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RETRACTED ARTICLE: Preparation of novel pirfenidone microspheres for lung-targeted delivery: in vitro and in vivo study

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Abstract: The aim of this study was to develop and characterize pirfenidone (PF)-loaded chitosan microspheres for lung targeting. The microspheres were prepared using the emulsion-solvent evaporation method and characterized by assessing morphology, particle size, and zeta potential. The microspheres had a spherical nature with highly smooth and integrated surfaces. The particle size of microspheres was 4.6±0.[illegible] μm, and the zeta potential was 20.3±1.4 mV. The in vitro release results indicated that the obtained formulation of PF could reach the state of sustained release with a biphasic drug release pattern. It was observed that there was no significant difference in both the percentage of entrapment efficiency and that of drug release before and after the stability study. In vivo, the calculated relative bioavailability indicated greater pulmonary absorption of PF when it was encapsulated in microspheres. According to histopathological studies, no histological change occurred to the rat lung after the administration of PF-loaded chitosan microspheres.

Keywords: pirfenidone, chitosan, microspheres, in vitro release

Introduction

Pirfenidone (PF) is an orally effective, pyridine, synthetic compound that has been used for the treatment of idiopathic pulmonary fibrosis (IPF) in many countries around the world.[1] Although the mechanism of action of PF is not very clear, the existing in vitro data and animal models of pulmonary fibrosis show that PF has antifibrotic, anti-inflammatory, and antioxidant properties and it is classified as an immunosuppressant.[2–8] The antioxidant properties of PF may contribute to its anti-inflammatory effects, and these, in turn, may account for some of PF's antifibrotic effects.[9,10] To date, several PF clinical studies have been conducted in patients with IPF. Its evaluation in Phase II and Phase III clinical trials led to the approval of PF for the treatment of IPF in Japan in 2008 and in Europe in 2011,[11,12] and PF is now indicated for the treatment of patients with IPF (when forced vital capacity is ≥50% of predicted value, carbon monoxide diffusing capacity is ≥35% of predicted value and a 6-minute walk test distance is ≥150 m).[13]

In vivo, administration of PF in fed and fasted states reduces overall exposure, maximal plasma concentration (C_{max}) values (reduced by 50% when administered with food), and the rate of absorption.[14–16] In clinical studies, PF binds mainly to plasma albumin in the human body with a mean binding rate of 50%–58%, but it was not widely distributed in other tissues. The results of a population pharmacokinetic study showed that the apparent oral steady state volume of distribution of PF was about 70 L.[14–16]

Because of the above pharmacokinetic properties and its narrow therapeutic index, the use of oral PF is very difficult in a clinical management setting and requires

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frequent serum concentration monitoring. The developed PF intravenous dosage that comes from the laboratory may contain a solubilizing agent, which has been reported to be a potentially toxic agent, and so we limited the use of this formulation. To overcome these limitations of PF, local immunosuppression by the inhaled route of administration is a potential approach to directly target the smaller airways, with improved bioavailability for the lung tissue, minimal or even zero systemic side effects, and improved pulmonary function.[17]

Chitosan (CS) is a polysaccharide polymer, similar in structure to cellulose. CS has been proven to be a useful and effective carrier for microspheres drug delivery through the inhalation route due to its low toxicity, biocompatibility, biodegradability, and mucoadhesive properties as well as macromolecule permeation enhancement.[18,19] It has been confirmed that spray-dried CS microspheres have a low toxicity in cell lines of human origin from airway and alveolar regions of the pulmonary tract; this is an encouraging index for the safe use of the dry powder inhalation.[20]

The main objective of this study was to develop and characterize PF-loaded CS microspheres (PFCSMs) for lung targeting. The developed formulations were characterized by assessing morphology, particle size, and zeta potential.

Materials and methods

Materials

PF (≥95%) was purchased from BeierKa Biopharm Co., Ltd. (Wuhan, People's Republic of China). Low-molecular-weight CS of 75%–85% deacetylation was purchased from Hengshuo Pharma Co., Ltd. (Wuhan, People's Republic of China). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were obtained from Sigma (Sigma-Aldrich, Shanghai, People's Republic of China). All other reagents and solvents were at least of analytical grade. Distilled water was used in all the experiments.

Preparation of PF-loaded CS microspheres

An emulsion-solvent evaporation method described previously in the literature was used to prepare the PFCSMs.[21] Briefly, 100 mg of PF was dissolved in 5 mL of methylene chloride, and 300 mg CS was dissolved in a surfactant solution (10 mL of 2.5% w/v aqueous solution of polyvinyl alcohol). Afterward, the PF solution was added dropwise into a 10 mL CS solution in a 100 mL flask with stirring at 400 rpm. This was followed by homogenization for 1 minute (220 W, 2 s/cycle). First, oil in water crude emulsion was obtained using a probe sonicator. The sonication step was repeated twice until the desired size was obtained. After sonication, the produced PF-loaded CS emulsion was then stirred overnight in a fume hood in an uncovered beaker to allow for the evaporation of methylene chloride. The solid microspheres were collected after storage under vacuum for 48 hours at room temperature.

Morphology

The external morphology of microspheres was observed with field-emission scanning electron microscopy (SEM). Lyophilized microspheres were mounted onto stubs using double-sided adhesive tape with conductive effect and analyzed using SEM. In SEM study, electrons are transmitted from specimen surface. The picture was taken under inert condition with an electron microscope (magnification ×200).

Characterization

To determine the PF content, microspheres were dissolved in acetonitrile and PF extracted followed by HPLC analysis. Briefly, 20 mg of PFCSMs was dissolved in 10 mL acetonitrile. This suspension was vigorously mixed by vortexing to get a clear solution and then separated and filtered through a 0.45 mm filter to remove the polymeric debris. The clear solution was analyzed for PF content using the HPLC method. The percentages of drug loading (DL) and entrapment efficiency (EE) of the microspheres were calculated using the following formula:

$$\%\mathrm{DL} = \frac{\text{Weight of drug in microspheres}}{\text{Weight of microspheres}} \times 100$$

$$\%\mathrm{EE} = \frac{\%\mathrm{DL}}{\%\text{Theoretical loading}} \times 100$$

Particle size

A nanoparticle size analyzer (Mastersizer 3000; Malvern Instruments, Malvern, UK) was used to determine the particle size of microspheres. To calculate volume mean diameter, PFCSMs suspended in ethanol and sonicated for 2 minutes were used as the sample. No dissolution or agglomeration of the sample was obtained from the measurements, and each sample was determined in triplicate.

In vitro release

The release of PF was evaluated by incubating the PF suspensions or PFCSMs (20 mg) in 50 mL of phosphate buffer solution (pH 7.4 at 37°C±0.5°C) via the dialysis bag method.[21]

At time intervals of 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 hours, the collected supernatants (500 μL) were passed through a 0.22 μm filter membrane and analyzed for PF content using reverse-phase HPLC. The chromatographic conditions refer to the method reported previously by Meng and Xu.[22]

Stability studies

According to the International Council on Harmonization guidelines, an accelerated stability study has to be carried out on the pharmaceutical dosage form at 40°C±2°C/75%±5% relative humidity (RH). For the present study, developed formulations were subjected to accelerated stability study. The formulations were placed in a stability chamber at 40°C/75% RH for a period of 30 days and then removed. After that, the %EE was determined and in vitro release studies carried out. The %EE and dissolution profiles were compared with the %EE and drug release profile of the same formulation before the stability studies.

In vivo studies

All animal study protocols were approved by the Institutional Animal Care and Use Committee at the Linyi Tumor Hospital and adhered to the guidelines of the Institutional Animal Care and Use Committee. Twelve Sprague Dawley rats weighing 200–250 g (approximately 7 weeks old) were purchased from the animal center. Animals were housed in microisolator cages under positive-pressure ventilation maintained in closed-shelf and laminar-flow racks to avoid contact with pathogens, odors, or noises. The animals were housed under standard laboratory conditions. Sterilized food and water were available ad libitum. Before the experiment, the rats were kept in a state of fasting for 8 hours. Twelve Sprague Dawley rats were randomly selected and divided into two groups (six in each group). The rats were anesthetized using urethane, which was administered through an intraperitoneal injection with the rats lying in supine position. According to a previously published method, the trachea was exposed, and one section of PE-240 polyethylene tubing was inserted into the tracheal incision.[23] The sample was administered into the rat lung using a dry-powder inhaler. The powder administration was made by insufflation of 3 mL of air contained in a syringe. The insufflator was weighed before and after powder filling as well as after administration to determine the actual amount of sample released and aerosolized into the lung. The blood sample was collected from the PE-10 polyethylene tubing embedded into the caudal vein of the rats.

Group 1 rats were administered a single intratracheal dose of native PF (10 mg/kg); Group 2 rats were administered a single intratracheal dose of PFCSMs (10 mg/kg). Blood samples (0.5 mL) were collected into heparinized tubes from the caudal vein at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hours after administration. Plasma samples were immediately centrifuged at 4,000× *g* for 10 minutes and frozen at −70°C until analysis. At the end of pharmacokinetic studies, samples of lung, liver, and kidney were collected from the sacrificed animals of both groups. Tissue samples were washed in ice-cold saline, blotted with a paper towel to remove excess fluid, and stored at −20°C until analysis.

At the end of in vivo studies, samples (lung, liver, and kidney) for histological analysis were prepared by immersion in 4% phosphate-buffered paraformaldehyde before washing overnight in running water, dehydrating through alcohol and clearing in xylene, and then embedding in paraffin wax. The cut sections (5 μm in thickness) were stained with hematoxylin and eosin and observed using SEM (magnification ×500). Saline was used as the control.

Statistical analysis

The obtained data was analyzed using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA) and expressed as mean ± standard deviation. Comparisons between two sets of data were made using Student's *t*-test for unpaired data. When multiple comparisons against a single control were made, one-way analysis of variance was used, followed by the Tukey–Kramer multiple comparisons test. Differences were considered significant when $P<0.05$.

Results and discussion

Physicochemical characterization

The PFCSMs were prepared successfully. The SEM revealed that the microspheres were homogeneous in size and had spherical shape with smooth and integrated surfaces (Figure 1). In addition, the microspheres showed relatively porous surfaces, which was due to the rapid evaporation of residual solvents such as methylene chloride during the

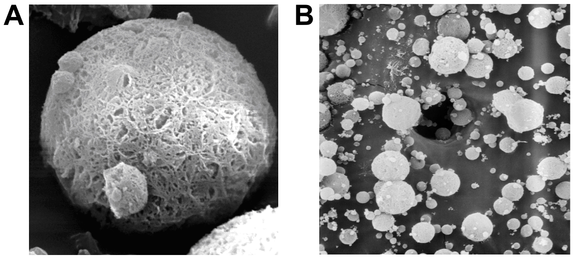

Figure 1 Scanning electron microscopy images of PF-loaded CS microspheres.
Notes: (**A**) Magnification ×5,000; (**B**) magnification ×500.
Abbreviations: PF, pirfenidone; CS, chitosan.

drying process. Currently, pulmonary delivery of PF is mainly via the dry-powder inhaler. The preparation of PF differs quite significantly in terms of the techniques and pharmacokinetic properties. The present study adopted the oil-in-water method to prepare the PF microspheres because the drug-saturated water solution used as the aqueous phase can effectively prevent the drug in the organic phase from diffusing out to the aqueous phase, thus greatly increasing the drug-loading capacity and encapsulation efficiency of the microspheres.

The volume mean diameter values of the prepared PFCSMs fall in the range of 4.31 to 4.78 µm. The mean particle size of microspheres was 4.6±0.3 µm, and the zeta potential was 20.3±1.4 mV, which indicated that there was a large number of positive charges on the surface of microspheres. Previous literature has reported that the higher the absolute value of the zeta potential (more than 15 mV) is, the stronger the electrostatic repulsion between particles will be, which makes the dispersal system more stable without aggregation and sedimentation.[24] The %EEs and %DL of PF in the prepared formulations were determined using HPLC. The data reveal that the %EEs values of the loaded PF fall in the range of 78.4% to 86.2% and the %DL, 5.4% to 6.7%.

In vitro release

The release profiles of PF from suspensions and PFCSMs are illustrated in Figure 2. As can be seen from the figure, the microspheres showed a fast initial release of PF (about 25%) within the first 2 hours and reached a plateau within 4 hours, which was followed by a relatively slow release. At the end of the experiment, over 61% of PF was completely released. At the early stage, a burst release phenomenon was observed because the drug molecules were encapsulated on the surface of microspheres, which may easily diffuse out initially. The in vitro release profile of microspheres was best explained using the Korsmeyer–Peppas model with highest r^2=0.9853. The values for zero-order, first-order, Higuchi, and Weibull model were found to be 0.8214, 0.8419, 0.8928, and 0.9128, respectively.

Stability studies

According to the International Council on Harmonization guidelines, an accelerated stability study was conducted. After being stored at 40°C and 75% RH, developed formulations appeared to be stable as the dried [illegible] showed no collapse or contraction. It was observed that there was no significant difference in both the %EE and that of drug release before and after the stability study. In addition, particle size and zeta potential measurements showed no change in stability during the storage procedure. Hence, both formulations were found to be stable.

In vivo studies

PFCSMs were intratracheally administered in rats to evaluate their absorption. The plasma concentration–time profiles of PF after intratracheal administration of the PFCSMs and native PF in rats are shown in Figure 3. Pharmacokinetic parameters (Table 1), such as C_{max}, the time of maximum concentration (T_{max}), and the area under the curve (AUC_{0-t}), were used to assess potential differences in the pulmonary absorption. After administration, the plasma concentration of

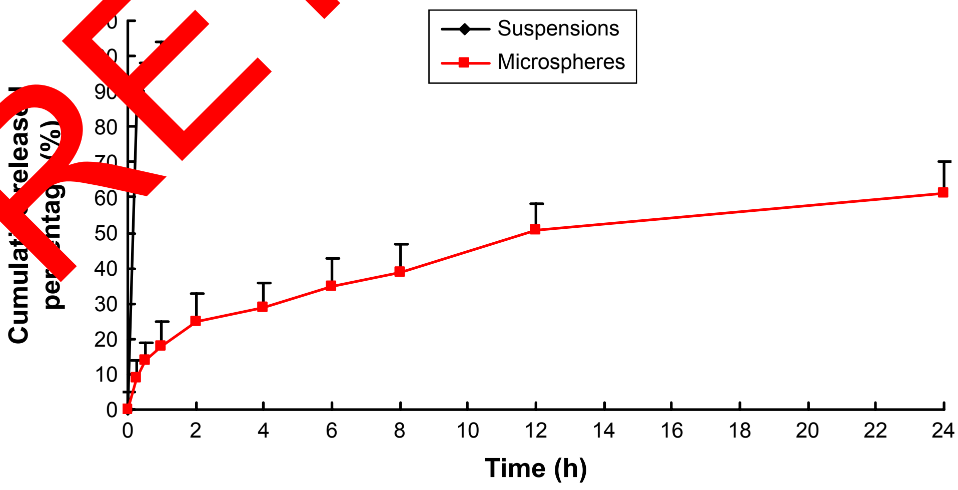

Figure 2 In vitro drug release profiles of PF-loaded CS microspheres and free PF (n=6).
Abbreviations: PF, pirfenidone; CS, chitosan.

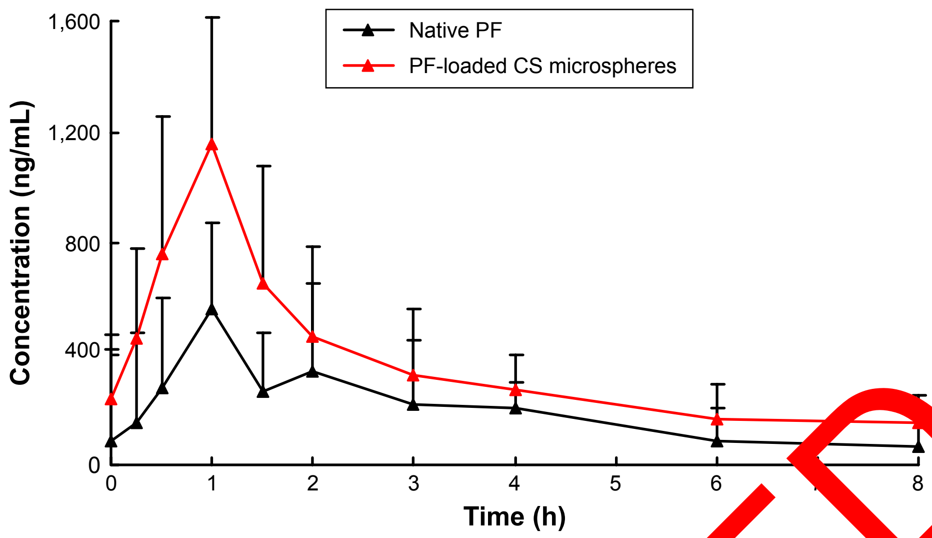

Figure 3 Plasma concentration–time profiles of PF after a single intratracheal administration of native drug and microspheres to rats (n=6).
Abbreviations: PF, pirfenidone; CS, chitosan.

native PF increased rapidly and reached a C_{max} of 562 ng/mL in 60 minutes (T_{max}). PFCSMs spray-dried powders gave a C_{max} of 1,154 ng/mL in 60 minutes, which was significantly different from that of the native PF ($P<0.05$). For PFCSMs, the AUC_{0-t} was 2,899.8±259.6 ng·h/mL, which was higher than that of native PF. The major obstacle against the development of sustained pulmonary drug delivery formulations is that the most appropriate aerodynamic size for particles to be respirable (0.5–5 μm) is also the optimum size for the rapid uptake by the macrophages in the alveolar region. Therefore, in this study, the particle size of PFCSMs was suitable for pulmonary drug delivery and could significantly improve the AUC related to native PF. As we all know, CS has been reported to be able to improve the absorption of macromolecules by opening the tight junctions of epithelial cells. In addition, CS nanoparticles have been used to increase the uptake of active molecules across mucosal surfaces.[25,26]

In some targeted formulations such as microspheres, most of the drugs and excipients would be accumulated in specific tissues (lung), and therefore, it was necessary to verify the biocompatibility and safety of these tissues and the microspheres formulation. Compared to the placebo group, the microsphere formulations did not show any degenerative changes in the cytoarchitecture of the tissue (lung), as shown in Figure 4. Throughout the experiment, the rats that were given PFCSMs did not develop any histological changes in the tissue of lung.

Table 1 Pharmacokinetic parameters of PF after a single intratracheal administration of native drug and microspheres to rats (n=6)

Parameters	Intratracheal	
	Native drug	Microspheres
T_{max} (h)	1.3±0.4	2.1±1.1
$t_{1/2}$ (h)	2.1±0.8	7.8±1.4*
C_{max} (ng/mL)	956.5±125.7	1,568.7±172.7*
AUC_{0-t} (ng·h/mL)	1,581.4±165.3	2,899.8±259.6*
$AUC_{0-\infty}$ (ng·h/mL)	1,985.1±171.4	3,362.5±315.5*
MRT (h)	5.3±1.7	9.5±1.4
CL (L/h)	11.2±2.4	2.9±0.6*

Notes: Native drug and microspheres; $*P<0.05$. Data presented as mean ± SD.
Abbreviations: PF, pirfenidone; $t_{1/2}$, half-life time; C_{max}, maximal plasma concentration; AUC, area under the curve; MRT, mean residence time; CL, clearance; T_{max}, the time of maximum concentration.

Conclusion

In this study, PFCSMs were prepared using the emulsion-solvent evaporation method and characterized by assessing morphology, particle size, and zeta potential. The microspheres had a spherical nature with highly smooth and integrated surfaces. The particle size of microspheres was 4.6±1.7 μm, and the zeta potential was 20.3±1.4 mV. The in vitro release results indicated that the obtained formulation of PF could reach a state of sustained release with a biphasic drug release pattern. It was observed that there was no significant difference in both the %EE and that of drug release before and after the stability study. In vivo, the calculated relative bioavailability indicated greater pulmonary absorption of PF when it was encapsulated in microspheres. According

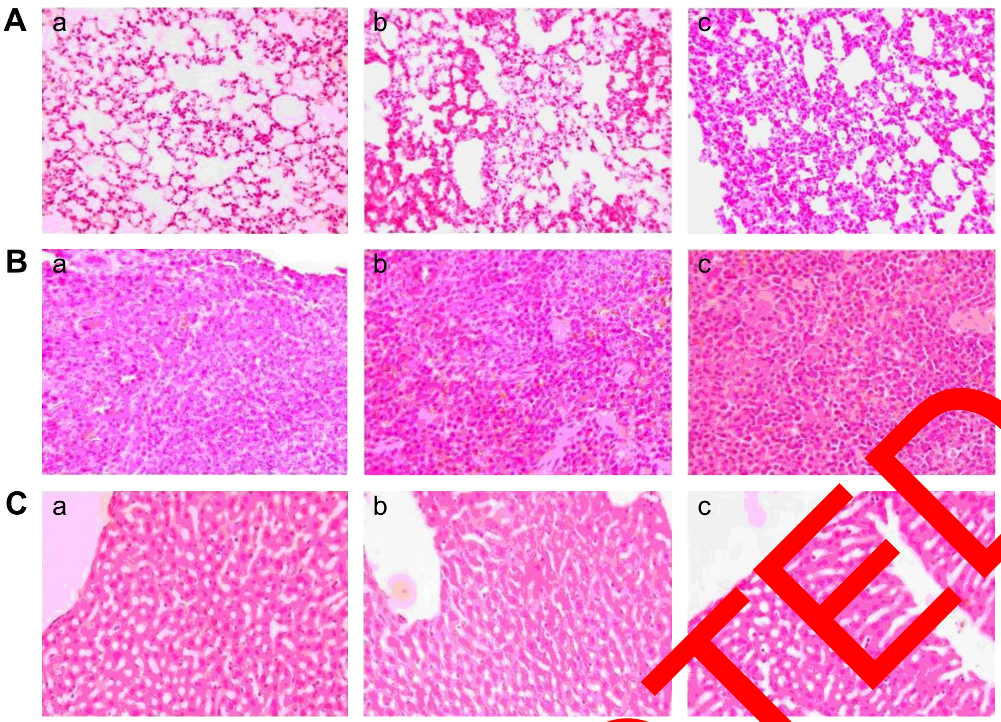

Figure 4 Histopathological studies of lung (**A**), liver (**B**), and kidney (**C**) in (a) control group; (b) native PF; (c) PF-loaded CS microspheres. Magnification ×500.
Abbreviations: PF, pirfenidone; CS, chitosan.

to histopathological studies, no histological change occurred to the rat lung after the administration of PFCSMs.

Disclosure

The authors report no conflicts of interest in this work.

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