Preparation of Ginkgolide B Polybutylcyanoacrylate Nanoparticles Suspension Powder-Injection

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Ginkgolide B polybutylcyanoacrylate nanoparticles suspension were prepared using polybutylcyanoacrylate as drug carrier by interface polycondensation method and the trehalose was selected as cryoprotectant in forming the powder injection. The result showed that the encapsulation efficiency and the drug loading of the drug nanoparticles suspension were $96.74 \pm 1.25\%$ and $5.74 \pm 0.46\%$ respectively and the powder injection of the ginkgolide B polybutylcyanoacrylate nanoparticles suspension exhibited good appearance, re-dispersion and high stability at the room temperature. The high loading of ginkgolide B polybutylcyanoacrylate in nanoparticles suspension might successfully solve the insolubility of ginkgolide B in aqua solution and be possible used as a brain targeted candidate drug form.

Key Words: Ginkgolide B, Nanoparticles, Polybutylcyanoacrylate.

INTRODUCTION

Ginkgolides A, B, C, J, K, L and M and bilobalide are rare terpene trilactones that have been isolated from leaves and root bark of the Chinese tree *Ginkgo biloba*¹⁻⁴. They were found to be specific and selective antagonists of platelet activating factor, of which the ginkgolide B (GB) is the most potent one⁵⁻⁸. A reduction of postischemic neurological disturbances and brain edema in the gerbil was achieved with ginkgolide B and the compound reduced the accumulation of free fatty acids in the postischemic gerbil brain^{9,10}. Studies showed that ginkgolide B possesses many beneficial effects such as antiinflammatory⁶, anti-allergic¹¹, antioxidant¹² and neuroprotective effects¹³, so it presents significant therapeutic action on many diseases such as thrombosis, acute pancreatitis, cardiovascular and other diseases. But due to its low solubility in aqueous solution, it is imperative to increase the Ginkgolide B's solubility or dispersity in aqueous solution to form a pharmaceutical acceptable form in clinical use.

The alkylcyanoacrylate was synthesized as adhesion agent by the US Eastman Company early in 1949, but only until the 1970s, Couvreur carried out its research in pharmacokinetics¹⁴. As one of the alkylcyanoacrylate compounds, polybutylcyanoacrylate (PBCA) had long been attracted scientific researchers for its good biocompatibility, biodegradable and low toxicity¹⁵.

Using PBCA nanoparticles as drug nanocarrier can be possible to carry active ingredient across the blood-brain barrier (BBB)¹⁶⁻¹⁹ for its long circulating time in blood. The object of the present study was to prepare the GB-PBCA nanoparticles by interface polycondensation method and to select the best cryoprotectants to ensure a steady dosage form in clinical use. The entrapment efficiency and loading rate of GB-PBCA nanoparticles were also investigated.

EXPERIMENTAL

α -Butyl cyanoacrylate was purchased from BeiJing ShunKang Science and Technology Development Co. Ltd., China; Ginkgolide B (>99 %) from Shanghai Tauto Biotech Co. Ltd., China; Dextran 70 from Sigma-Aldrich Co.; The Chromatographic grade Methanol from Fisher Co.

The R-114 rotary evaporator was from BuCHI Corp.; Malvern 3000HS laser particle analyzer from Malvern Co. England; JEM-2010 transmission electron microscope from JEOL Ltd., Japan; FreeZone 12L freeze drier from LABCONCO, USA; Sciex API 3000TM LC/MS/MS from Applied Biosystems, Foster City, CA, USA; Shimadzu LC-10ADVP, Shimadzu, Japan.

HPLC and MS conditions: The content of ginkgolide B in GB-PBCA nanoparticles was detected on LC/MS/MS

apparatus (Applied biosystems, Foster City, CA, USA). The chromatography condition was as follows: Waters XTerra RP18 (100 mm × 2.1 mm ID, 5 µm) was used as chromatographic column and the Phenomenex RP18 (4 mm × 2.0 mm ID, 5 µm) was joined as guard column. The mobile phase containing methanol and water (70:30, v/v) was filtered through a 0.5 µm pore size membrane before use. The chromatography was performed isocratically at a flow rate of 0.3 mL/min at room temperature. The injection volume of sample was 20 µL each time.

All mass spectrometric measurements were performed on a Sciex API 3000™ equipped with an electrospray source. The instrument was operated in the positive mode and coupled to the outlet of the HPLC column *via* PEEK tubing. The temperature of the heated capillary was 380 °C. Source voltage was set at 4.2 kV. Auxiliary gasification gas, nebulizer gas, curtain gas, collision gas, declustering potential, focusing potential, entrance potential, collision energy, collision cell exit potential, were set at the following values: 7000, 6, 10, 8, -43, -400, -10, -22 and -10, respectively. Full scan data, centroid product ion scan as well as MRM scan data (dwell time 20 ms) were collected when analyzing samples. Formation of product ions in the tandem MS experiments was done in several scan events. Product ions were obtained for the precursor ions with *m/z* 423.3 → 367.0 within a scanning time of 2 min. The entire scanning time was 2 min. Fig. 1 showed a typical HPLC spectrum of the reference substance of ginkgolide B.

Fig. 1. HPLC spectra of the reference substance of ginkgolide B

Preparation of GB-PBCA nanoparticles: The GB-PBCA nanoparticles suspension was prepared by interfacial polymerization method as follow: The oil phase was formed by adding 0.0200 g ginkgolide B into 2 mL ethyl acetate and then mixing with 0.8 mL α -butyl cyanoacrylate in 1 mL acetone together under continuous stirring. The aqueous phase was obtained by dissolving the 0.10 g dextran-70 into 50.0 mL water. Then, the oil phase was added dropwisely into aqueous solution under continuously stirring for 0.5 h, followed by filtering out bulk and precipitated by G3 sintered glass funnel. Finally, the GB-PBCA nanoparticles suspension could be obtained by removing the organic solvents under reduced pressure.

Accuracy and precision and recovery and standard curve: Accuracy and precision of intra-day (in 3 days) and inter-day were analyzed by six replicates of six batches at 3 different concentration levels (the ginkgolide B concentration were 20, 40 and 100 ng/mL respectively in methanol). The coefficients of variation (CVs) at each concentration level were calculated as the accuracy. The intra day and inter day precision was tested by one-way analysis of variance (ANOVA).

Precision was expressed as the relative standard deviation (RSD). The results showed the precision of intra-day analysis at three different concentration levels were $0.52 \pm 0.04 \%$, $0.76 \pm 0.11 \%$, $1.12 \pm 0.13 \%$, respectively and that of the inter-day analysis were $0.63 \pm 0.08 \%$, $0.62 \pm 0.06 \%$ and $1.21 \pm 0.14 \%$, respectively.

To determine the methods average recovery, blank nanoparticles solution was condensed by Vivaflow™ 200 ultrafiltration, from which 1.0 mL filtered fluid was collected and mixed with 100 µL ginkgolide B stocked solution (100 ng/mL). The methods recovery could be calculated by the ratio of measured value and addition value, which was $100.9 \pm 0.2 \%$ in this experiment. The plus average recovery test was designed by dissolving 10 mg ginkgolide B and 100 mg lyophilized blank nanoparticles powder together in methanol. It could be calculated by the ratio of measured value and addition value, which was $98.9 \pm 0.7 \%$ in the result.

Ginkgolide B standard was dissolved in 0.20 µg/mL methyl and was diluted to concentration of 10, 40, 80, 120, 160 and 200 ng/mL and the samples were prepared and analyzed by LC-MS as described in the section of materials and methods. The regression equation of ginkgolide B was: $Y = 4076.6 C + 9921.7$, $r = 0.9995$, where the X was the concentration of ginkgolide B and Y was the responding peak area, which indicated good linear correlations between concentrations and analytical responses. The calibration curve linearity of diltiazem hydrochloride was over the range 10 to 200 ng/mL. The lower limit of quantification (LLOQ) of ginkgolide B methyl was 10 ng/mL.

Entrapment efficiency and loading rate: For the drug entrapment efficiency tests, the entrapped drug nanoparticles suspension was separated by Vivaflow™ 200 ultrafiltration. The free ginkgolide B in 1.0 mL filtered fluid was extracted by 2.0 mL ethyl acetate and vibrated on vortex for 1 min, followed by centrifuging at speed of 9000 rpm for 10 min. The residue below the oil phase was separated under N₂ flow and dissolved in mobile phase for sample injection. The entrapment efficiency (%) of drug can be calculated by the following equation:

$$\text{Drug efficiency (\%)} = \frac{\text{mass of drug in nanoparticles}}{\text{mass of drug used in formulation}} \times 100 \%$$

For loading rate tests, 0.10 mg lyophilized samples were weighted and ultrasonic extracted by 2 mL ethyl acetate, then centrifuged at speed of 9000 rpm for 10 min. The combined oil phase was dried by N₂ at room temperature and redissolved by mobile phase for sample injection. The loading rate can be calculated by the following equation:

$$\text{Drug loading rate (\%)} = \frac{\text{mass of drug in the formulation}}{\text{mass of the formulation}} \times 100 \%$$

Stability of nanoparticles suspension and selection of skeleton agent: Needle-like crystals sediment appeared time dependent after they were kept for 2 weeks at room temperature or even at 4 °C, which suggested that the GB-PBCA nanoparticles were unstable in liquid form for clinical use. The ideal method was to keep them in solid lyophilized powder form.

To select the cryoprotectant of lyophilized powder of the nanoparticles, the skeleton agent such as sorbierite, sucrose,

TABLE-1
LYOPHILIZATION RESULT OF NANOPARTICLES WITH DIFFERENT SKELETON AGENT

Cryoprotectant	Concentration (% w/v)	Solid appearance	Dispersion time	Clarity	Particle size (nm)
Mannitol	1.25	o	20s	#	216.8
	2.5	o	21s◆	#	186.3
	5.0	o	22s◆	#	176.3
	7.5	Full but off wall	26s◆	#	148.6
	10.0	Full but porous	26s◆	#	163.2
Trehalose	1.25	o	22s	*	175.5
	2.5	o	21s	*	138.0
	5.0	o	28s	*	153.4
	7.5	Full but porous	37s	*	151.2
	10.0	Full but off wall	35s	*	129.8
Sorbierite	1.25	§	Cant disperse	Layered	-
	2.5	§	45s◆	#	189.6
	5.0	§	49s◆	#	178.9
	7.5	§	52s◆	#	209.6
	10.0	§§	50s◆	#	242.8
Sucrose	1.25	o	30s	#	165.7
	2.5	o	65s	#	152.1
	5.0	o	45s	#	146.3
	7.5	Full but off wall	27s	#	120.4
	10.0	§§	Cant disperse	Layered	-

o indicate the appearance of the product looked full; # indicate the dispersion appeared white and opaque; * indicate the nanoparticles lyophilized powder dispersed in injective water appeared light blue opalescent. ◆ indicate the dispersion must be with the help of ultrasound; § indicate the product looked shrink and collapse. §§ indicate a severe collapse

mannitol and trehalose were mixed with the GB-PBCA nanoparticles suspension separately. For the lyophilize process, the 2 mL sample solution was transferred into 7 mL glass antibiotic bottle and then placed in the lyophilizer machine. The thermal gradient in the cooling unit was set to 0.5 °C/min until it was cooled to -34 °C and kept for 24 h, then adjusted the thermal gradient unit to 0.07 °C/min, 0.04 °C/min, 0.07 °C/min in sequence until it was finally heated to 25 °C for 10 h in vacuum. The appearance and re-dispersion of the powder were adopted as parameters to assess the effect in the form of powder.

Characterizations of nanoparticles: Size distribution and zeta potential may play important roles in determining the fate of nanoparticles after administration²⁰. The diameter and size distribution of the prepared nanoparticles were measured at a Malvern 3000 HAS Zeta Sizer using the PCS and ELS analysis, respectively. The experiments were carried out at 25 ± 0.05 °C and distilled water was used as dispersant solution. For Zeta-potential measurement, the nanoparticles were diluted in distilled water at pH 6.5, using a He-Ne laser light ($\lambda = 633.0$ nm) and a scattering angle of 90° with a 256-channel correlator.

The TEM of the nanoparticles microphotographs were observed at JEM-1200EX. To obtain the samples for TEM observations, one drop of nanoparticles aqua dispersion suspension was placed on a copper grid covered with nitrocellulose membrane and nitrogen gas-dried at 25 °C before negative staining with 1 % phosphotungstic sodium solution. The photographs could be stored and gathered from the linked computer.

RESULTS AND DISCUSSION

Selection of cryoprotectant: Generally, nanoparticles show a poor long-term stability due to different physical and

chemical factors that may destabilize the system²¹. For these reasons, special excipients must be added to the suspension of nanoparticles before freezing to protect these fragile systems²². Although the GB-PBCA nanoparticles suspension could be steady kept at room temperature for 2 weeks, they were still not appropriated to clinical use. The most commonly used process which allows to converting solutions or suspensions into solids of sufficient stability for distribution and storage in the pharmaceutical field is freeze-drying²³. Usually, cryoprotectants are added to the formulation to protect the submicronic particles²⁴. In general, the kind of cryoprotectant and their concentration must be optimized to ensure a maximum stabilization of nanoparticles. Usually, a freeze-thawing study should be realized before freeze-drying to select the best cryoprotectant which is able to conserve the properties of nanoparticles²⁵.

For selecting the best skeleton protective agent, the parameters including the content of cryoprotectant in liquid, the powder appearance, the re-dispersion time in injective water, the clarity of the re-dispersion liquid and the particle size were mainly investigated. The lyophilizate was dispersed in 2 mL injective water in bottle to investigate the re-dispersion effects including the dispersing time, whether the powder could be dispersed freely in water or must be with the help of ultrasound. The immobilization of nanoparticles within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against collapse from the mechanical stress of ice crystals^{26,27}. Table-1 showed that the effects of solid appearance, the re-dispersion time, the re-dispersion clarity and the particle size of different kinds and concentration of the cryoprotectant. By contrast, the trehalose as skeleton agent in 1.25-5.0 % could keep the powder a good solid appearance, a quick dispersion time in injective water, a light blue opalescent disperse liquid and a reasonable range particle size.

Trehalose seems to be a preferable cryoprotectant for biomolecules²⁸. It has many advantages in comparison over other sugars as: less hygroscopicity, an absence of internal hydrogen bonds which allows more flexible formation of hydrogen bonds with nanoparticles during freeze-drying²⁹, low chemical reactivity and finally, higher glass transition temperature T_g ³⁰. Report revealed that 2 % of trehalose was not sufficient to protect the nanoparticles³¹, as size measurements of reconstituted nanoparticles showed an increase in their average diameter and polydispersity index. But in this experiment, the 2.5 % trehalose was full enough to could ensure a quick dispersion time and relatively small size in narrow distribution, so it was appropriated as cryoprotectant.

Particles size and morphology: Fig. 2 showed a typical transmission electron microscope image of the GB-PBCA nanoparticles. The nanoparticles were clearly well separated and essentially no aggregation could be seen, indicating successful capping of PBCA on the nanoparticle surfaces.

Fig. 2. Morphology of GB-PBCA nanoparticles observed by TEM [Top: nanoparticles solution ($\times 70000$), bottom: redissolving solution of nanoparticles after lyophilization ($\times 100000$)]

Fig. 3 described the size distribution of the particles diameter of GB-PBCA. Before freeze drying, the average diameter, the zeta potential and polydispersity index of GB-PBCA nanoparticles aqua suspension were 114.0 nm, -29.3 mv and 0.0094 respectively. But after freeze drying, those values increased to 138.5 nm, -22.3 mv and 0.11 respectively, which meant that the trehalose as cryoprotectant in the freeze drying process could only slightly affect the basic physical morphology of the particles.

Fig. 3. Size distribution of the particles diameter of PBCA-GB (left: solution state; right: redispersion state)

Entrapment rate and loading capacity: The mean entrapment and drug loading capacity of three batches were $96.74 \pm 1.25 \%$ and $5.74 \pm 0.46 \%$ respectively, which indicated

a high entrapment efficiency and drug loading capacity of the ginkgolide B in PBCA nanocarriers. The high loading capacity of nanoparticles showed that the encapsulation of nanoparticles could effectively solve the low solubility of ginkgolide B in aqua solution, which will broaden its use in aqua dosage form.

Conclusion

The high encapsulation efficiency and loading rate of GB-PBCA nanoparticles with a uniform distribution were successfully prepared using interfacial polymerization. By selecting 2.5 % trehalose as the cryoprotectant, a well re-dispersed GB-PBCA nanoparticles suspension with diameter 138.5 nm and a freeze drying powder injection form could be obtained successfully. The character of PBCA as drug carrier to carry ginkgolide B over blood-brain barrier will be further investigated.

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