Preparation and characterisation of ginkgolide nanoparticles via the emulsion solvent evaporation method

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Ginkgolide is the first choice for treatment of cardiovascular and cerebrovascular diseases. However, its conventional dosage form (ginkgolide injection) can easily produce side effects and adverse reactions. Hence, developing a new, safe and efficient nanometre dosage form is imperative. Ginkgolide nanoparticles (GLNs) were prepared via the emulsion solvent evaporation method, and their mean hydrodynamic diameter, surface zeta potential, and surface morphology were characterised. The work studied four factors that were able to influence the properties of GLNs and discussed the preparation mechanism. The results showed that the prepared GLNs were nanomaterials. The optimum preparation conditions were with a W/O of 5.0, C_{PVA} of 6.0%, P of 250 W and T of 4.0 min. The GLN that was prepared under optimum conditions exhibited good dispersity and stability. Its surface zeta potential was −20.97 mV, single particle size was 20–50 nm, and the preparation yield was 73.51%. The pharmacokinetics of GLN was studied in detail, and the C_{max} and $AUC_{0\to\infty}$ increased by 63.29 and 67.56%, respectively, compared with ginkgolide injection. This study lays a foundation for the research and development of new nano-dosage forms of ginkgolide and has an important value.

1. Introduction: Ginkgolide is a type of platelet-activating antagonist factor and possesses a strong physiological activity [1, 2]. For the past few years, it has become the preferred natural medicine for the treatment of cardiac–cerebral vascular diseases and was favoured by doctors and patients. The annual sales have amounted to $5 billion and was still increasing at a rate of 10% [3, 4]. Many researches have been made for ginkgolide. However, they focused on the pharmacology and efficacy, and there are very few studies on the dosage form. Thus, the priority is still given to injection, which has a low drug utilisation rate, severe side effects, and adverse reactions, and this causes a serious influence on the actual use and effects [5–7].

The dosage form has an important effect on the drug effect. Nanotechnology provides a new way to research dosage forms and receives wide attention [8–10]. Using nanotechnology to pelletise drugs can greatly improve the solubility and dispersion of hydrophobic drugs. Numerous studies show that nanoscale drug particles can cross the blood–brain barrier and gather in lesions, which significantly improved drug's bioavailability and efficacy, and significantly reduced side effects and adverse reactions [11–15]. Significant research results on paclitaxel, adriamycin, ibuprofen, isradipine have been reported [16–21]. Thus, nanocrystallisation has become a new method to create safe and effective dosage forms. It is generally believed that ginkgolide nanoparticles (GLNs), which are similar to most hydrophobic drugs, can also significantly improve bioavailability and efficacy, reduce side effects and adverse reactions, and decrease the cost of treatment. However, there are no reports on GLN at present. Hence, we firstly report the preparation of GLN to lay a foundation for research and development of safe and effective new nanometre dosage forms in the future.

In this study, GLNs were directly prepared via the emulsion solvent evaporation method. Four factors, the volume ratio of polyvinyl alcohol (PVA) solution and ginkgolide solution (W/O), the concentration of PVA (C_{PVA}), ultrasonic power (P) and ultrasonic time (T), which could influence the properties of GLNs, were studied in detail. A laser particle size analyser (LPSA), scanning electron microscope (SEM), and transmission electron microscope (TEM) were used to test the physical properties of the GLNs. Then, the pharmacokinetics of GLNs in rats was studied in detail.

2. Methods

2.1. Materials: PVA, ethyl acetate, dichloromethane, acetone, and ginkgolide of analytical standards were purchased from Sigma-Aldrich Co. LLC. (Shanghai, China). Ginkgolide was purchased from Shanghai Yuanye Biological Technology Co., Ltd (Shanghai, China). Ginkgolide injection was obtained from Baiyu Pharmaceutical Inc. (Chengdu, China). Wistar rats were purchased from Laboratory Animal Center of Zunyi Medical College. Other materials were obtained from Aladdin Industrial Inc. (Shanghai, China). All chemicals were directly used as received without further purification.

2.2. Design of experiment: We studied the influences of W/O, C_{PVA}, P and T on the properties of GLNs (mean hydrodynamic diameter, polydispersity, and surface zeta potential). Each factor was set to five levels, as shown in Table 1.

2.3. Preparation of GLNs: According to the experimental design, 5.0 g of ginkgolide was added to 100.0 ml of mixed organic solution (the volume ratio of ethyl acetate, dichloromethane and acetone was 1:1:1, respectively), stirred for 30 min and filtered to remove the undissolved solids. Then, ginkgolide solution was added to PVA solution at room temperature, stirred for 5 min at 300 rpm until a milky white opaque emulsion formed. Next, the emulsion was treated with an ultrasonic method (ultrasonic method: operate 5 s, pause 10 s). After ultrasonic treatment, the emulsion was transferred to a rotary evaporator and evaporated for 30 min at 35°C until the organic solvent was completely removed. Then, the mixture was centrifuged for 15 min at 15,000 rpm, and the precipitation was cleaned with deionised water. The centrifugation and cleaning operations were repeated 10 times until PVA was completely removed.

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Table 1 Experimental design

Factors	Levels				
W/O	1.0	3.0	5.0	7.0	9.0
C_{PVA}	2.0%	4.0%	6.0%	8.0%	10.0%
P, W	50	150	250	350	450
T, min	1.0	2.0	3.0	4.0	5.0

2.4. Characterisation: The LPSA (Melvin – ZS 90, 25°C, automatic program) was used to determine the particle size distribution, polydispersity, and surface zeta potential. Scanning electron microscopy (SEM, Hitachi S-4800, voltage: 2.0 kV) and transmission electron microscopy (TEM, Hitachi J EOL2100CXII, accelerating voltage: 200 kV, current: 96 μA) were used to determine the surface morphology and structure. A high performance liquid chromatograph (Agilent 1200) and a mass spectrometer (MS, Agilent 1946D) were used to determine the plasma concentration.

2.5. Determination of yield: GLN was freeze-dried by a vacuum freezing dryer. The quality of GLN freeze-dried powder was determined, and the preparation yield was calculated

$$\text{yield} = \frac{m_f}{m_i} \times 100\%,$$

where m_i is the quality of ginkgolide initially added and m_f is the quality of GLN freeze-dried powder.

2.6. Stability tests: The stabilities of GLN were investigated at room temperature and at 4°C. GLN properties (mean hydrodynamic diameter, polydispersity, and surface zeta potential) were measured every 30 days.

2.7. Pharmacokinetics research: Six Wistar rats (3 male and 3 female, 200 ± 20 g) were selected to study the pharmacokinetics, and intragastric administration was adopted. These rats were fasted 12 h before dosed and the dosage was 5 mg/kg (5 mg ginkgolide per kg rat). After dosed for 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 3.00, 6.00, 9.00 and 12.0 h, 0.2 ml blood was acquired from orbital venous, respectively. Then, the blood was placed in a centrifuge tube containing heparin and centrifuged (3000 rpm) 10 min to separate plasma. Liquid chromatograph–MS was used to measure the plasma concentration of the processed plasma [22, 23]. DAS 2.1 pharmacokinetic software was used to calculate the pharmacokinetic parameters. Ginkgolide injection was used as a blank control.

3. Results and discussion

3.1. Effects of W/O on GLN properties: The GLN properties (mean hydrodynamic diameter, polydispersity, and surface zeta potential) as a function of W/O were studied when C_{PVA} was 6.0%, P was 250 W, and T was 3.0 min. The results are shown in Table 2.

As shown in Table 2, W/O has a significant impact on the GLN properties. In our experiment, all GLNs had good and similar dispersibilities. However, when W/O is <5.0, the particle size decreases rapidly and the surface zeta potential obviously increases along with an increase in W/O, which makes GLNs more stable. When W/O is >5.0, the particle size enlarges, surface zeta potential decreases, and the stability of GLNs becomes worse. An oil-in-water microemulsion was used as a reactor in our experiment, and GLN was prepared by removing the organic solvent via evaporation. Therefore, the stability of the microemulsion and the size of the emulsion droplet are important factors influencing the properties of GLN [24]. W/O is also an important factor influencing the properties of GLN [25]. When the volume of ginkgolide solution remains constant, the amount of the oil phase in a single emulsion droplet and the emulsion droplet size reduce with an increase of W/O, which causes the size of GLN to decrease. However, when W/O is more than a certain range, the amount of the oil phase in a single emulsion droplet is too small. The emulsification becomes difficult, and the stability of the system is poor, which causes a bigger size and a worse dispersity of GLN. The optimum W/O is 5.0.

3.2. Effects of C_{PVA} on GLN properties: The experiment investigated the properties of GLNs (mean hydrodynamic diameter, polydispersity, and surface zeta potential) as a function of C_{PVA} when W/O was 5.0, P was 250 W, and T was 3.0 min. The results are shown in Table 3.

As shown in Table 3, C_{PVA} has an evident impact on GLN properties. In our experiments, all GLNs exhibited good and similar dispersibilities. When C_{PVA} is <4.0%, the particle size enlarges and the surface zeta potential diminishes along with an increase in C_{PVA}. Then, with an increase in C_{PVA}, the particle size decreases, and the surface zeta potential increases. When C_{PVA} is >8.0%, the particle size rapidly enlarges, the surface zeta potential obviously decreases, and the stability of GLNs becomes worse. PVA is a surface active agent and dispersant, and it disperses the oil phase in the water phase evenly [26]. Hence, C_{PVA} has an important influence on the emulsion droplet's stability and size. At low

Table 2 Effects of W/O on GLN properties

W/O	1.0	3.0	5.0	7.0	9.0
mean diameter, nm	281.28 ± 3.41	195.44 ± 12.20	177.50 ± 6.54	186.29 ± 8.38	231.45 ± 11.21
PDI	0.247 ± 0.019	0.214 ± 0.021	0.185 ± 0.014	0.226 ± 0.023	0.231 ± 0.083
surface zeta potential, mV	−10.24 ± 0.92	−16.17 ± 0.41	−18.43 ± 0.31	−17.51 ± 0.74	−12.23 ± 0.87

Data are shown as the mean ± standard deviation of three replicate measurements.

Table 3 Effects of C_{PVA} on GLN properties

C_{PVA}	2.0%	4.0%	6.0%	8.0%	10.0%
mean diameter, nm	181.78 ± 6.07	205.94 ± 4.58	179.17 ± 7.23	248.77 ± 10.05	257.92 ± 20.53
PDI	0.203 ± 0.039	0.281 ± 0.019	0.197 ± 0.013	0.347 ± 0.019	0.349 ± 0.007
surface zeta potential, mV	−17.72 ± 0.40	−15.85 ± 0.37	−18.31 ± 0.24	−12.45 ± 0.95	−11.48 ± 0.60

Data are shown as means ± standard deviations of three replicate measurements.

Table 4 Effects of *P* on GLN properties

P, W	50	150	250	350	450
mean diameter, nm	297.85 ± 15.09	236.24 ± 5.37	178.84 ± 7.94	174.99 ± 10.84	178.28 ± 9.88
PDI	0.311 ± 0.003	0.253 ± 0.006	0.197 ± 0.012	0.186 ± 0.034	0.193 ± 0.020
surface zeta potential, mV	−11.45 ± 0.95	−15.85 ± 2.30	−18.15 ± 0.27	−18.52 ± 0.40	−18.21 ± 0.44

Data are shown as means ± standard deviations of three replicate measurements.

Table 5 Effects of *T* on GLN properties

T, min	1.0	2.0	3.0	4.0	5.0
mean diameter, nm	231.34 ± 4.36	185.54 ± 4.56	174.99 ± 10.84	167.57 ± 9.19	174.65 ± 7.96
PDI	0.313 ± 0.014	0.263 ± 0.012	0.183 ± 0.026	0.141 ± 0.048	0.230 ± 0.051
surface zeta potential, mV	−11.45 ± 0.35	−16.85 ± 2.30	−18.48 ± 0.20	−20.52 ± 0.40	−18.61 ± 0.44

Data are shown as means ± standard deviations of three replicate measurements.

concentrations, the solubility of the water phase to oil phase enhances with an increase in C_{PVA}. The number of emulsion droplets sharply increases, and the amount of the oil phase in a single emulsion droplet reduces, thus reducing the emulsion droplet size and decreasing GLN's size. However, when PVA reaches a certain concentration, the number of emulsion droplets is too larger, and the probability of collision among adjacent emulsion droplets increases. A larger emulsion droplet is formed, which leads to a worse particle dispersity and a bigger mean hydrodynamic diameter. The optimum C_{PVA} is 6.0%.

3.3. Effects of P on GLN properties: We also investigated the properties of GLNs (mean hydrodynamic diameter, polydispersity, and surface zeta potential) as a function of *P*, when W/O was 5, C_{PVA} was 6.0%, and *T* was 3.0 min. The results are shown in Table 4.

As shown in Table 4, *P* has a significant impact on GLN properties. When *P* is <250 W, the particle size and polydispersity rapidly decrease, and the surface zeta potential obviously increased with an increase in *P*. When *P* is >250 W, there are few differences among GLNs. *P* is an important factor that influences the emulsification effect [27]. In general, the emulsion droplet dispersion is more uniform and the emulsification effect is better with an increase in *P*. When the emulsion reaches a certain degree, the influence of *P* on the particle size and stability of emulsion droplet weaken with an increase in *P*. As a result, the particle size and dispersity of GLN are nearly unchanged when there is an improvement in *P*. The optimum *P* is 250 W.

3.4. Effects of *T* on GLN properties: The properties of GLNs (mean hydrodynamic diameter, polydispersity, and surface zeta potential) as a function of *T* were investigated when W/O was 5, C_{PVA} was 6.0%, and *P* was 250 W. The results are shown in Table 5.

As shown in Table 5, *T* has a significant impact on GLN properties. When *T* is < 4.0 min, the particle size and polydispersity decrease rapidly, and the surface zeta potential obviously increased with an increase in *T*. When *T* is >4.0 min, the particle size and polydispersity enlarge and surface zeta potential decreases, and the stability of GLNs becomes worse. *T* is an important factor that also influences the emulsification effect [28]. The emulsion droplet dispersion is more uniform and the emulsification effect is better with an extension of *T*. However, when the emulsion reaches a certain degree, the influence of *T* on the particle size and stability of the emulsion droplet weaken with an extension of *T*. Thus, the particle size and dispersity of GLN are nearly unchanged when *T* is extended. The optimum *T* is 4.0 min.

3.5. Property characterisation: The above experiment studied the main factors influencing the properties of GLN and determined the optimum value of each factor. Then, we prepared GLN by employing all the optimum conditions (W/O was 5, C_{PVA} was 6.0%, *P* was 250 W and *T* was 4.0 min), and it was selected as a sample for the following research. Property characterisations were determined using LPSA, SEM, and TEM. According to the results, we observed that GLN was yellow, clarified, translucent, and there was an obvious Tyndall phenomenon (see Fig. 1). GLN is spherical or axiohitic, good dispersive, and negatively charged, and it has a polydispersity index (PDI) of 0.145, a surface zeta potential of −20.97 mV, and a single particle size of 20–50 nm (see Figs. 2–4). The preparation yield was 73.51%.

3.6. Stability tests: Stability is very important in view of the potential applications. The stabilities (mean hydrodynamic

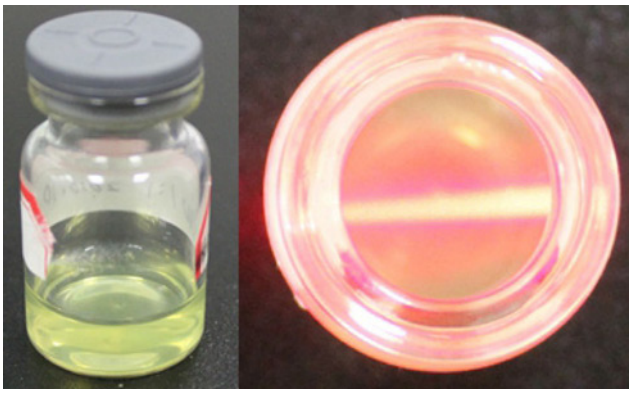

Fig. 1 *Outside images of GLN*

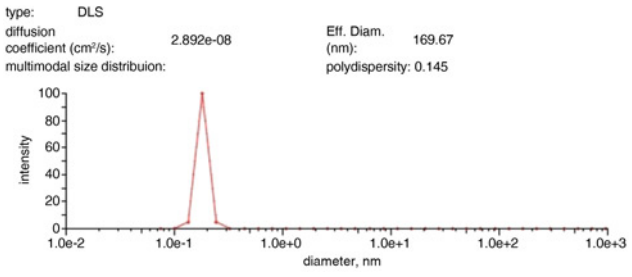

Fig. 2 *Particle size distribution image of GLN (each sample was tested three times; test temperature: 25°C; test way: automatic program)*

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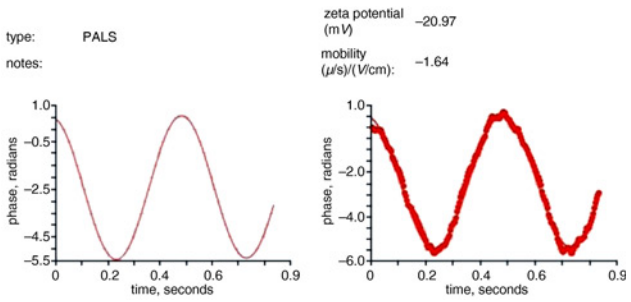

Fig. 3 *Surface zeta potential image of GLN (each sample was tested three times. Test temperature: 25°C; test way: automatic program.)*

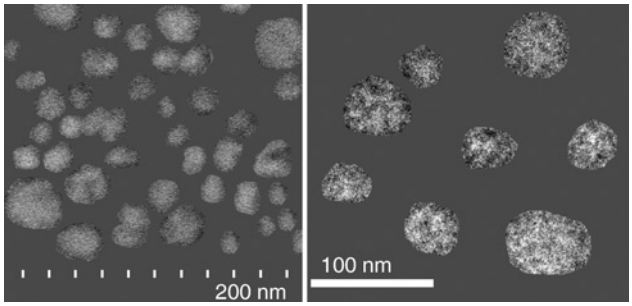

Fig. 4 *SEM image (left) and TEM image (right) of GLN (the voltage of SEM is 2.0 kV, and the voltage and current of TEM are 200 kV and 96 μA, respectively)*

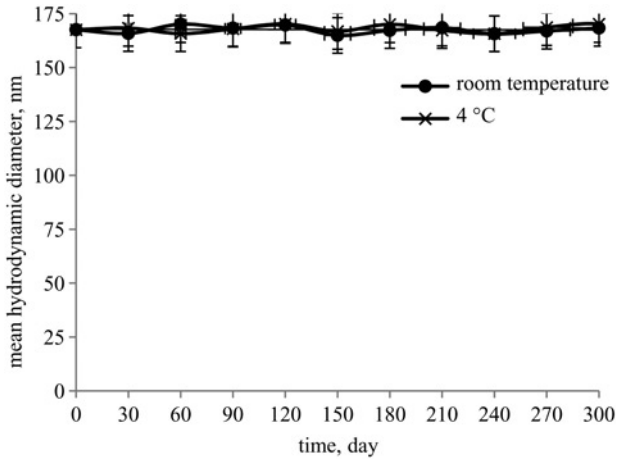

Fig. 5 *Mean hydrodynamic diameter of GLN (GLN was stored in the dark and each sample was tested three times. Test temperature: 25°C, test way: automatic program)*

diameter, polydispersity, and surface zeta potential) of GLN against storage were researched every 30 days at room temperature and at 4°C.

As shown in Figs. 5–7, there was no obvious change in the mean hydrodynamic diameter, polydispersity or surface zeta potential when GLN was stored at room temperature and at 4°C after 300 days. The results show that GLN exhibited good stability.

3.7. Pharmacokinetics research: Pharmacokinetics is an important index for drug properties, and we studied the pharmacokinetics of GLN in rats. The plasma concentration – time curve and pharmacokinetic parameters are shown in Table 6 and Fig. 8.

The plasma concentration – time curve and pharmacokinetic parameters showed that both ginkgolide injection and GLN were

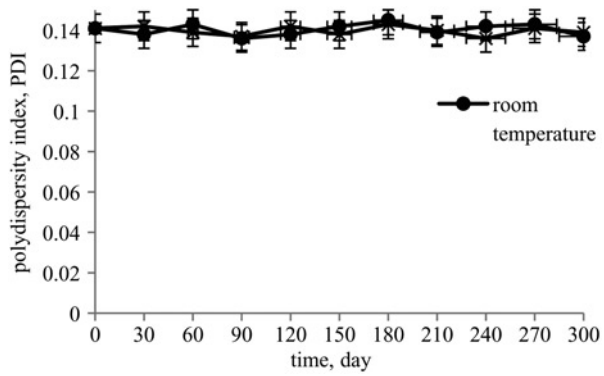

Fig. 6 *Polydispersity of GLN (GLN was stored in the dark, and each sample was tested three times; test temperature: 25°C; test way: automatic program)*

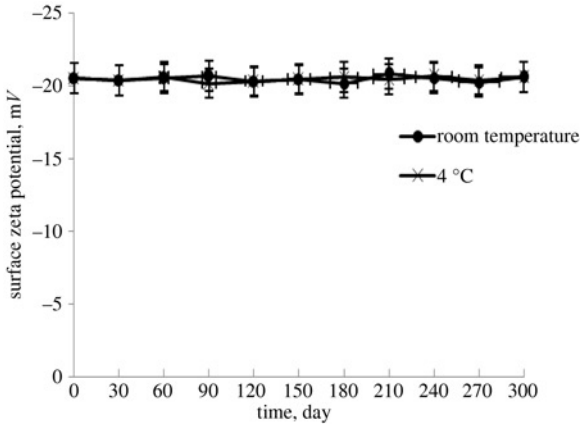

Fig. 7 *Surface zeta potential of GLN (GLN was stored in the dark, and each sample was tested three times; test temperature: 25°C; test way: automatic program)*

Table 6 Main pharmacokinetic parameters of the plasma after administered with GLN

Parameters	Unit	Ginkgolide injection	GLN
C_{max}	μg/l	863.87 ± 30.32	1429.31 ± 67.69
T_{max}	H	1.17 ± 0.26	1.42 ± 0.20
$AUC_{0\to t}$	μg/(l h)	3061.62 ± 165.32	5241.83 ± 104.88
$AUC_{0\to\infty}$	μg/(l h)	3182.007 ± 186.39	5331.86 ± 103.60

Data are shown as means ± standard deviations of six rats.

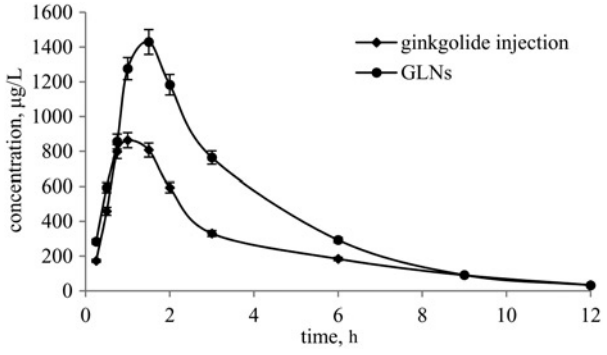

Fig. 8 *Time curve for ginkgolide plasma concentration (six rats were tested according to the experimental design, and each plasma was tested three times)*

quickly absorbed. Despite the absorption velocity of GLN was little slower than ginkgolide injection, the absorption rate was significantly improved, and its C_{max} and $AUC_{0\rightarrow\infty}$ increased by 63.29 and 67.56%, respectively. Therefore, the bioavailability was significantly improved. Meanwhile, compared with ginkgolide injection, the attenuation speed of GLN was slower, which could maintain a higher plasma concentration for a protracted period and improve the efficacy in clinical treatment [29].

4. Conclusion: In this research, GLNs were prepared via the emulsion solvent evaporation method. W/O, C_{PVA}, *P,* and *T* have significant impacts on GLNs' properties, and the optimum preparation conditions are: a W/O of 5.0, a C_{PVA} of 6.0%, a *P* of 250 W and a *T* of 4.0 min. The GLN, which was prepared under the optimum conditions, was clarified, translucent, spherical or axiohitic, good dispersive and negatively charged. Its PDI is 0.145, surface zeta potential is −20.97 mV, single particle size is 20–50 nm, and preparation yield is 73.51%, it also has a good stability at room temperature and at 4°C. Compared with ginkgolide injection, the C_{max} and $AUC_{0\rightarrow\infty}$ of GLN increased by 63.29 and 67.56%, respectively. This research proved that nanocrystallisation of ginkgolide could improve the bioavailability and efficacy.

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