



Research Article

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Preparation and evaluation of potent cardiovascular drug-Loaded PLGA microspheres based on early-stage preparation discovery concept

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ABSTRACT

Ensuring sufficient drug solubility is a crucial problem in pharmaceutical-related research. For water-insoluble drugs, various formulation approaches are employed to enhance the solubility and bioavailability of lead compounds. The goal of this study was to enhance the dissolution and absorption of a new cardiovascular lead compound, 26PH-1. Early-stage preparation discovery concept was employed in this study. Based on the concept, 26PH-1-loaded poly lactic co glycolic acid (PLGA) microspheres hold great potential as a drug delivery system for improving drug solubility and bioavailability. In this study, we used Shirasu Porous Glass (SPG) premix membrane emulsification technique characterized with high trans-membrane flux and size controllability to prepare uniform-sized PLGA microspheres. By optimized trans-membrane pressure and PVA concentration in external aqueous phase, uniform-sized PLGA microspheres with small size (around 5.3 μ m) were successfully obtained. Our results showed that using ultrasonication to form primary emulsion, microspheres with high encapsulation efficiency and appropriate in vivo release were achieved, the particle size determination was employed to confirm the formation of the microspheres. Scanning electron microscopy demonstrated that 26PH-1 was converted into an amorphous form. Finally, LC-MS/MS method for determining the concentration of 26PH-1 in plasma demonstrated that the bio-stability in microspheres was preserved during the preparation process.

Keywords: bioavailability; dissolution; early-stage preparation discovery concept(EPDC); PLGA microspheres; LC-MS/MS; 26PH-1;

INTRODUCTION

During drug discovery and development work, the permeability and solubility of drugs can be the limiting factors in *vivo* absorption. Poor solubility can cause drugs to dissolve very slowly in the gastrointestinal tract, which leads to a low bioavailability. Since permeability is an intrinsic drug property, various strategies have been developed with the aim of improving the dissolution rate. In addition to chemical modification, various formulation approaches are important and effective methods of enhancing solubility[1,2]. In this study, we chose a potent cardiovascular compound, 26PH-1, to serve as a model of water-insoluble drug and investigate the effect of PLGA microsphere formulation.

Early-stage preparation discovery concept (EPDC) is a promising method for screening new drugs. It is a specific novel approach in pharmaceutical research for drug discovery. In order to improve the disadvantageous physicochemical properties of lead compounds, the methods based on EPDC could be effective and might rescue some potential drugs in an early stage of their development.

26PH-1 is a newly discovered anti-cerebrocardiac ischemia lead compound. It is composed of two molecules trimethylpyrazine (TMP) and vanillic acid (Fig.1.), both of which are extracted from Chinese herbal medicine, conjugated via ester bond and ether bond. 26PH-1 was reported to be a bioactive molecule. It can significantly promote the growth of primary neuronal cells and improve neurological damage after cerebral ischemia in rats[3,4]. However, as a potential cardiovascular drug, a major problem still remains to be resolved. 26PH-1 is a water-insoluble drug. The water solubility of it is less than 1.0 $\mu\text{g/ml}$, which leads to poor bioavailability. Therefore, it is important to establish effective methods of enhancing 26PH-1 dissolution.

Many pharmaceutical methods can be employed to improve drug dissolution, including microemulsion, solid dispersions, cyclodextrin inclusion complexes and microspheres. In this investigation, we prioritize microspheres because an ideal PLGA microspheres formulation have reasonably high drug encapsulation efficiency, high drug loading capacity and sustained release of the loaded drug[5,6,7]. In recent years, Shirasu Porous Glass (SPG) membrane emulsification technique has been developed to prepare uniform-sized PLGA microspheres[8,9]. This technique is divided into two types: conventional membrane emulsification and premix membrane emulsification[10,11,12]. The former is usually employed to prepare microspheres with large size (from a few to tens of microns). However, it is time-consuming in mass production-scale due to low flux. Therefore, premix membrane emulsification has been developed[13]. In this process, coarse double emulsions are firstly prepared under stirring. Then they are extruded and broken into smaller uniform droplets through SPG membrane due to shear stress induced by trans-membrane pressure (Fig.2.).

In this paper, to prepare microspheres, PLGA was selected as a polymeric carrier while 26PH-1 was selected as a model water-insoluble drug. SPG premix membrane emulsification technique combined with double emulsion ($W_1/O/W_2$)-solvent evaporation method was employed, in the same time characterized by scanning electron microscopy (SEM) and the particle size determination. In addition, in vivo bioavailability of the microspheres was investigated by LC-MS/MS.

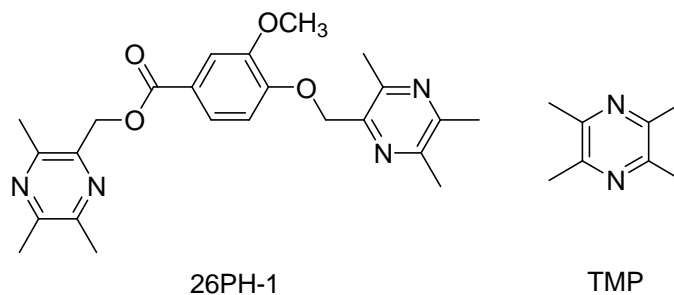


Fig.1. Structures of 26PH-1 and tetramethylpyrazine (TMP)

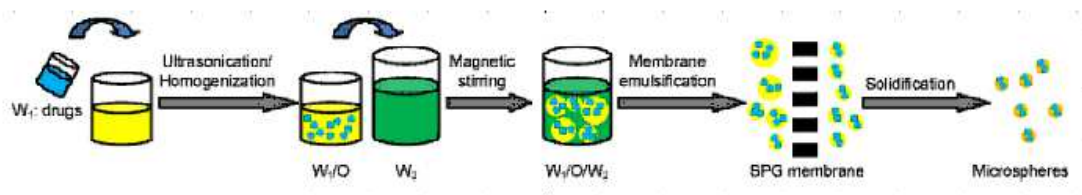


Fig.2. Preparation process of microspheres using SPG premix membrane emulsification technique

EXPERIMENTAL SECTION

2.1 Materials

26PH-1 powders (internal standard, 97%) were obtained from Beijing University of Chinese Medicine (Beijing, China). Its synthesis route has been reported previously[3]. PLGA with ratio of lactide-glycolide 75/25 (Mw 13kDa) was purchased from Lakeshore Biomaterials (Birmingham, USA). SPG membranes were purchased from SPG Technology Co., Ltd. (Japan). All other reagents and solvents, such as dichloromethane, methanol, were of analytical grade. SPF male Wister rats (250 \pm 10g) were provided by the Vital River Laboratory Animal Technology Co., Ltd. (Animal production license SCXK (Beijing) 2006-009).

2.2 Preparation of microspheres

The 26PH-1-loaded PLGA microspheres were prepared by SPG premix membrane emulsification technique combined with double emulsion ($W_1/O/W_2$)-solvent evaporation method (Fig.2.). First, 0.5g W_1 emulsified 30mL organic solvent (methylene dichloride: acetone=1:1,) containing PLGA (6.7%, w/v) by ultrasonication for 1min on

120W in ice bath to form W₁/O. Next, the W₁/O emulsion was poured into 120mL W₂ and NaCl (0.9%, w/w) to form coarse double emulsions (W₁/O/W₂). Then, the coarse emulsion droplets were achieved by extruding the coarse double emulsion through SPG member (5.3μm) with proper pressure. After that, the emulsion droplets were solidified for 2h at room temperature. At last, the microspheres were collected by centrifugation, washed with distilled water for 3times and obtained after freeze-drying.

2.3 Surface morphology observation and size distribution measurement

The shape and surface morphology of PLGA microspheres were observed by a Hitachi S-4800 field emission scanning electron microscope (Hitachi High-Technologies Corp; Tokyo, Japan). Particle size distribution was referred as Span value and calculated as follows:

$$\text{Span} = \frac{D_{v,90\%} - D_{v,10\%}}{D_{v,50\%}}$$

Where $D_{v,90\%}$, $D_{v,50\%}$ and $D_{v,10\%}$ are volume size diameters at 90%, 50% and 10% of the cumulative volume, respectively. The smaller Span value indicates the narrower size distribution.

2.4 In vivo bioavailability study

Eight Wister rats, weighing 250±10 g, were randomly divided into three groups, three rats, three rats and two rats, respectively. All the animals were fasted for 12h prior to initiation of the experiment. The drug was administrated by intragastric method and the tail vein injection method (250mg/kg body weight), while physiological saline was used as control. A total of 1.0 mL of blood was collected from the orbital venous plexus 2h, 4h and 6h after the drug administration. Blood samples were placed into heparinized tubes. After centrifugation, the obtained plasma was stored at -20°C until further study. Four hundreds microliters of plasma was used for quantification, and this step was followed by the addition of 600μL of acetonitrile. After being vortexed (VORTEX SHAKER, QL-861; Jiangsu, China) for 1min, the mixture was centrifuged at 1000rpm for 5min at 4°C. The supernatant was then withdrew and evaporated under ventilation with compressed air at room temperature. The residue was redissolved in 500μL of acetonitrile by vortex. The mixture was centrifuged at 1000rpm for 3min at 4°C, and 10μL of the supernatant was injected in the LC-MS/MS system for quantification.

LC-MS/MS analysis of plasma samples

The concentration of 26PH-1 in plasma was quantified by LC-MS/MS (Agilent LC-MSD-Trip-XCT_plus, Agilent Co., Ltd; USA;). The mobile phase used was acetonitrile and 1% acetic acid-water (10:90, v/v), and the signal was monitored at 254nm. The flow rate was maintained at 1.0mL/min, column temperature was kept at 25°C, and the sample volume was 10μL.

The acquisition parameter is as follows: ion polarity (positive), ion source type (ESI), dry temperature (350°C), nebulizer (35psi), dry gas (11.0L/min). All the animal experiments were performed according to the Guidelines for Animal Experimentation, University of TCM, Beijing.

RESULTS

3.1 Microsphere characterization

All the samples of 26PH-1 microspheres prepared using the optimized method and conventional W₁/O/W₂ method were characterized by analytical techniques. Scanning Electron Microscopy was performed to visualize the morphology of 26PH-1-loaded PLGA microspheres. However, 0.5g W₁ was emulsified 30mL different organic solvent. These results demonstrated that 26PH-1 was emulsified 30mL methylene dichloride: acetone (1:1, v/v) smooth surfaces and narrow size distributions (Fig.3. and Fig.4.). We can choose SPG membrane with proper pore size to prepare microspheres with intended size readily according to this relationship.

3.2 In vivo bioavailability

The chemical structure of 26PH-1 is shown in Fig.1. The full mass spectra of in MS infusion experiment showed the protonated molecular ions [M+H]⁺ at m/z 437.2, 284.9, 150.5 and 134.6, respectively (Fig.5.). 26PH-1 and its metabolites in rat plasma were formed via the following metabolic pathway (Fig.6.).

In our in vivo rat tests, we randomly divided into three groups, group 1 (ig), group 2 (tail vein injection) and blank group respectively. The full validated method was finally applied to a pilot animal study aimed at quantifying 26PH-1 in rat plasma by two groups. We found that the pharmacokinetic profile of 26PH-1 was highly improved by PLGA microspheres, as the C_{\max} and T_{\max} were all significantly enhanced or prolonged compared with pure 26PH-1 drug (no detected in plasma). Plasma samples were collected 2, 4 and 6h after the drug administration and the plasma

concentration-time profiles were shown (Fig.7.). 26PH-1 was always detectable in two groups, while plasma concentration of group1 reached peak levels between 4 and 6h post-administration times and slowly began to decline thereafter. On the contrary, plasma concentration of group 2 quickly reached peak levels in 2h and slowly decline thereafter.

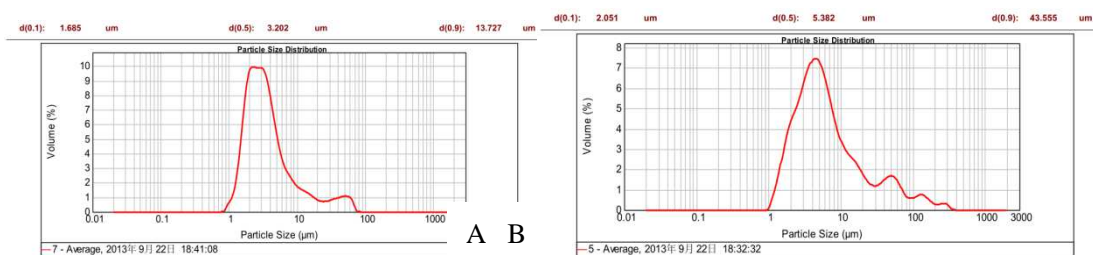


Fig.3. The particle size determination of 26PH-1-loaded PLGA microspheres (A,B used different organic solvent, dichloride: acetone and dichloride, respectively.)

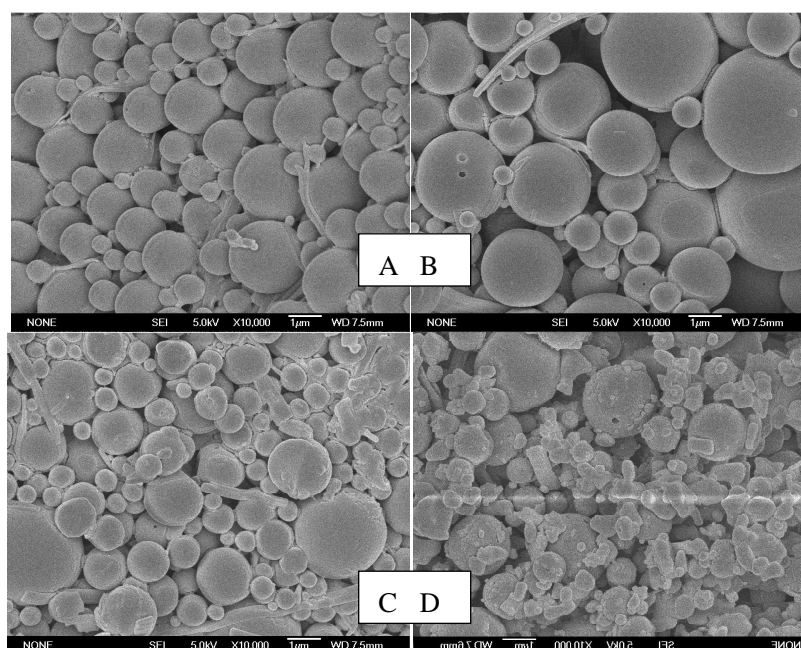


Fig.4. Scanning electron microscopic photography of the PLGA microspheres in different organic solvents. dichloride: acetone(A), dichloride (B), dichloride: diethyl ether(C) and ethyl ether(D)

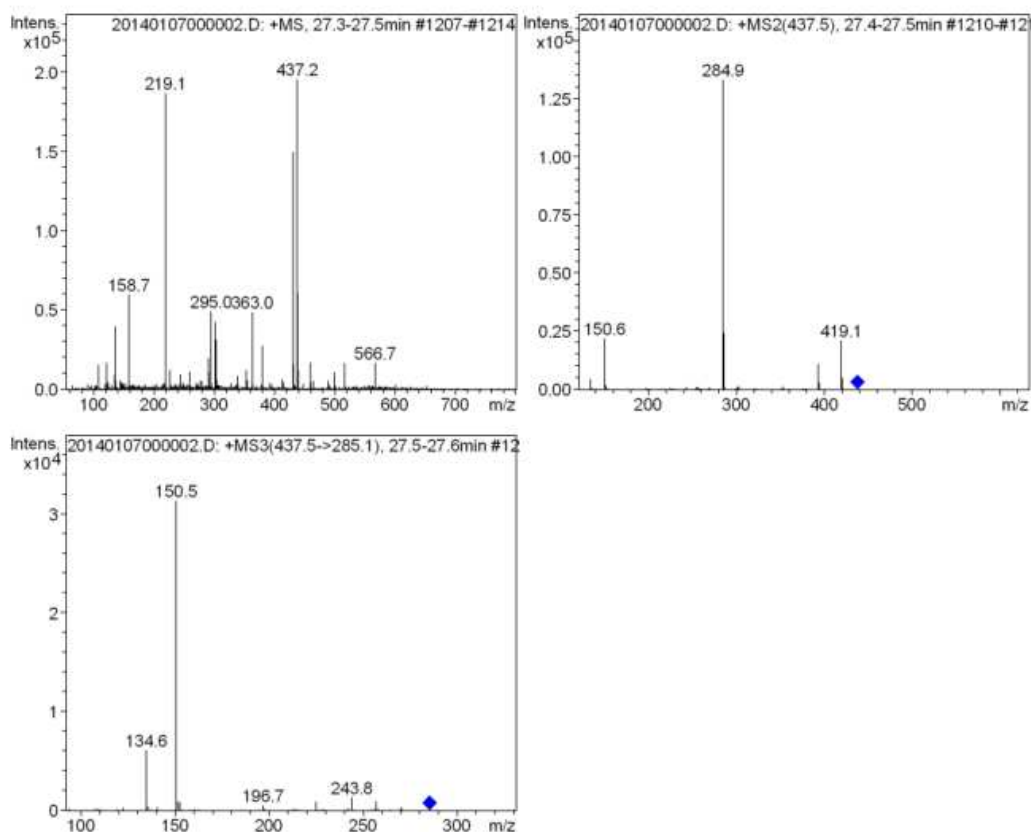


Fig.5. ESI-MS direct infusion analysis of 26PH-1 loaded-PLGA microspheres (positive ion-mode): full MS spectrum (m/z :50-800), full MS/MS spectrum of the parent ion at m/z 437.5 and full MS/MS/MS spectrum of the parent ion at m/z 284.9

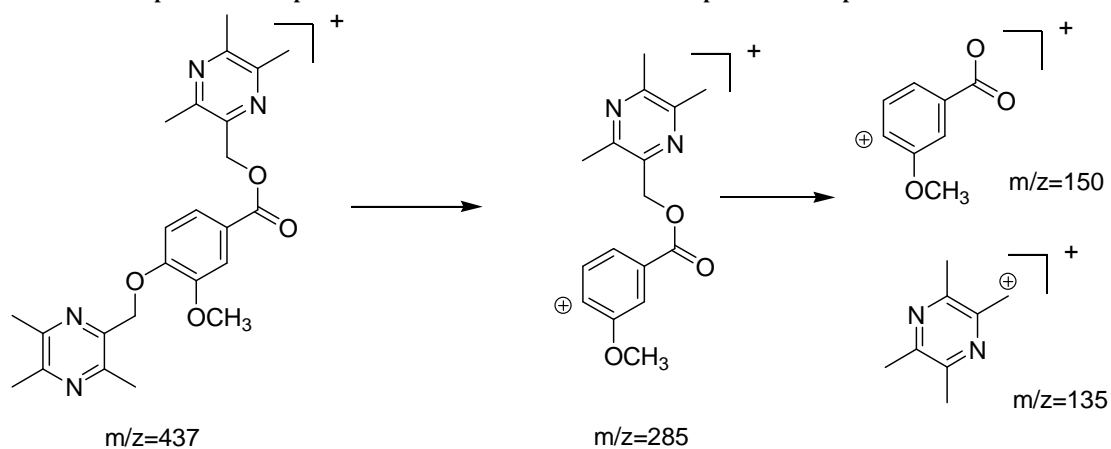


Fig.6. Proposed metabolic profile of 26PH-1 loaded PLGA microsphere in rat plasma

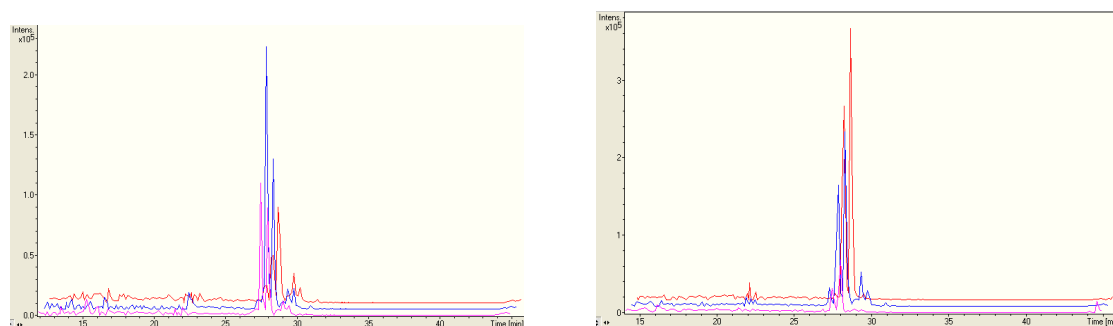


Fig.7. MRM chromatograms (positive ion-mode) of microsphere in rat plasma divided two groups
red line, blue line and pink line represent 2h, 4h and 6h, respectively

DISCUSSION AND CONCLUSION

During the drug development research, Early-stage preparation discovery concept (EPDC) is an effective method which is worth considering. This method refers to carrying out pharmaceutical work normally performed later at the candidate compound discovery stage, in order to improve the physicochemical properties of leading compounds and the improve efficiency of the drug discovery. In general drug development process, the pre-formulation and formulation stages are always performed following early-stage drug discovery, while we intend to introduce these steps into the discovery stage. Some leading compounds often have to be abandoned at an early research stage because of undesirable physicochemical properties. A formulation method based on EPDC can improve the unfavorable drug properties and influence drug bio-fate, such as distribution, metabolism, *in vivo* absorption and bioavailability, or by reducing drug toxicity and special accumulation. Our work aimed to improve the physicochemical properties and *in vivo* absorption of a lead compound, 26PH-1, through EPDC.

There are many ways being introduced into EPDC. In author's opinion, at the rapid screening stage, the early preparation technology could not only save research time but also be useful for the similar structure compounds. So it is very important for pharmaceutical scientists to design compound delivery formulation with the minimal number of trials and the least. The minimum types of experiments and the relatively unique process are needed. So PLGA microspheres technique was chosen by us firstly.

In this study, we have successfully prepared the uniform-sized microspheres with large size (about 20 μm) by SPG premix membrane emulsification technique. 26PH-1-loaded PLGA microspheres were obtained using ultrasonication to form W₁/O. By SEM analysis, 26PH-1 was observed to form long-term release microspheres, and then can persist at injection sites to realize to sustained efficacy without passing through most biological barriers in the body. The *in vivo* experiments confirmed the high dissolution rate of 26PH-1- loaded PLGA microsphere. Both group1 and group 2 experiments were all significantly improved by formulation of the drug as microspheres.

In herbal extracts and chemical modification studies, there are many compounds that have similar structures and physicochemical properties. PLGA microsphere systems may enhance the dissolution rate of novel compounds with similar structures, such as the $-\text{C}=\text{O}-\text{O}-$ link function group observed in 26PH-1. The employed of microsphere systems is a good direction in EPDC and may increase drug screening efficiency.

The microsphere based on PLGA technique was successfully employed for the improvement of the dissolution and absorption of a water-insoluble compound. 26PH-1-loaded PLGA microsphere was made, both the drug's solubility and bioavailability were highly improved. Research based on EPDC can increase the efficiency of discovery of potential new drugs. Additionally, we have proved that the whole preparation process had no influence on the bio-stability of 26PH-1 by LC-MS/MS analysis.

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