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Controlled release of huperzine A from biodegradable microspheres: *In vitro* and *in vivo* studies

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Abstract

The objective of the present work was to further study the *in vitro* characteristics, *in vivo* pharmacokinetics and pharmacodynamics of huperzine A (HupA) loaded biodegradable microspheres designed for sustained release of HupA over several weeks. A conventional o/w emulsion-solvent evaporation method was used to incorporate HupA, which is of interest in the palliative treatment of Alzheimer's disease (AD), into end-group uncapped poly(D,L-lactide-*co*-glycolide) (PLG-H). A prolonged *in vitro* drug release profile was observed, with a complete release of the incorporated drug within 5–6 weeks. The *in vivo* pharmacokinetics study of HupA loaded microspheres showed sustained plasma HupA concentration–time profile after subcutaneous injection into rats. The pharmacodynamics evaluated by determination of the activity of acetylcholinesterase in the rat cortex also showed a prolonged pharmacological response. Both the *in vitro* release and *in vivo* pharmacological responses correlated well with the *in vivo* pharmacokinetics profile. The results suggest the potential use of HupA-loaded biodegradable microspheres for treatment of AD over long periods.
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Keywords: Biodegradable microspheres; Huperzine A; Poly(D,L-lactide-*co*-glycolide); Controlled release

1. Introduction

Alzheimer's disease (AD) has become a major public health issue and a forth-leading cause of death among adults in developed nations as the proportion of elderly increases in the population. Studies focusing on the pathogenetic mechanism have revealed that cholinergic abnormalities are associated with the disturbance of cognitive function in patients with AD and inhibition of the brain acetylcholinesterase (AChE) to increase the synaptic concentration of acetylcholine (ACh) may improve the cognitive dysfunction (Cummings and Mendez, 1997; Parnetti et al., 1997). Huperzine A (HupA), an alkaloid isolated from the Chinese herb *Huperzia serrata* (*Thunb*) *Trev*, is a potent, highly specific and reversible cholinesterase inhibitor. Studies suggested that HupA is one of the most promising agents for palliative therapy of cognitive deficits in patients with AD. At present, HupA is available in the market as either tablet or capsule formulation, and daily oral administration is required. Though oral administration is convenient for most patients, it is extremely difficult for AD patients suffered from mental disorder to secure the therapy. A sustained release dosage form for long periods of time avoids daily administration, and is therefore the best way to improve patient compliance and to secure the therapeutic efficiency.

Biodegradable microspheres have been widely used for an injectable depot formulation of various small molecular weight drugs, peptides and proteins, and poly(D,L-lactide acid) (PLA) and poly(D,L-lactide-*co*-glycolide) (PLG) are the most widely used and well-characterized materials for the preparation of biodegradable microspheres (Chen et al., 1997; Park et al., 1998; Roullin et al., 2002; Liu et al., 2003). In this paper, the *in vitro* characteristics, *in vivo* pharmacokinetics and pharmacodynamics of the drug loaded microspheres were further evaluated and their correlations were discussed in detail.

2. Materials and methods

2.1. Microspheres

HupA loaded microspheres were fabricated and the drug content of the microspheres was measured as described previously

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(Gao et al., 2006). The surface morphology and internal structures of the microspheres were observed with an SHIMADZU scanning electron microscope (SHIMADZU SSX-550, Japan). The average particle size and size distribution of the microspheres were measured by laser diffractometry using a Beckman Coulter LS32 (Beckman Coulter, USA).

2.2. Methods

2.2.1. DSC analysis

To investigate the physical state of HupA and the polymer in the microspheres, thermal analysis was performed by differential scanning calorimetry with a Perkin-Elmer DSC-7 (Norwalk, USA). Under nitrogen flow, samples were first scanned from room temperature to 100 °C at a heating rate of 10 °C/min, and subsequent scan was performed from 0 to 300 °C at the same heating rate followed by cooling the sample to below zero.

2.2.2. Stability

The microspheres were stored in bottles at 4 °C, room temperature (20 ± 5 °C) and 37 °C for 6 months, respectively. The surface morphology, drug content and *in vitro* drug release behavior were studied.

2.2.3. In vitro release study

The *in vitro* drug release experiments were performed in phosphate buffer solution (PBS pH 7.4), using the dialysis method as described in previous report (Gao et al., 2006). To study the resistance of the dialysis membrane to drug permeation, HupA-PBS (pH 7.4) solutions of different concentration (5, 10 and 25 μg/ml) was, respectively, put into the dialysis tubes in stead of microspheres suspensions, and the drug release from the dialysis bag was studied. All experiments were carried out in triplicate.

2.2.4. Pharmacokinetics and pharmacodynamics study

2.2.4.1. Animals. Male Wistar rats (provided by Shenyang Pharmaceutical University Experimental Animal Center) weighing ∼300 g were used to evaluate *in vivo* performance of HupA loaded microspheres. All animal experiments complied with the requirements of the National Act on the use of experimental animals (People's Republic of China). The animals were maintained under constant environmental conditions (22 ± 1 °C, 50 ± 5% relative humidity). Food and water were available *ad libitum*.

2.2.4.2. Pharmacokinetics study. For the pharmacokinetics study, drug loaded microspheres were injected subcutaneously at the back of rats ($n = 6$) after reconstitution with a viscous aqueous vehicle (1% carboxymethylcellulose, w/v and 0.5% Tween-80, v/v). Blood samples were collected from the orbital vein at specific time points into heparinized tubes, and plasma was separated immediately by centrifugation and stored at −20 °C until assay.

Plasma drug levels were determined as previously described (Gao et al., 2006).

2.2.4.3. Pharmacodynamic study. For the pharmacodynamic study, blank microspheres or drug loaded microspheres were administered to rats ($n = 12$) as described in Section 2.2.4.2 at 1 h, 8 h, 1 day, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks and 6 weeks before they were sacrificed. Brains were extirpated rapidly and the cerebral cortexes were dissected out on ice and homogenized in 19 volumes of ice-cold sodium phosphate buffer (75 mM, pH 7.4). The homogenates were subjected to centrifugation (3500 rpm × 10 min) and supernatant were collected appending for assay. AChE activity in the homogenate supernatant was assayed according to the instructions of the AChE Assay Kit (Nanjing Jiancheng Bioengineering Institution, Nanjing, China). Protein concentration of the homogenate supernatant was measured by the Coomassie brilliant blue protein-binding method. AChE activity was expressed as the percentage of inhibition by comparison with mean values obtained from the blank microspheres treated rats.

3. Results and discussion

3.1. Microspheres characterization

End-group uncapped PLG was selected to encapsulate HupA due to its special encapsulation capacity to huperzine A as reported in a previous paper of our group (Gao et al., 2006). It was reported that the ionic interaction between basic amino residue of the drug and uncapped carboxylic acid end-group of the polymers played a key role in the drug encapsulation, which was in consistent with several other reports (Ogawa et al., 1988; Heya et al., 1991; Okada et al., 1994b; Okada, 1997). In the present work, PLG-H 75/25 (IV = 0.22) was selected to be the encapsulating matrix for its relatively higher encapsulation efficiency of HupA and faster degradation rate. Microspheres prepared by optimal experimental conditions were spherical in appearance and dispersed well. SEM observation of micro-

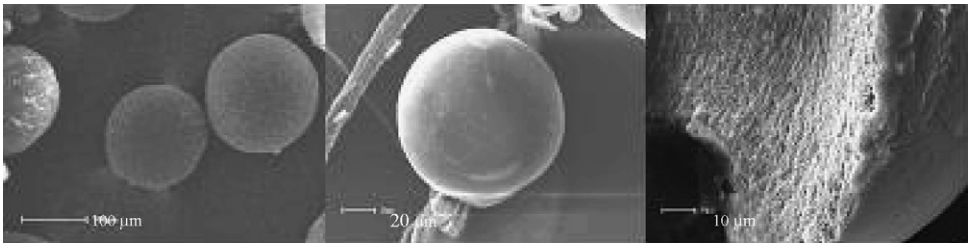

Fig. 1. Scanning electron micrographs of HupA loaded microspheres.

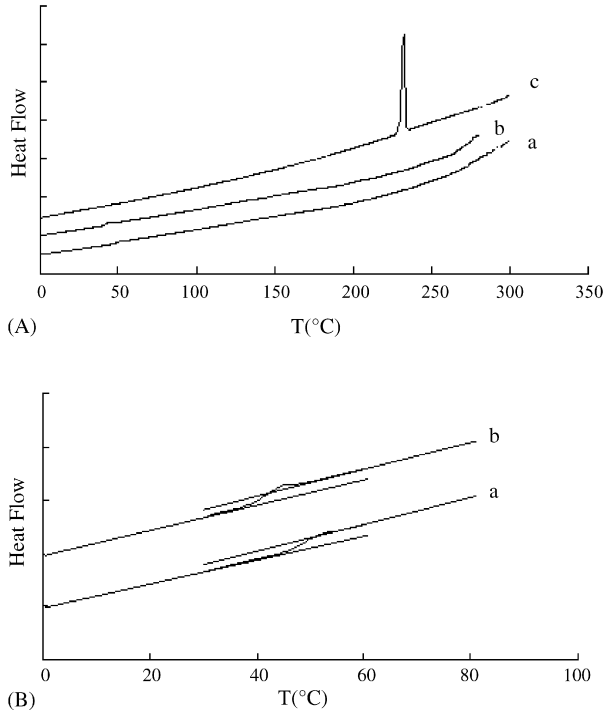

Fig. 2. (A and B) DSC thermograms of the drug loaded microspheres (a), blank polymer (b) and plain HupA crystals (c).

spheres revealed a smooth surface structure and porous internal structure, as shown in Fig. 1. The mean diameter of the microspheres was 142.8 ± 57.98 μm and the average drug loading and encapsulation efficiency were 5.36 ± 0.28 and 59.66 ± 3.14%, respectively.

3.2. DSC study

DSC thermograms of the drug loaded microspheres, blank polymer and plain HupA crystals are presented in Fig. 2(A). The absence of the endothermic peak of T_m of HupA at 232.2 °C in the DSC traces for the drug loaded microspheres suggested that HupA dispersed homogeneously in the microspheres and no crystallization of HupA occurred during the microspheres preparation process.

T_g of the drug loaded microspheres was elevated from 41.1 °C for the blank material to 48.3 °C, as shown in Fig. 2(B). Similar result was observed by other authors (Okada et al., 1994a,b). They hypothesized that the polymer molecules were arranged around the drug cores in a similar way to surfactant molecules in a micelle, due to ionic interaction between the basic amino acids of the drug and the terminal carboxylic anions of the polymers, formed a rigid structure, which was responsible for the elevated T_g. A barrier against diffusion of the drug was thus created by the hydrophobic long alkyl chains of the polymers which assure the prolonged drug release.

3.3. The stability of HupA microspheres

There were no notable changes in the surface morphology and drug content of the HupA loaded microspheres after 6-month storage at 4 °C or room temperature (Table 1). However, at 37 °C, aggregation was observed. The *in vitro* release profiles of microspheres before and after 6-month storage are shown in Fig. 3, no significant change has been observed. Since the polymer is degraded via hydrolysis, the microspheres are recommended to be stored under dry and cool conditions.

Table 1
Effects of storage temperature on the HupA loaded microspheres after 6 months (mean ± S.D., $n = 3$)

Storage conditions (°C)	Drug content (S.D.) (%)		Characteristics
	Before storage	After storage	
4	5.36 ± 0.28	5.31 ± 0.33	White powder
20 ± 5	5.36 ± 0.28	5.22 ± 0.29	White powder
37	5.36 ± 0.28	–	White, aggregated

3.4. In vitro drug release

The *in vitro* release profile and kinetic studies are important in order to establish a correlation with the *in vivo* pharmacokinetics performances. Dialysis method was used in the present studies. The resistance of the membrane to the diffusion of free drug was studied, and it was revealed that the drug was completely released within 10 h from the HupA-PBS pH 7.4 solutions in the dialysis tube (Fig. 4). The *in vitro* release of HupA from the microspheres depended greatly on the type of release medium; the effects of several factors, such as pH, buffer strength were studied in finding an appropriate *in vitro* release condition. Fig. 3 shows the *in vitro* drug release profile in PBS pH 7.4. There was only moderate burst release as only less than 10% percent of the total drug was released within the first 24 h. A sustained release profile of HupA was observed for the following 4 weeks. The release profiles of HupA from the microspheres in PBS pH 7.4 were fitted using the Higuchi, zero and first order kinetics models. The kinetic model equations and calculated coefficients are listed in Table 2. Higuchi's model was selected to describe the kinetics of the HupA dissolution profile due to the highest correlation coefficient.

3.5. Pharmacokinetics study

For pharmacokinetics study, drug loaded microspheres (containing about 3 mg of HupA) were injected subcutaneously to

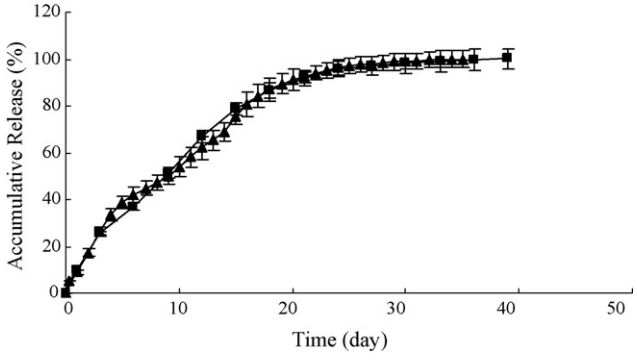

Fig. 3. *In vitro* release of HupA loaded microspheres in PBS pH 7.4 at 37 °C before (▲) and after (■) storage at 4 °C for 6 months (mean ± S.D., $n = 3$).

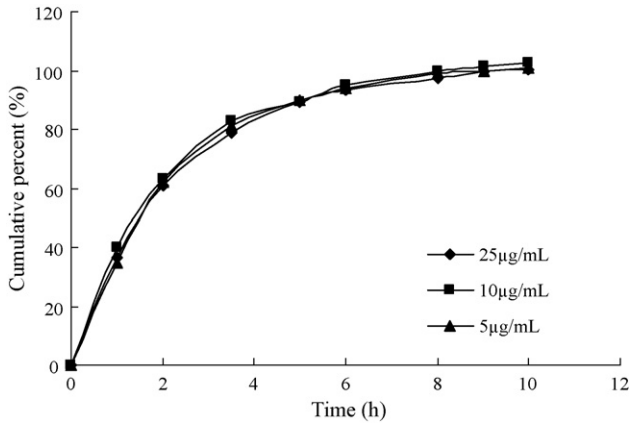

Fig. 4. Cumulative percent of HupA in the outside of the dialysis tube containing different concentration of HupA-PBS pH 7.4 solutions ($n = 3$).

Table 2
Coefficients of *in vitro* HupA release calculated from different mathematical models

Mathematical model		R^2
Zero order	$Q_t = k_{ro}t$	0.8646
First order	$\log(y_\infty - y) = -k_{r1}t/(2.303 + \log M)$	0.8944
Higuchi	$Q = k_H t^{1/2}$	0.9628

each rat ($n = 6$). HupA plasma level was monitored for more than 6 weeks. The HupA level reached a C_{max} within about 1 week (Fig. 5) and then gradually decreased for the followed 4 weeks, then it exhibited another increasing of drug level before it approached to zero after about 6 weeks. The later increasing of drug level was resulted from a second burst release, which might due to the collapse of microspheres and uptake of the degraded fragments by macrophage *in vivo* as was reported that the macrophage cell could take up particles smaller than 10 μm (Anderson and Shive, 1997; Tabata and Ikada, 1990), and there was no obvious residue of the injected microspheres at the end of the pharmacokinetic study.

Fig. 6 compares the *in vitro* and *in vivo* HupA release profiles, the later was plotted as cumulative area under plasma HupA curve normalized as percent of the total area between days 0 and 46 (total area under the curve was 168.62 ng d/mL). The overall *in vitro* release rate was faster than the estimated *in vivo* release. We attributed the faster *in vitro* release to the relatively high drug water solubility, small molecular weight of the drug, as

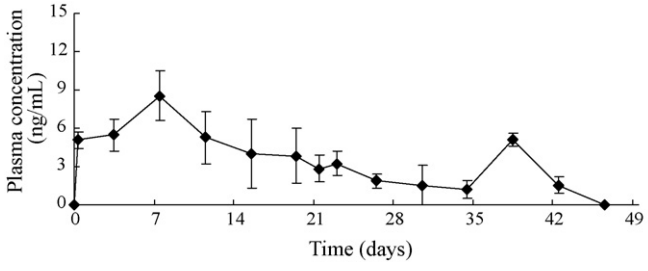

Fig. 5. Plasma levels of HupA in rats after subcutaneous injection of drug loaded microspheres (containing 3 mg of HupA) (mean ± S.D., $n = 6$).

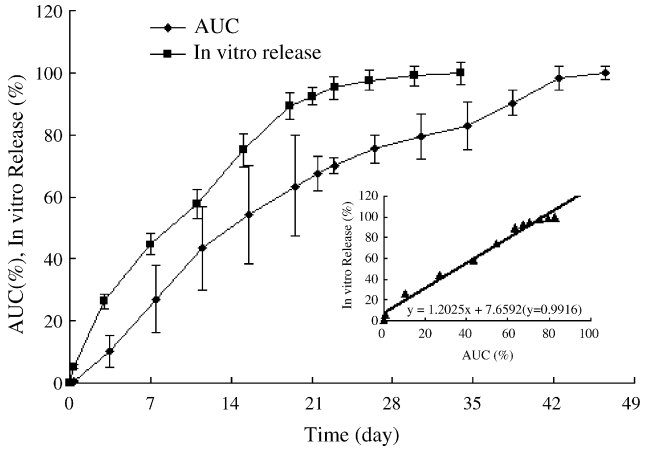

Fig. 6. *In vitro* release of HupA loaded microspheres in PBS pH 7.4 at 37 °C ($n = 3$) and comparison with *in vivo* release profile ($n = 6$) (the later was plotted as cumulative area under plasma HupA curve normalized as percent of the total area under the curve); *in vitro–in vivo* correlation plot (small panel).

well as the bulk aqueous solution surrounding the microspheres during *in vitro* release (Gao et al., 2006). However, the *in vitro* release rate of HupA correlated rather well with the estimated *in vivo* release ($\gamma = 0.991\,6$) ($n = 12$, $P < 0.001$), as shown in Fig. 6, small panel.

3.6. Pharmacodynamics study

The principle of indirect cholinomimetic therapy using ChEIs is to reduce ACh breakdown in the central nervous system by inhibiting AChE. The resulting increase in extracellular ACh concentration could reverse the central cholinergic hypofunction that is a major feature of AD (Giacobini and Becker, 1994; Liang and Tang, 2004). Liang and Tang (2004) reported that the doses caused similar increases of ACh levels also caused similar degrees of AChE inhibition. Thus, in the present study the pharmacodynamic responses of the drug loaded microspheres were evaluated by determination of the activity of AChE in the brain after a single subcutaneous injection of drug loaded microspheres into the back of the rats.

Injection of blank microspheres produced no significant change in the levels of AChE activity in cerebral cortex collected from any tested rat, but injection of HupA loaded microspheres invariably caused a significant inhibition on the AChE, as shown in Fig. 7. The results were similar to the pharmacokinetics profile, it reached a maximum pharmacodynamics response within 1 week, and produced continuous inhibition on the activity of AChE for the followed 4 weeks with an inhibition between 10 and 20% before it increased to another high level at the 6th week. This later increasing inhibition was corresponding to the second burst release observed in the pharmacokinetics study. The AChE inhibition percentage was plotted against the plasma HupA level obtained from pharmacokinetic studies. The slope and correlation coefficient of the linear regression was 0.2844 and 0.9564 ($n = 9$, $P < 0.001$), respectively, indicating positive correlation between the AChE inhibition percentage and HupA plasma concentrations (Fig. 8). The results suggest that the AChE inhibition may be used to explore the pharmacodynamic

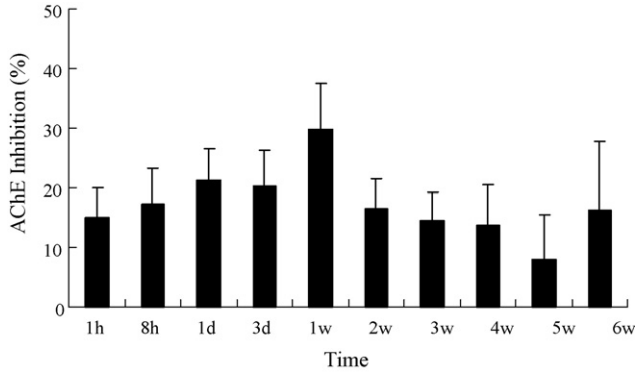

Fig. 7. The AChE inhibition percentage at different time points after subcutaneous injection of drug loaded microspheres (mean ± S.D., $n = 12$).

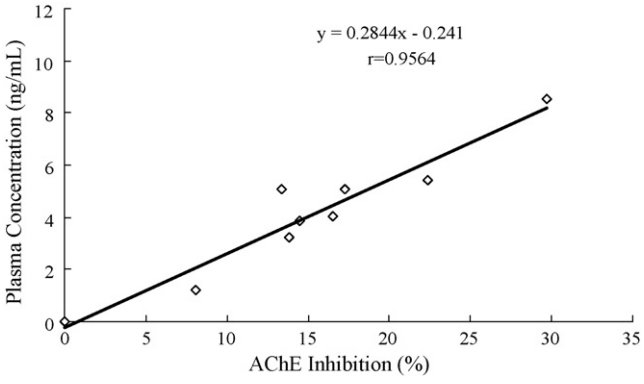

Fig. 8. The AChE inhibition percentage *vs.* HupA plasma concentration plot.

responses of various formulations of HupA. The established pharmacokinetics–pharmacodynamic correlation may also be utilized to predict the pharmacological response of other dosage forms of HupA once the plasma concentrations have been determined.

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