PREPARATION PROCESS FOR ORAL DISSOLVING FILM OF GINKGOLIDE B FOR TREATMENT OF ALZHEIMER'S DISEASE

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ABSTRACT

Objective: *Ginkgolide B (GB) has been applied to cardiovascular diseases in clinic with its anti-oxidative and anti-aging effects. GB plays a neuroprotective role in models of various diseases. A study showns that Aβ1-42 induces oxidative damage to the cellular biomolecules, which are associated with AD pathology, and are protected by the pre-treatment of GB against Aβ-toxicity. We prepared the oral dissolving film (ODF) of GB by tape casting. The influence of film forming material, plasticizer and defoamer on the performance of the ODF of GB was studied. ODF has better adherence and is easy to take, which provides certain reference value for future nerve agents.*

Methods: *The preparation parameters were as follows: 35mg GB was dissolved with 70ml of distilled water and mixed well, which was followed by the addition of 0.5g of glycerol as the plasticizer. After complete dissolution, 2g of hydroxypropyl methylcellulose was added as the film forming material. An ODF solution was formed by mixing, during which two to three drops of edible defoamer were added. After defoaming, the solution was coated onto the carrier sheet and left to stand until complete drying. Then the film was cut into a proper size.*

Results: *Experiments showed that the ODF of GB could completely dissolve in 280s and 80% of GB was released in 1min. The degree of release reached100% in 3min. This drug was pasted onto the supralingual area of 0.6×1.3mm with a single dose of 1mg/kg for the rats. Pharmacokinetic parameters were measured, and the clearance rate CL (L/h) was calculated as 2.156, Cmax (ng•mL-1): 13.52, Tmax: 4h.*

Conclusion: *The AUC for the ODF of GB was larger by about 1.77-fold as compared with the intragastric administration of GB. The bioavailability of ODF of the drug was higher than other conventional oral preparations.*

Keywords: *Alzheimer's Disease, oral dissolving film, ginkgolide B, pharmacokinetics.*

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Introduction

Modern society has witnessed an increasing incidence of brain diseases due to the accelerated ageing of the population. According to a survey by World Health Organization (WHO), cerebrovascular diseases are among the top three leading causes of death in 40 countries. Bureau of Disease Control and Prevention of National Health and Family Planning Commission of the People's Republic of China has reported[(1)] that over 80% of the elderly people have brain lesions affecting the vascular and neural functions to varying degrees. This usually means a great economic burden for both the society and family. Senile dementia and Parkinson's syndrome caused by stroke sequelae and brain dysfunction adversely impact the life quality and independent living ability of China's elderly people.

Ginkgo leaf, containing ginkgolides, has a high medicinal value, and ginkgolide B (GB) is especially known for its high bioactivity[(2)]. In vitro studies have found that GB fulfills many physiological roles, such as inhibition of endothelial dysfuntion and thrombosis[(3, 4)]. GB is believed to have a treatment

value for cardiovascular and cerebrovascular disease and possess anti-inflammatory and anti-oxidative activities in addition to many pharmacological effects[5-7]. Many studies have shown that GB has neuroprotective effect on degenerative dementia and neurosensory disorders[8-13]. Ming-Yang and others found that Ginkgo biloba extract (GBE) and one of its main ingredients, ginkgolide B (GB) promoted cell cycle exit and neuronal diferentiation in NSCs derived from the postnatal subventricular zone (SVZ) of the mouse lateral ventricle[14]. Wang Li and others found that GB could alleviate hypoxia-induced neuronal damage in rat hippocampus by inhibiting oxidative stress and apoptosis[15]. GB may also be a potential therapeutic drug for atherosclerosis and osteoporosis[16, 17].

GB is a liposoluble substance, and can penetrate the blood brain barrier. It has been applied to cerebral ischemia, kidney transplantation, burns and asthma, as well as ischemic encephalopathy including acute ischemic stroke[18-26]. Supplement of GB may be a potential way to improve neurodegenerative diseases or brain injury.

Oral dissolving film (ODF) is a novel drug delivery system, prepared by the mixing and processing of the drug with appropriate film forming material. The size and thickness of an ODF are comparable to those of a stamp. It can dissolve fast and release the drug in saliva without drinking water after pasting it onto the tongue[27]. The first patent for ODF was granted in 1964 (GB1061557). In 2001 Listerine, a mouth freshener film, was developed by Pfizer, and in 2003, the first OTC ODF of anaesthesin by InnoZen went on the market[28]. ODFs usually possess the following advantages. The local action drugs delivered in ODF can directly act on the lesions with a higher bioavailability; the drugs for systemic action can be directly absorbed by the oral mucosa, thus avoiding the first pass effect. Since there is no need for swallowing down with water, neither is there a risk for blocking the throat. And so this formulation is particularly suitable for children and elderly people. ODFs are easy to carry.

There will be no worries about choking or inhalation as with fast disintegrating tablets or 1yophilizedwafer. Finally, ODFs are easy to prepare and less costly. However, there are also some defects with ODFs, such as limited drug loading amount, and only highly active drugs can be prepared into ODFs. How to mask the bitter taste the drugs is another important issue[29, 30]. ODFs are composed of drug, film forming material, plasticizer, and pigments, and sometimes a small amount of surfactant to enhance the bioavailability of the drug, as well as excipients such as stabilizer and thickener[31].

The commonly used preparation methods of ODFs include:

• Tape casting:

The components (film forming material, plasticizer, and defoamer) are dissolved or dispersed in solvent and spread over the carrier sheet until drying and film forming;

• Hot-melt extrusion technique:

The active component of the drug is mixed evenly with thermoplastic film forming materials (film forming materials and plasticizer) and other excipients (defoamer and surfactant), followed by drying and film forming.

There is no need for solvent and water and the preparation is simple and efficient[32]. The commonly used film forming materials are hydroxypropyl methylcellulose and polyoxyethylene[33]. Glycerol, PEG 400 and glycerol triacetate are usually used as the plasticizers[34]. Defoamers include peanut oil, rapeseed oil, sesame oil, Span 65, methyl silicone oil and edible defoamers. Surfactants include linear alkylbenzene sulfonates (LAS) and sodium alcohol ether sulphate (AES). Edible pigments are usually used as the colorants[35, 36].

In this study, the formulation was designed and optimized with GB as the active drug and an ODF of GB was prepared. Based on test on the release degree in simulated saliva, the process parameters that influenced the quality of the ODF were analyzed. The pharmacokinetics of the prepared drug after a single dose were studied using the protocol for non-clinical drugs. Then a comparison was made with the conventional oral formulations that had gone onto the market, and the drug release features of the ODF were assessed. Our research findings lay the basis for the development of safe, convenient, efficient and high-quality drug formulations.

Materials and methods

General information

GB, content ≥98% (Shanghai Jinsui Bio-Technology Co., Ltd.), hydroxypropyl methylcellulose (Beijing Gaohuaweiye Food Additive Co., Ltd.), polyoxyethylene (Union Carbide, USA), glycerol (Shenyang Aohua Biochemical Reagent Co., Ltd.), food defoaming agent (Beijing Gaohuaweiye Food Additive Co., Ltd.), other reagents (Beijing Reagent Company), distilled water (self-prepared).

LA series electronic balance (Changshu Bailing Balance Instrument Co., Ltd.); LK-Q1036 ultrasonic cleaner (Zhuhai Lingke Automation Technology Co., Ltd.); magnetic stirrer mixer SH-2 (Qun' an Experimental Device Co., Ltd.); multi-head magnetic stirrer (Shanghai Qige Industrial Co., Ltd.); oven (Guangzhou Daxiang Electronic Machinery Equipment Co., Ltd.); TU-1901 ultraviolet spectrophotometer (Beijing Purkinje General Instrument Co.,Ltd.); ZRS-8G intelligent dissolution tester (Tianjin University Radio Factory); drug stability test chamber (Shanghai Boxun Industry & Commerce Co., Ltd.).

Analytical method of GB

GB reference standard of 5mg was weighed precisely and placed into a 10ml volumetric flask. It was dissolved in water until reaching the mark on the flask with proper shaking. Gradient solutions of 0.025mg/ml, 0.05mg/ml, 0.075mg/ml and 0.1mg/ml were prepared respectively. Absorbance was measured at 216nm using the ultraviolet spectrophotometer[28].

The regression equation y = 0.444x + 0.41 R=0.99994 was derived and the linear range was 0.025-2mg/ml.

Preparation of ODF of GB

An appropriate amount of GB was weighed according to the prescription and added with distilled water and plasticizer.

When GB was fully dissolved under mixing, the film forming material was slowly added and a gel was formed by proper mixing. A few drops of defoamer were added during the mixing. After the defoaming was complete, the solution was spread over the carrier and left to stand. The film was cut into proper size after complete drying.

Tensile test

The film was cut into specimens of 40 mm×15 mm with smooth edges and no notch or damage. Then the specimen was placed between the grips of a universal testing machine.

The longitudinal axis of the specimen coincided with the central line of the upper and lower grips. The grips were adjusted to a proper tightness to avoid slipping or fracture of the specimen.

The distance between the two grips was 20mm. The tensile test machine was started with a rate of 50 mm/min. The maximum load and elongation at break were read when the specimen fractured.

Dissolution measurement of GB

To measure the dissolution of GB, artificial saliva, 2.38g NaHPO4, 0.19g KH2PO4, 8g NaCL and 15ml of water were used. The pH value was adjusted to 5-7 with phosphoric acid. The specimen was cut into a size of 1 cm×1 cm, and 6 patches of film were used for the experiment. The films were held with paper clips. The third method of dissolution test in the appendix XC of the second volume of Chinese Pharmacopoeia 2010 Edition was performed, and 100ml of artificial saliva was used as the dissolution medium. Counting began from the moment that the specimen came into contact with the artificial saliva at a rotational speed of 304/min. Sampling was performed at 0.5, 1, 1.5, 2 and 3min, respectively, for 1ml each time. The sample was passed through a 0.22 um filter membrane. The filtrate was analyzed and the dissolution curve was plotted.

Drug stability test

Measurement of disintegration time:

The film was cut into a size of 1 cmx1 cm. The disintegration time was measured according to the Appendix XA standards for measuring disintegration time in the second volume of Chinese Pharmacopoeia 2010 Edition. The time needed for the film to completely disintegrate and to pass through the filter membrane was recorded.

Lighting stability test:

The ODF was placed on the dish inside the illumination incubator under the illuminance of 2000LX for 7 days. Sampling was performed on d1, d3, d5 and d7, respectively. The disintegration time was measured.

Temperature stability test:

The ODF was placed in the drug stability test chamber for 7 days at 20°C, 40°C and 60°C, respectively. Sampling was performed on d1, d3, d5 and d7, respectively, and the disintegration time was measured.

In vivo pharmacokinetic study of ODF of GB in rats

Time points for blood sampling and sample preparation

Each group had 6 rats, which were fasted for 12h overnight and weighed. After anesthesia was induced using 10% chloral hydrate, the drug was administered according to the body weight. About 0.5ml of the blood sample was drawn from the ophthalmic venous plexus before administration and at 5min, 15min, 30min, 1h,

2h, 4h, 8h and 12h post-administration, respectively. The blood sample was collected into an anticoagulant (heparin) tube, which was centrifuged to obtain the plasma. Hydrochloric acid was added into the plasma (100μL 1mol/L hydrochloric acid per 1ml of plasma) and the plasma was stored at -20°C prior to use.

Plasma sample processing

Into 0.1ml of the acidified plasma, 10μL of fluconazole as the internal standard (10μg/mL) was added with proper mixing. Then 1ml of ethyl acetate was added with vortex mixing for 2min. This was followed by centrifugation at 6000r/min for 5min and 800μL of the supernatant was collected. In a 40°C water bath, the sample was blown dry in N2 gas. The residue was redissolved in 200μL of acetonitrile with volution for 30s. The liquid phase separated by centrifugation was collected into a flask.

Conditions of HPLC-MS

Shimadzu 8050 HPLC-MS system was used, consisting of Shimadzu 30AD liquid chromatograph (LC-30A dual pump, SIL-30AC automatic sample introduction system, photodiode array detector SPD-M30A, CTO-20AC column) and 8050 triple quadrupole mass spectrometer. Liquid phase separation was performed using Phenomenex Kinetex® C18 column (100 mm × 2.1 mm i.d.; 2.6 μm). The mobile phase contained acetonitrile (A) and 0.1% benzoic acid aqueous solution (B). The flow rate was 0.5 mL·min–1 with gradient elution, A:B ratio being 35:65. The column temperature was maintained at 30°C, and the injection volume was 1 μL. $y = 0.0125x + 0.0024$, $R^2 = 0.9998$, and the linear range was 0.5-50 ng/ml. The conditions for MS are as follows: dry gas flow rate 10.0 L·min–1; nebulizer gas flow rate 3.0 L·min–1; flow rate of the heater 10.0 L·min–1; interface voltage 3kV; detector voltage 1.8kV; heater temperature at the interface 300°C; temperature of desolvation tube 250°C; temperature of the heating module 400°C; ESI ion source, multiple reaction monitoring (MRM). Ionization parameters included ion pair information, Q1 energy, Q3 energy, collision energy and dwell time, as shown in Table 1.

Compound	Parent ion-product ion	Dwell time	Q1	CE	Q3
GB	423.20-367.25	100	10	15	10
Fluconazole	305.20-191.25	100	16	12	20

Table 1: Optimized parameters of LC-MS/MS for GB and internal standard.

Results

Influence of film forming material and film forming performance

Into 35mg of GB 70ml of distilled water and 0.5g glycerol as plasticizer were added with sufficient mixing. Then 2g of the film forming material was slowly added.

Two different film forming materials were used in this experiment, namely, hydroxypropyl methylcellulose and polyoxyethylene.

The mixture was left to stand and spread onto the carrier after the defoaming was complete.

The film was then air dried in a well-ventilated place. Hydroxypropyl methylcellulose is a commonly used film forming material, and the resulting film has uniform color and luster, contains air bubbles and can be easily stripped from the mold.

In contrast, the film prepared by using polyoxyethylene as the film forming material has poor ductility and muddy color, and therefore it is not suitable for industrial production.

In this study, hydroxypropyl methylcellulose was used as the film forming material.

The ODFs prepared by using any of the two film forming materials were poor in strength and ductility, and plasticizer was needed in the prescription.

Influence of plasticizer on the film forming performance

Into 35mg of GB 70ml of distilled water and 0.5g glycerol as plasticizer were added with sufficient mixing.

Two plasticizers were used, glycerol and polyethylene glycol. After the plasticizer was fully dissolved, 2g of hydroxypropyl methylcellulose was slowly added and the mixture was left to stand until the defoaming was complete.

Then it was air dried in a well-ventilated place. The influence of two plasticizers on the ductile strength of the films is shown in Table 2.

Plasticizer	Maximum load /N	Elongation at break/%
Glycerol	46.77±3.68	6.51±2.34
Polyethylene glycol	38.28±7.31	5.89±0.48

Table 2: Influence of two plasticizers on the ductile strength of the films.

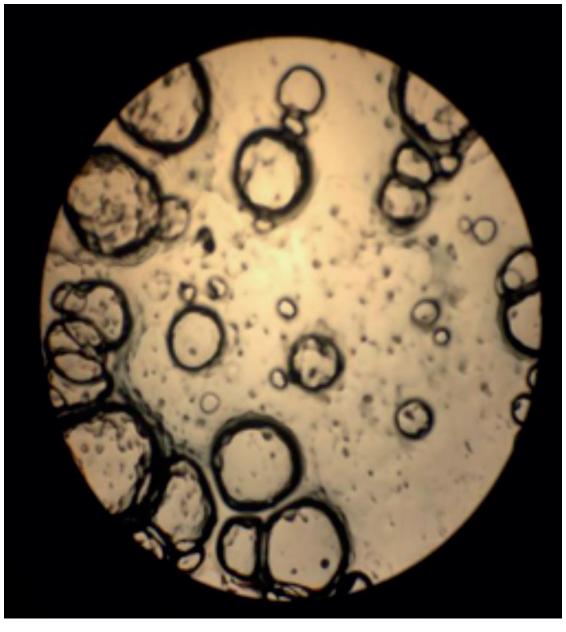

Figure 1: Micrograph of films prepared by glycerol as plasticizer.

Influence of defoamer on the film forming performance

The following defoamers were added, respectively: peanut oil, rapeseed oil, sesame oil, Span 65, silicone oil and edible defoamer.

Among them, peanut oil, rapeseed oil and rapeseed oil achieved a good defoaming effect, though the films prepared by these defoamers were darker in color and less palatable. The defoaming effect was less satisfactory with Span 65 or silicone oil. In contrast, only two drops of edible defoamer were sufficient to make all the bubbles disappear in the films, and so the edible defoamer achieved the best defoaming effect.

The addition of hydroxypropyl methylcellulose would cause air bubbles in the films, and the edible defoamer achieved the best defoaming effect if added immediately after the appearance of the air bubbles. We observed that a large amount of air bubbles disappeared quickly once the edible defoamer came into contact with the air bubbles. Only two or three drops of edible defoamer added at an early stage of film forming were enough.

Based on the above analysis, the optimized preparation process was determined as follows: 35mg of GB was added with 70ml of distilled water with proper mixing. Then 0.5g of glycerol was added as plasticizer and mixed until complete dissolution. Next 2g of hydroxypropyl methylcellulose was slowly added as the film forming material, and a gel was formed with proper mixing. Two to three drops of edible defoamer were added during mixing. The ODFs of GB prepared are shown in Fig. 2.

After magnification of the films by 1-3 folds according to the prescription, the properties of the ODF of GB showed no significant changes. The disintegration time was less than 280s, indicating stability of the preparation process.

Figure 2: ODF of GB.

Stability test of the ODF of GB

Lighting stability test

Four batches of ODFs of GB were prepared according to the prescription and optimized parameters, with 10 films in each batch (2cmx2cm).

The ODF was placed on the dish inside the illumination incubator under the illuminance of 2000LX for 7 days and the sampling was performed on d1, d3, d5 and d7, respectively.

The disintegration time was measured, and the results are shown in Table 3.

Day	Disintegration time (s)			
	First batch	Second batch	Third batch	Forth batch
Day 1	254±3	278±11	248±2	264±21
Day 3	298±14	265±23	255±7	298±2
Day 5	243±8	268±17	272±11	298±5
Day 7	280±5	298±4	305±16	340±7

Table 3: Disintegration time of ODF of GB in lighting stability test.

As shown by the dissolution curves in Figure 3, there were no significant differences in the disintegration times of ODFs of GB across the four batches. This indicated high lighting stability of the films. In the dissolution test of the films, 80% of GB was released within 1min, and the degree of release reached up to 100% in 3min.

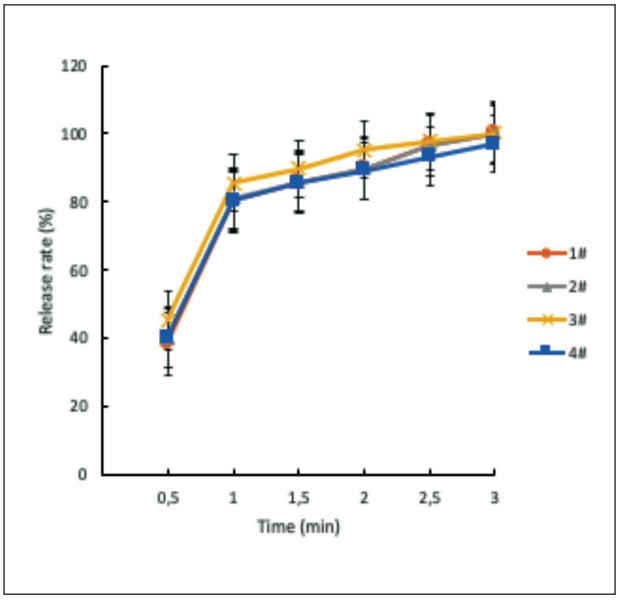

Figure 3: Dissolution curves of four batches.

Temperature stability test

The ODF was placed in the drug stability test chamber for 7 days at 40°C and 60°C, respectively. Sampling was performed on d1, d3, d5 and d7, respectively, and the films were observed.

Under the temperature of 40°C and 60°C, a large number of films were softened and dissolved on d3. Only thin and soft films in small quantities were left on d7.

This indicated low resistance of the ODFs of GB to high temperature, and therefore the films should be kept in a cool place.

In vivo pharmacokinetic study in rats

The ODFs of GB prepared in this study were pasted to the supralingual area of rats. The area of drug administration was 0.6×1.3mm and the dose was 1.0mg/kg.

For intragastric infusion the dose was 1mg/kg. PKPlus (version 9.5.0004) was used for plasma drug concentration-time analysis for each sample, and the pharmacokinetic parameters were calculated.

The plasma drug concentration-time curves are shown in Figure 4. The pharmacokinetic parameters calculated by using non-compartment model are shown in Table 4.

Pharmacokinetic parameters	Intragastric administration	ODF
$AUC_{0\text{-}12}$/ug.h/ml	0.067±0.051	0.121±0.060
$AUC_{0\text{-}y}$/ug.h/ml	0.069±0.053	0.122±0.060
AUMC (area under the first moment curve)	0.418±0.31	0.831±0.410
MRT (mean retention time)/h	6.027±4.00	6.820±2.38
C_{max} (peak concentration)/ng/ml	13.8	13.52
T_{max} (peak time) /h	1	4
Vd (volume of distribution)/L	17.37±9.56	14.70±0.004
CL (clearance rate)/L/h	2.882±0.69	2.156±0.75

Table 4: Pharmacokinetic parameters of GB after a single dose 1.0mg/kg to rats in PO and ODF formulation.
Each value was expressed as mean±S.D. (n=6). $^{*}p<0.05$ as compared with PO.

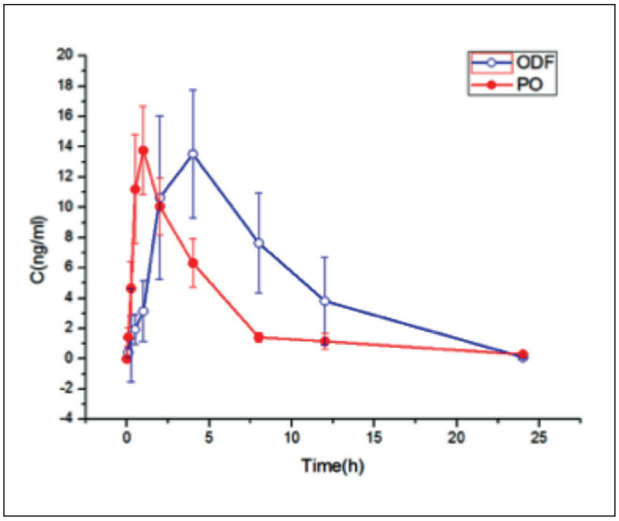

Figure 4: Plasma drug concentration-time curves.
A single dose of 1 mg/kg GB was given in ODF (∘) or by intragastric infusion (•). Each value was expressed as mean±S.D. (n=6).

Discussion

Prepare the oral dissolving film (ODF) of GB by tape casting, the influence of film forming material, plasticizer and defoamer on the performance of the ODF of GB was studied. The addition of two plasticizers had little impact on the casting of the films and the films were easily stripped from the mold. As seen from the Table 2 above, the strength and ductility of the films prepared using glycerol as plasticizer were better than those of the films prepared by polyethylene glycol. Therefore, glycerol was chosen as the plasticizer. As shown in Figure 1, the films prepared by glycerol as the plasticizer contained a large amount of air bubbles, which affected the appearance of the film product. Therefore,

defoamer was needed in the prescription. The total plasma concentration of GB administered in ODF was apparently higher than that by intragastric infusion. AUC for the ODF of GB was about 1.77-folds that of intragastric infusion, which suggested higher bioavailability of ODF of GB. Tmax was also much higher with the ODF, indicating the delayed release of GB. For either pathway of administration, Vd was lower than 20L.

Thus GB given in either pathway was mainly found in the plasma and extracellular fluid, and the difference in the administration pathway did not change the drug distribution in rats. A reduction in CL suggested a slower clearance of the drug and hence a longer retention time in rats. In a word, ODF was conducive to maximizing the efficacy of GB.

GB is generally used to prevent or treat ischemic stroke and senile dementia. ODF is a convenient pathway of administration without the need to wash the drug down with water. The film can dissolve on the tongue and it is not easily spit out after adhesion. So this formulation is especially suitable for the elderly people with swallowing difficulty. Drug released from the ODFs is directly absorbed by the oral mucosa, thus avoiding the first pass effect and enhancing bioavailability. ODF is attracting an increasing attention in recent years due to its simple preparation and low cost. Many companies have developed ODFs for drugs that are usually delivered in liquid preparation, capsules, tablets and fast disintegrating tablets. In our study, the ODF for GB was easy to prepare and had a high stability under low temperature. In vivo pharmacokinetic study indicated the ODF of GB had a higher bioavailability than other preparations and this formulation had a promising\ market prospect.

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