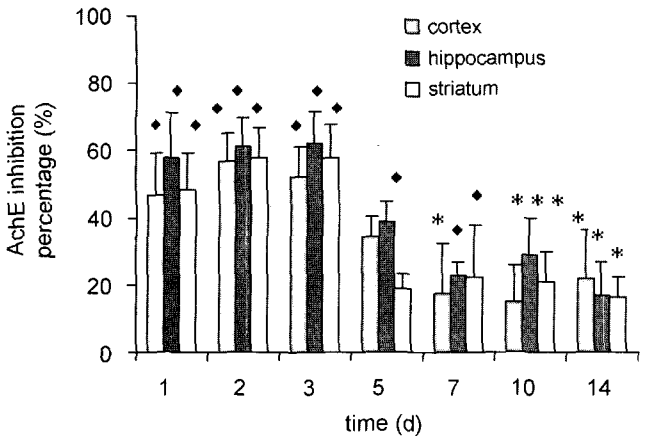

Fig. 7. Time course of AchE inhibition percentage after intramuscularly injection huperzineA microspheres (Mean±S.D., n=6) * and ◆ expressed $p<0.05$ and $p<0.01$ compared with saline control group

Pharmacodynamic studies

Huperzine A-loaded PLGA microspheres exerted significant AchE inhibition in hippocampus, cortex and striatum during 14 days (Fig. 7). Stronger AchE inhibition percentage in hippocampus, cortex and striatum was seen in the initial 3 days after i.m. injection microspheres and became weaker after day 5. It was consistent with drug plasma concentration-time curves and *in vivo* release profiles. It was reported that 10%~20% inhibition of AchE activity in the cortex could be maitained for about 6 hours after oral administration huperzine A solution 0.36 μg/kg (Wang *et al.*, 1998). Huperzine A microspheres significantly prolonged the duration of action and enhanced the effects. But it was not very satisfactory that inhibition of AChE activity could not keep at a comparative constant level although effect could maintain for 14 days. In order to avoid some possible side effects such as fasciculations or other cholinergic signs, it should be considered to chose more safer and effective administration dose.

CONCLUSIONS

O/W emulsion solvent evaporation method was applied successfully to fabricate huperzine A loaded PLGA microspheres. Huperzine A could be prolonged and almost completely released from PLGA microspheres *in vivo* with a significant inhition of AchE activity in rats brain region for 14 days. The present work demonstrated the feasibility of controlled delivery of huperzine A utilizing PLGA-based microspheres.

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