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Formulation screening and freeze-drying process optimization of Ginkgolide B lyophilized powder for injection

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Abstract

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was increased from -15.0℃ to 20.0℃ at 1.0 ℃/min, maintaining 4 hours, and the chamber pressure was raised up to 80 Pa. In these lyophilization process conditions, the products complied with relevant provisions of the lyophilized powders for injection, meanwhile, the reproducibility was satisfactory. Post-freezing annealing had no significantly beneficial effects on shortening the freeze-drying cycle and improving the quality of GB lyophilized powder.

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maintaining 4 hours, and the chamber pressure held at 10 Pa; (4) shelf temperature was increased from -15.0℃ to 20.0℃ at 1.0 ℃/min, maintaining 4 hours, and the chamber pressure was raised up to 80 Pa. In these lyophilization process conditions, the products complied with relevant provisions of the lyophilized powders for injection, meanwhile, the reproducibility was satisfactory. Post-freezing annealing had no significantly beneficial effects on shortening the freeze-drying cycle and improving the quality of GB lyophilized powder.

KEY WORDS: Ginkgolide B, formulation screening, freeze-drying process optimization, collapse temperature

INTRODUCTION

Ginkgo biloba is among the oldest living trees, with a long history of use in traditional Chinese medicine. In recent years, the extracts of ginkgo biloba leaf have been widely sold as herbal medications worldwide. The most unique components of the extracts are the terpene trilactones: ginkgolides and bilobalide. Ginkgolide B (GB) is one kind of ginkgolides, and it is the most potent inhibitor of the platelet-activating factor receptor (PAFR)（1）. PAFR is a highly active mediator in the human body and has been implicated in various disease states（2）. GB has been postulated to include improvement of memory, increased blood circulation, as well as beneficial effects to sufferers of Alzheimer's disease（3,4）and cisplatin-induced ototoxicity（5）.

GB is a diterpene with a cage skeleton consisting of six five-membered rings (Fig.1): a spiro[4.4]-nonane carbocyclic ring, three lactones, and a tetrahydrofuran ring, with a relative molecular weight of 424.4 g/mol （6）. GB is a white crystal, and can be dissolved in acetone, ethanol, methanol, ethyl acetate, tetrahydrofuran, dioxane, acetic acid, trifluoroacetic acid, acetonitrile, pyridine as well as dimethyl sulfoxide, and slightly dissolved in ethyl ether and water (the solubility in water of 2.5×10^{-4} mol/L(0.106mg/ml)). GB cannot be dissolved in hexane, benzene, chloroform and carbon tetrachloride（7,8）. Under neutral or acidic conditions, all lactonic rings of GB are closed; under alkaline conditions (pH at 7.5 to 12), some lactonic rings are opened because of hydrolysis. If the alkalinity increases, the majority of lactonic rings are opened to form salts. However, the GB without hydrolysis is the biologically active form, this is one of the reasons why some GB formulations can not reach the expected clinical efficacy （9,10）.Under physiological conditions, the lactonic rings of GB are partially hydrolyzed, the

original form only accounts for 34 percent at equilibrium（11）.

Ginkgo biloba products are offered today in many different preparations sometimes without any kind of scientific background and control. However, in evidence-based medicine and all clinical investigations and treatments, ginkgo biloba should only be used in the form of standardized ginkgo biloba extracts (e.g. EGb 761®, LI 1370®) defined by a special composition and manufacturing process（12）. Since GB is poorly soluble in water and gastric fluid environment, resulting in low dissolution and bioavailability through oral administration, it limits the development of oral preparations to some extent（13）. Lyophilized formulations can be injected after reconstitution, so that higher bioavailability can be ensured（14）. Furthermore, the loss of the active ingredient is reduced during the freeze-drying production. In general, lyophilized formulations are easy to transport and long-term storage. In order to develop GB to lyophilized powder for injection, the a five step procedure has been carried out in this paper: (1) solvent, surfactant, excipient and solution of PH screening for formulation; (2) process preparation: choosing GB API adding order, mixing temperature, added amount of active carbon and absorption time; (3) freeze-drying optimization of process parameters for freeze stage, primary drying stage and secondary drying stage; (4) Verification experiments of lyophilization process; (5) Effects of annealing on lyophilization rate and product quality.

MATERIALS AND METHODS

Materials

GB (the purity≥98%) was purchased from Nanjing Dierge Medical and Technological Co., Ltd.(Jiangsu, China). Tween 80, Tween 20, Tween 40, Poloxamer 188, Cremophor El, glucose, lactose, mannitol, dextran20, sucrose and L-arginine were purchased from Aladdin Reagent Database Inc. (Shanghai, China), which were analytical grades. Methanol was obtained from Xingke Solvent Inc. (Shanghai, China), which was HPLC grade. Water for injection was supplied from GMP Training Center of China Pharmaceutical University.

Formulation Screening of GB Lyophilized Powder for Injection

Specification Determination

Only several ginkgo biloba preparations were approved in China. On the basis of the ginkgo biloba injection produced by Chengdu Baiyu Pharmaceutical Co., Ltd.(Sichuan, China), each vial contains 2 mL solution and 10 mg terpene lactones as active pharmaceutical ingredient (API), in

which, GB probably accounts for thirty-four percent, being equal to 1.7 mg/mL. Therefore, each vial contained 2 mL GB solution after reconstitution in this study, and the concentration of GB was 2.0 mg/mL.

Establishing a Standard Curve between Concentrations and Peak Areas of GB

GB standards were accurately weighed and placed in a volumetric flask, then dissolved with methyl alcohol. The obtained solution was diluted by methyl alcohol to prepare standard solutions with different concentrations, such as 0.5×10^3 mg/L, 1×10^3 mg/L, 2×10^3 mg/L, 4×10^3 mg/L, 6×10^3 mg/L, then the contents of GB were determined with HPLC (Shimadzu Co., Ltd, China). The chromatographic conditions: the column was Agilent ZORBAX SB-C18 (4.6×150 mm, 5 μm), the column temperature was 25℃, the mobile phase was methanol-water (50:50), the velocity of flow was 1.0 mL/min, the detection wavelength was 220 nm, and the injection volume was 20 μL.

The concentrations of GB and their correspondent peak area data were shown in Table 1, the standard curve was shown in Fig. 2. The results showed that from 0.5×10^3 mg/L to 6×10^3 mg/L, and three experiments were repeated at each concentration to get the standard deviation of peak area. As a result, the linear relationship between concentrations and peak areas was good. The sampling precision test was done as follows: the reference solution (the concentration of 4×10^3 mg/L) was sampled six times, and the peak areas were recorded, and RSD was 0.15% by calculation.

Table 1 GB standard curve data

GB con. /mg·L^{-1}	500	1000	2000	3000	4000	5000	6000
Peak areas	291028± 1896	612131± 2130	1207230± 4213	1793174± 6472	2458279± 8745	3075164± 11824	3591077±14658
Regression equation	$y = 606.49x - 1634.4$, $R^2 = 0.9993$（n=7）						

Note: “n=7” means the number of GB concentrations to fit the regression equation of 7.

Solvent Selection

The most commonly used solvent in lyophilized powder for injection is water, but GB is poorly soluble in it. Therefore, mixed solvents were considered. Among solvents with ability to dissolve GB, ethyl alcohol, propylene glycol, glycerin, PEG 200 and PEG 400 had higher safety, which could be mixed with water to form co-solvents. It was found that the solubility to GB of the

co-solvents increased with their increased volume concentration. However, when volume concentrations of ethyl alcohol, propylene glycol, glycerin, PEG 200 were above 10 percent, the frozen solid could easily spray during primary drying. Because their melting point were relatively low, which were hard to be fully frozen. Only PEG 400 had relatively high melting point, and the above phenomenon did not easily happen. So PEG 400 and water for injection as the co-solvent was chosen.

Solubilization Method Selection

When poorly water-soluble drugs were prepared to lyophilized powders for injection, commonly solubilized methods included adding surfactants and latent solvents, adjusting pH, inclusion technique, emulsified or micro-emulsification etc. when we design an experiment, in general, we should consider the operating conditions and procedure, the cost of experiment, the kind of equipment to be used and the easiness to carry out the experiment. In this study, according to physical and chemical properties of GB and characteristics of lyophilized powders, adding surfactants and adjusting pH were chosen to increase the solubility of GB because these two methods are easy to fulfill, efficiency in cost and only HPLC instrument is used for experiments, the other methods of increasing the solubility of GB will be discussed in the future.

Surfactants for injection provided by FDA include Tween 80, Tween 20, Tween 40, Poloxamer 188, Cremophor EL, etc. The concentration of the above surfactants were mixed with 2mg GB, respectively, then 1 mL water for injection was added, ultrasonic processing was carried out for 10 minutes. Clarities of the obtained solution containing Poloxamer 188 or Cremophor EL were better than the others. It found that moulds produced easily in the Poloxamer 188 solution. So the Cremophor EL was more suitable, and its safe dose was large（15）.

GB was soluble and stable in concentrated acid, but poorly soluble in weakly acid solution. In weakly alkaline solution, its lactone rings occurred partial hydrolysis, with the increase of pH, the more lactone rings opened. From injection aspects to consider, it was suitable to adjust the pH of GB solution to be acidulous or neutral. The solution (which consisted of PEG 400, Cremophor EL and water) pH value was around 6.0,. Na_2CO_3, $NaHCO_3$, phosphate, meglumine, L-arginine, etc. could be used as the basic pH adjusting agents. L-arginine played an important therapeutic effect on atherosclerosis, which could promote vasodilation and angiogenesis, as well as inhibit the

aggregation of platelets and granulocytes（16）. Therefore, L-arginine was chosen as the pH adjusting agent.

Excipient Screening

The desired appearance of lyophilized powder should be an intact and porous cake structure. Furthermore, the color should be uniform. In order to obtain a better appearance, some excipients were added into API to provide a lyophilized skeleton. The following five excipients were used in this experiment: glucose, lactose, mannitol, dextran 20 and sucrose. The above excipients were separately added in the PEG 400 and Cremophor EL solution, and the dosage of each excipient was 6% (g/mL). Then **solution appearance** and **lyophilized product appearances** of above obtained solutions were compared, and the results were shown in Fig.3 and Table 2. By comprehensive comparison, mannitol as the excipient was the best because the structure by mannitol as excipient is intact and porous, that means there is no defect (no collapse, no crack on the surface, no shrink, no spray phenomenon and no collapse at the bottom etc.).

Table 2 Screening results of five excipients

excipients	**solution appearance**	after standing for 12 h	**lyophilized product appearances**
glucose	clear and transparent	clear and transparent	serious collapse on one side
lactose	clear and transparent	clear and transparent	slight collapse and cracks on the surface
mannitol	clear and transparent	clear and transparent	intact and porous cake structure
dextran 20	clear and transparent	precipitation of crystals after 8 h	shrinking into a huddle
sucrose	clear and transparent	precipitation of crystals after 4 h	collapse near the bottom, forming an inverted circular table

Formulation Screening by Orthogonal Design

After the determination of solubilization methods and excipients, the formulation of GB lyophilized powder for injection was screened by orthogonal design. The following four items were as investigation factors: A-PEG 400 concentration (mL/mL); B-Cremophor EL concentration (mL/mL); C-mannitol concentration (g/mL); D-solution pH. GB content in the prepared solution before freeze-drying and its reconstitution time after freeze-drying were as two evaluation indexes.

The GB content was determined through external standard method of HPLC, building a $L_9(3^4)$ orthogonal table.

Freeze Drying Process Optimization of GB Lyophilized Powder for Injection

Determination of Glass Transition Temperature of Maximally Freeze-concentrated Solution

In the freeze-drying process, three stages are included, they are freezing, primary drying and secondary drying. In the freezing stage, the lowest temperature could be confirmed on the basis of freezing point (including eutectic temperature or glass transition temperature), and the highest temperature during the primary drying was confirmed on the basis of collapse temperature. Material freezing point is usually determined through electric resistance method, freeze-drying microscope observation method and differential scanning calorimetry（17）.

According to the optimized formulation, the GB solution was prepared and the vials with GB solution were placed on the shelves of freeze drier (Shanghai Tofflon Co., Ltd., China). Two electrodes of the digital multi-meter were fixed on both sides in a beaker, and the solution temperature was determined by temperature probes of the freeze drier. Electric resistances of GB solution and corresponding temperatures were recorded as shown in Table 3. Then the temperature-resistance curve was obtained from data directly and data by regression(shown in Fig. 4), the temperature at the maximum curvature was the freezing point. The fitted curve was $R = a \times e^{b \times T} (where\ a = 0.225, b = -0.102)$ and the computational formula was as following（18）:

$$k=\left|\frac{R''}{(1+R'^2)^{\frac{3}{2}}}\right| \quad (1)$$

Where k is the slope of temperature-resistance curve, R is the solution resistance (MΩ), R′ is the first derivative of the solution resistance, R″ is the second derivative, T is the solution temperature (℃).

There was not a fixed melting point of GB solution by DSC 204 F1 (Netzsch Geraetebau GmbH, Germany).Therefore, where the freezing point was the glass transition temperature of maximally GB freeze-concentrated solution (Tg′). By calculation, the freezing point of GB solution was -17.6℃, while the freezing point was determined at -16.5℃ through freeze-drying microscope observation method. We can see the glass transition temperature measured by two method has a 1℃ difference, it proves that the measured transition temperature has a high accuracy. In general case, the lowest temperature during the freezing was about 10~20℃ lower

than Tg′. Therefore, the lowest freezing temperature was at -45.0℃ to ensure the material being fully frozen.

Table 3 Resistances of GB solution and corresponding temperatures

Temperature/℃	20.4	12.5	6.1	-0.2	-6.6	-12.3	-16.1	-20.4	-26.1
Electric resistance/MΩ	0.02	0.09	0.15	0.24	0.38	0.64	0.98	2.3	3.24

Determination of Collapse Temperature

During primary drying, if the product temperature is higher than the collapse temperature, the amorphous material will undergo viscous flow, resulting in loss of the pore structure obtained by freezing, which is defined as the collapse phenomenon by Pikal and Shah（19）. Collapsed dried products generally have a high residual water content and lengthy reconstitution times and may also present a loss of functional properties. Moreover, in the pharmaceutical industry, collapse is a normally cause for rejection of the vials due to the lack of material elegance. Since a small variation of temperature can greatly modify the primary drying time as well as the dried product structure, an accurate determination of the collapse temperature is critical for the process optimization（20）, the Freeze dry microscope usually is used for measuring collapse temperature.

Collapse temperature of GB lyophilized powder for injection was determined by the FDCS 196 freeze-drying microscope (Linkam Scientific Instruments Ltd., UK). 2 μL GB solution was taken and dropped between two glass cover slips. Then the freeze-drying stage was sealed, and an appropriate multiple objective was used to observe the solution. The solution was cooled from room temperature to -45.0 ℃ at 10.0 ℃/min, then the freeze-drying stage was moved to find the edge of the frozen solution, and the pressure in the stage maintained at about 20 Pa. Slow heating was followed, the movement of sublimation interface was observed, to judge at what temperature it began to collapse and collapsed completely, respectively. During -45.0℃ to -15.0℃, the sublimation interface moved from the edge of the glass slide to the center, there was a very clear sublimation interface between the drying zone and freeze zone. And at-14.0℃, the sublimation interface became not clear and there was a small quantity of viscous flow, resulting in loss of the pore structure, meaning -14.0℃ was the temperature of the onset of collapse. When the temperature increased to -13.0℃, the more viscous flow happened near the sublimation interface,

judging that -13.0℃ was the full collapse temperature. Heating up continually, much more viscous flow happened, and all were shown in Fig. 5.Hence, the highest temperature of primary drying was below -14.0℃.

Optimization of the Freeze-drying Process by Orthogonal Design

Firstly, various factors influencing GB lyophilized powder quality were confirmed from preliminary experiments, then freeze-drying process was optimized by orthogonal design. The following four items were as investigation factors: I: freezing temperature, cooling rate and duration; II: temperature changes during the primary drying, with gradient heating at 0.1 ℃/min; III: the primary drying time and pressure; IV: the secondary drying temperature and pressure. Appearance yield and residual water content were as two evaluation indexes, building a $L_9(3^4)$ orthogonal table. Residual water content was determined by V 20 Karl Fischer (METTLER TOLEDO, Switzerland). Qualified appearance criterions: it should be an intact and porous cake structure (no collapse, no crack on the surface, no shrink, no spray phenomenon and no collapse at the bottom etc.), without significant volume changes before and after lyophilization（21）.

RESULTS AND DISCUSSIONS

Optimized Formulation

The formulation was optimized by an orthogonal design using the software SPSS 19.0. Factor levels are shown in Table 4, orthogonal results shown in Table 5 and variance analysis results shown in Table 6 and Table 7. For the intuitively range analysis of GB content and reconstitution time in Table 5, the range values represented the influence order of the factors. Therefore, according to the range values in Table 5, the order of four factors for GB content was D＞B＞A＞C (A: PEG 400 concentration (mL/mL); B: Cremophor EL concentration (mL/mL); C: mannitol concentration (g/mL); D: solution pH). As for the evaluation index of GB content, the higher mean value under one factor (A, B, C, D) was required, so $D_1B_2A_1C_1$ was chosen as the optimized formulation. Similarly, for the reconstitution time of GB lyophilized powder, the influence order of the factors was B＞A＞D＞C. But the shorter reconstitution time was better, so $B_3A_3D_3C_1$ or $B_3A_3D_3C_2$ was chosen as the optimized formulation.

As shown in Table 6, through the variance analysis of GB content, only factor D had a significant influence ($p<0.01$), obtaining the highest GB content when factor D in level 1 in Table 5(The GB content increased from 2.5×10^{-4} mol/L(0.106mg/ml) to 1.914

(2×(94.7%+96.2%+95.8%)/3)mg/ml). As shown in Table 7, factor A, B, D had significant effects on GB lyophilized powder reconstitution time. And in Table 5, when factor A and B were in level 3, the reconstitution time was the shortest. On both GB content and reconstitution time respects by the variance analyses in Table 6 and Table 7, factor C had no significant effect on measurements. But the amount of mannitol was chosen 8% (g/mL), the obtained GB lyophilized powder was the most intact and porous by comparing 6% (g/mL) and 10% (g/mL). Through comprehensive analyses of the two evaluation indexes, the optimized formulation was $D_1B_3A_3C_2$, i.e. pH at 6.5, Cremophor EL in an amount of 16% (mL/mL), PEG 400 in an amount of 9% (mL/mL), and mannitol in an amount of 8% (g/mL).

Table 4 Factor levels

levels	factors			
	A/%	B/%	C/%	D
1	5	8	6	6.5
2	7	12	8	7.0
3	9	16	10	7.5

Table 5 Orthogonal design results of formulation screening

No.		levels				evaluation indexes	
		A	B	C	D	GB content/%	reconstitution time /s
	1	1	1	1	1	94.7	61
	2	1	2	2	2	86.6	58
	3	1	3	3	3	36.0	48
	4	2	1	2	3	36.8	52
	5	2	2	3	1	96.2	55
	6	2	3	1	2	87.2	48
	7	3	1	3	2	85.3	54
	8	3	2	1	3	38.3	46
	9	3	3	2	1	95.8	45
GB contents	mean 1	72.433	72.267	73.400	95.567		
	mean 2	73.400	73.700	73.067	86.367		
	mean 3	73.133	73.000	72.500	37.033		
	Range	0.967	1.433	0.900	58.534		
reconstitution time	mean 1	55.667	55.667	51.667	53.667		
	mean 2	51.667	53.000	51.667	53.333		
	mean 3	48.333	47.000	52.333	48.667		
	Range′	7.334	18.667	0.666	5.000		

Table 6 Variance analysis results of GB contents

sources of variance	sum of square of deviations	degree of freedom	F ratio	F critical value	P value
A	1.496	2	1.205	19.000	
B	3.082	2	2.481	19.000	
D	5944.569	2	4786.287	99.000	＜0.01
（C(error)	1.242	2	1.000		

Note: If F ratio> F critical value, P <0.01, representing the difference was significant. The effect of C (mannitol concentration)on GB content could be neglected, and its range value in Table 5 was minimum, so the factor C was chosen as error term.

Table 7 Variance analysis results of GB lyophilized powder reconstitution time

sources of variance	sum of square of deviations	degree of freedom	F ratio	F critical value	P value
A	80.889	2	90.989	19.000	＜0.05
B	118.222	2	132.983	19.000	＜0.05
D	46.889	2	52.744	19.000	＜0.05
C(error)	0.889	2			

Note: If F ratio> F critical value, P <0.01, representing the difference was significant. The effect of C (mannitol concentration) on reconstitution time could be neglected, and its range value in Table 5 was minimum, so the factor C was chosen as error term.

Confirmation of GB Solution Preparation Process

GB stability is affected by GB adding order, preparing GB solution temperature, amount of activated carbon and its adsorption time experiments. For example, the order of adding GB API will affect the transparent time of mixed solution; high temperature of preparing GB solution will cause the GB decompose; the over amount of active carbon added and more adsorption time of active carbon will cause the GB content decrease. So, GB adding order, preparing GB solution temperature, amount of activated carbon and its adsorption time experiments were set up one factor at a time to study their effects on GB stability. GB solution preparation process was confirmed as followings: 2.0 mg/mL GB was taken and added into the mixed solvent of 16% (mL/mL) Cremophor El and 9% (mL/mL) PEG 400, via ultrasonic treatment for 10 minutes. Then prescribed water for injection were added in it under electromagnetic stirring, and 8% (g/mL) mannitol was added the obtained solution, L-arginine was used to adjust the pH at 6.5. And 0.05%

(g/mL) activated carbon was added into the above solution, then under electromagnetic stirring(1000 r/min) for 35 minutes at 40.0℃, which was filtered through 0.22 μm PVDF membrane, finally filled and freeze-dried.

Optimized Freeze-drying Parameters

Through above, freeze-drying parameter optimization was carried out with SPSS 19.0 using an orthogonal design . Factor levels are shown in Table 8, orthogonal design results are shown in Table 9, and variance analysis results are shown in Table 10 and Table 11. For the intuitively range analysis of appearance yield and residual water in Table 9, the range values represented the influence order of factors. According to the range values in Table 9, considering the appearance yields, the influence order of the factors was I>II> III> IV (I: freezing temperature, cooling rate and duration; II: temperature changes during the primary drying, with gradient heating at 0.1 ℃ /min; III: the primary drying time and pressure; IV: the secondary drying temperature and pressure). As for the evaluation index of appearance yield, the higher mean value under one factor (I, II, III, IV) was required, so $I_1II_3III_1IV_1$ was chosen as the optimized freeze-drying parameters. Similarly, for the residual water content, the order of the factors was II> IV > I > III. Since the lower residual water content of GB lyophilized powder the better, II_3IV_3 I_1III_3 was chosen as the optimized freeze-drying parameters.

As shown in Table 10, through the variance analysis of appearance yields, the factor I and II had significant effects (P value <0.05). And in Table 9, when factor I was in level 1 and factor II in level 3, the appearance yield was the highest. As shown in Table 11, factor II and IV had significant effects on the residual water content. When factor II was in level 3 and factor IV in level 3, the residual water content of GB lyophilized powder was the lowest. On both appearance yield and residual water content respects, factor III had no significant effects. In fact, it is well known that shorter drying time can shorten the whole freeze-drying cycle and improve the productivity for enterprises, so III_1 (12h, 10 Pa) was chosen as the duration of primary drying. Based on the above considerations, I_1 II_3 III_1 IV_3 was the optimized freeze-drying parameters.

Table 8 Factor levels

levels	factors			
	I	II	III	IV
1	-45.0℃,0.5 ℃/min,2 h	-45.0℃→ -15.0℃	12 h,10 Pa	20.0℃,20 Pa
2	-45.0℃,1.0 ℃/min,2 h	-45.0℃→-30.0℃	15 h,10 Pa	20.0℃,50 Pa

3	-45.0℃,2.0℃/min,2 h	-30.0℃→-15.0℃ -45.0℃→-25.0℃ -25.0℃→-15.0℃	18 h,10 Pa	20.0℃,80 Pa

Table 9 Orthogonal design results of freeze-drying process

	No.	factors				evaluation indexes	
		I	II	III	IV	appearance yield/%	residual water content/%
	1	1	1	1	1	96.2	3.2
	2	1	2	2	2	98.3	2.4
	3	1	3	3	3	99.6	1.2
	4	2	1	2	3	90.6	2.6
	5	2	2	3	1	95.7	2.9
	6	2	3	1	2	97.2	1.8
	7	3	1	3	2	87.5	3.0
	8	3	2	1	3	93.9	2.3
	9	3	3	2	1	94.2	2.5
appearance yield	mean 1	98.033	91.433	95.767	95.367		
	mean 2	94.500	95.967	94.367	94.333		
	mean 3	91.867	97.000	94.267	94.700		
	Range	6.166	5.567	1.500	1.034		
residual water content	mean 1	2.267	2.933	2.433	2.867		
	mean 2	2.433	2.533	2.500	2.400		
	mean 3	2.600	1.833	2.367	2.033		
	Range′	0.333	1.100	0.133	0.834		

Table 10 Variance analysis results of appearance yields

sources of variance	sum of square of deviations	degree of freedom	F ratio	F critical value	P value
I	57.447	2	34.880	19.000	<0.05
II	52.607	2	31.941	19.000	<0.05
III	4.220	2	2.562	19.000	
IV(error)	1.647	2			

Note: If F ratio> F critical value, P <0.01, representing the difference was significant. The range value of factor IV in Table 9 on appearance yields was minimum, so it was chosen as error term.

Table 11 Variance analysis results of residual water content

sources of variance	sum of square of deviations	degree of freedom	F ratio	F critical value	P value

I	0.167	2	6.185	19.000	
II	1.860	2	68.889	19.000	<0.05
IV	1.047	2	38.778	19.000	<0.05
III(error)	0.027	2			

Note: If F ratio> F critical value, P <0.01, representing the difference was significant. The range value of factor III in Table 9 on residual water content was minimum, so it was chosen as error term.

Verification of Lyophilization Process

According to the previously optimized process, three batches of 300 mL GB solution were prepared and filled into 7 mL vials, and each vial contained 2 mL solution. These 100 vials were lyophilized in accordance with the optimized freeze-drying parameters, with the freeze-drying cycle of 20.7 hours. All products were intact and porous, the mean reconstitution time was (41±5) seconds, and the mean residual water content was (1.18±0.14)%. It showed that the optimized formulation and freeze-drying process could meet the specifications of lyophilized powder for injection, and with a good reproducibility.

Effects of Annealing on Lyophilization Rate and Product Quality

Some researches reported that annealing could improve lyophilization rate and shorten the freeze-drying cycle（22,23）. Hence, experiments were carried out to study the effects of annealing on lyophilization rate and product quality. The frozen solution was heated to above the eutectic temperature or glass transition temperature, but below the melting temperature held for a specified duration and then frozen again. In the experiments, GB solution has a glass transition temperature of -17.6℃, so the annealing temperature was set as two different temperatures of -14.5℃ and -8.0℃.

Annealing at -14.5 ℃

At normal atmospheric pressure, the GB solution was frozen from room temperature to -45.0℃, and then immediately heated to -14.5℃. This temperature was maintained constant for one hour, so that the lyophilization process continued following the above mentioned optimized freeze-drying parameters. By annealing at -14.5℃, the period of primary drying was shortened of about three hours, so that the whole cycle was 21.7 hours long. Compared to the GB lyophilized powder without annealing, the products annealed at -14.5℃ were looser, and the apertures were larger, even showing some cavities, and slight collapse occurred for about 1 percent of the

material, as shown in Fig. 6. In terms of residual water content, GB content, related substances and pH, both of the two had no significant differences.

Annealing at -8.0 ℃

Like the above annealing treatment, another batch of GB solution was annealed at -8.0℃ for one hour. The period of primary drying was shortened to about three hours, the freeze-drying cycle was 22.2 hours and the whole process parameters was automatically recorded by the lyophilizer. GB lyophilized powder in all vials shrinked up severely and had difficult in reconstitution, shown in Fig. 7. Compared to the GB lyophilized powder without annealing, the products annealed at -14.5℃ had no significant differences in terms of residual water content, GB content, related substances and pH.

To find the reason of annealing collapse at -14.5℃ and -8.0℃, decreasing freeze temperature and extending its time, decreasing the highest temperature and pressure of the primary drying measures were taken, only decreasing the highest temperature of the primary drying was helpful to improve the collapse. It could be deduced that the annealing treatment might improve the collapse temperature because of the changes of ice crystal morphology and size distribution. Thus, qualified GB lyophilized powder for injection could be obtained according to the optimized process, without annealing.

CONCLUSION

In this paper, the optimal formulation of GB lyophilized powder for injection was determined alongside with the optimised conditions for the complete formulation process. Firstly, solvent, solubilizer, excipient and pH screen were screened, PEG 400 in an amount of 9% (mL/mL), Cremophor EL in an amount of 16% (mL/mL), mannitol in an amount of 8% (g/mL) and pH at 6.5 were determined to make the solubility of GB improved of more than 18 times (from 0.106mg/ml to 1.914 mg/ml). Secondly, the preparing process was carried out by using a one factor at a time experimental design; thirdly, by orthogonal design, the optimized formulation was obtained. The optimized operation schedule for the process was: freezing from room temperature to -45.0℃ at 0.5 ℃/min, then holding for 2 hours; in the primary drying stage, heating the temperature from -45.0℃to －25.0℃ at 0.1℃/min, and then holding for 3 hours; heating the temperature from -25.0℃ to－15.0℃ at 0.1℃/min, and holding for 4 hours; in the whole primary drying stage

the chamber pressure was kept at 10 Pa; in the secondary drying stage, heating the temperature from -15.0℃ to 20.0℃ at 1℃/min, and holding for 4 hours, with the chamber pressure kept to 80 Pa. Then, the verification batch experiments have been carried out according to the above optimized protocol. A very satisfactory quality for GB lyophilized power for injection was achieved. Furthermore, post-freezing annealing process analysis has been carried out. The relevant discovery was that annealing is not beneficial for the processsince it did not allow to reduce the freeze-drying cycle and, what was worse, it caused some collapse on the material. Therefore, a complete process of formulation preparation of GB lyopilized power for injectionhas been given. This study would provide references for optimization technology of GB lyophilized powder for injection.

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Answer to Reviewer 1:

Thanks for your helpful suggestion to improve the paper. We have corrected the misunderstood according to your comments. On original page 6 and page 7, changed from “dissolution status” to “solution appearance”, and from ”lyophilized solution appearances” to “lyophilized product appearance”.

Figure Captions

Fig.1 The structure of Ginkgolide B

Fig. 2 The standard curve between concentrations and peak areas of GB

Fig. 3 Lyophilized product appearances of five excipients

Fig. 4 The temperature-resistance curve of GB solution

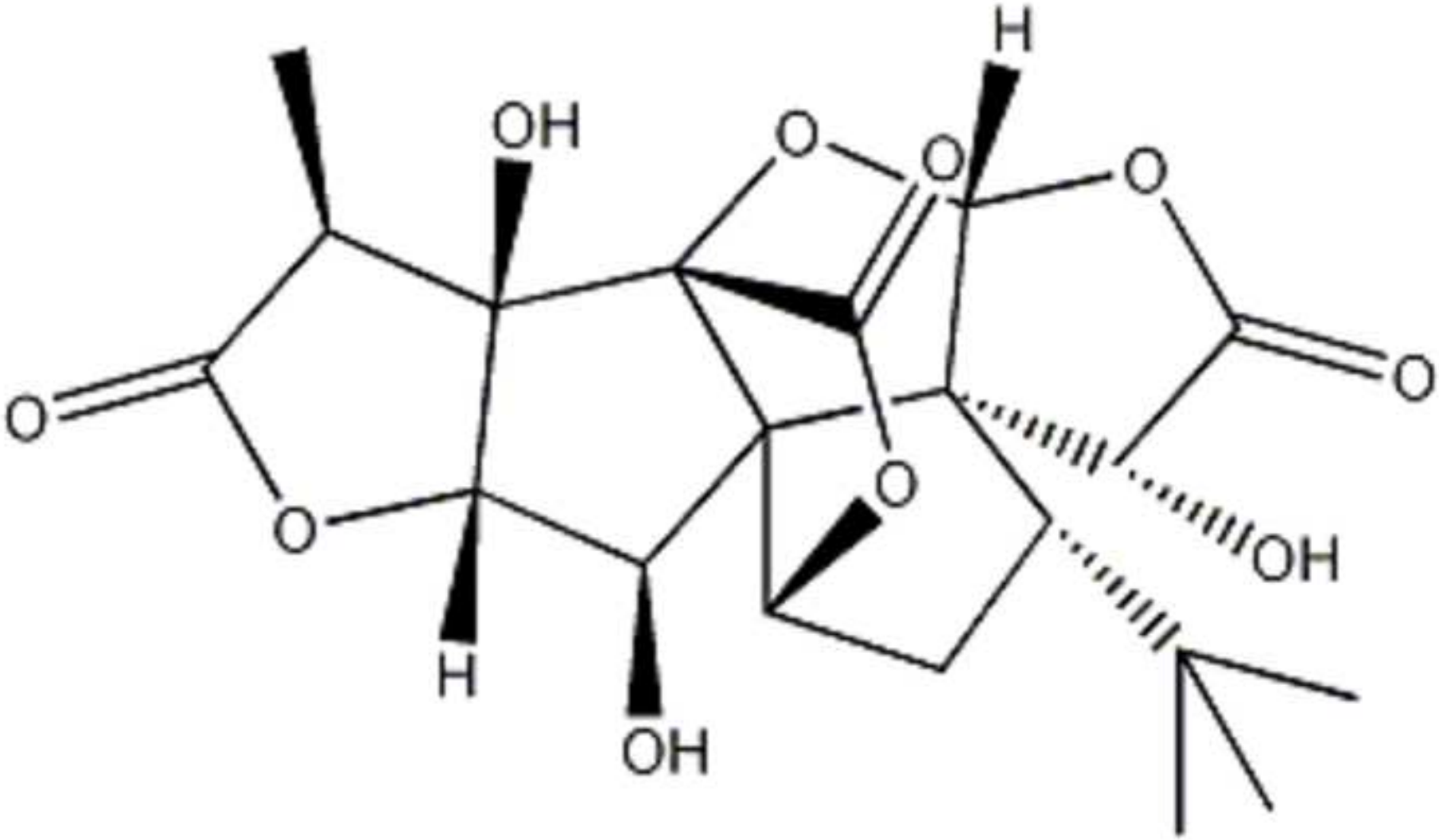
Fig. 5 Collapse process of GB lyophilized powder: a(at-15℃),b(at-14℃),c(at-13℃),d(at-12℃).

Fig. 6 The left vial was the appearance of GB lyophilized powder without annealing

Fig. 7 The left vial was the appearance of GB lyophilized powder without annealing

Figure 1

Click here to download Figure Fig.1 The structure of Ginkgolide B.bmp



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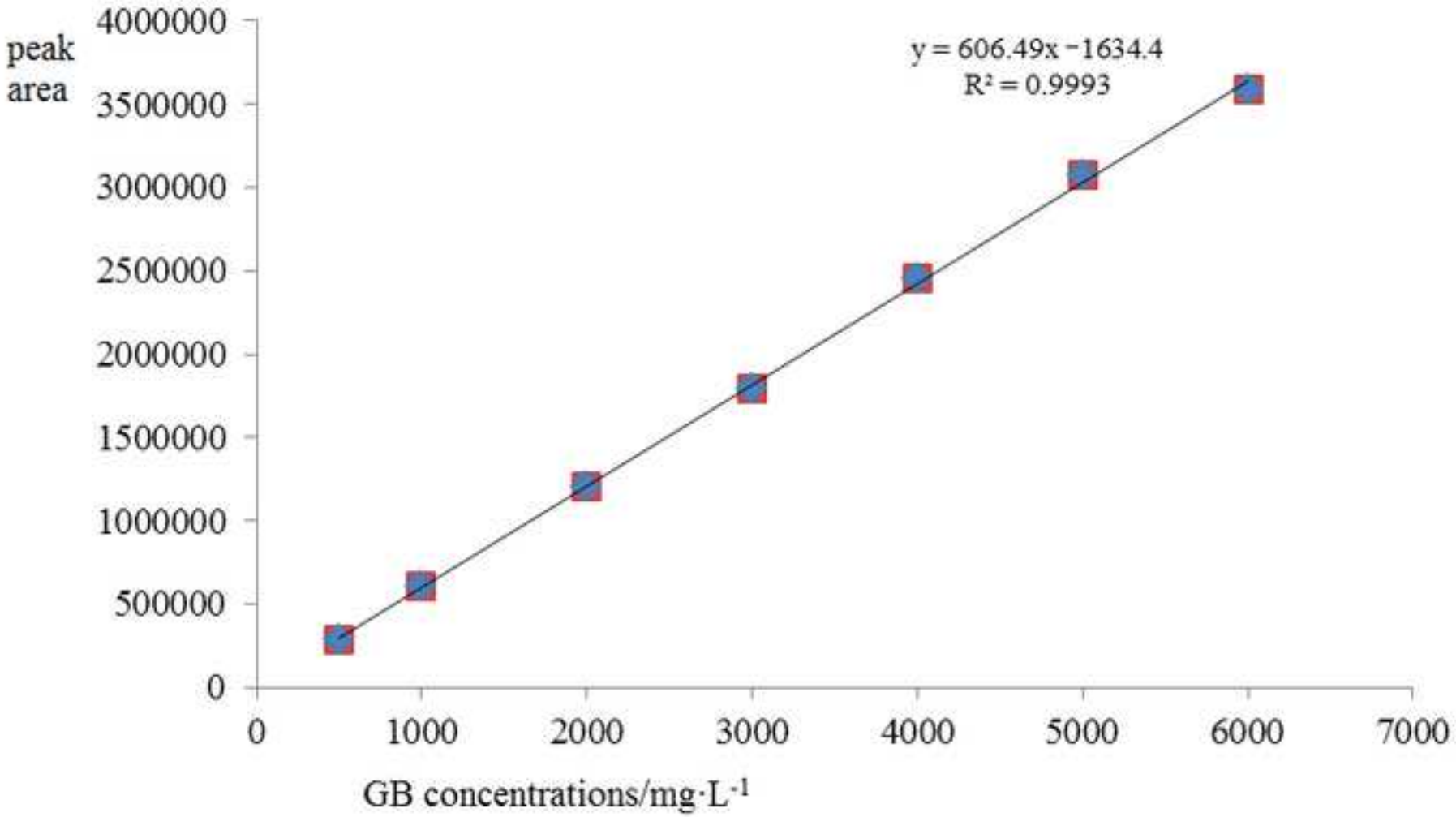

Figure 3

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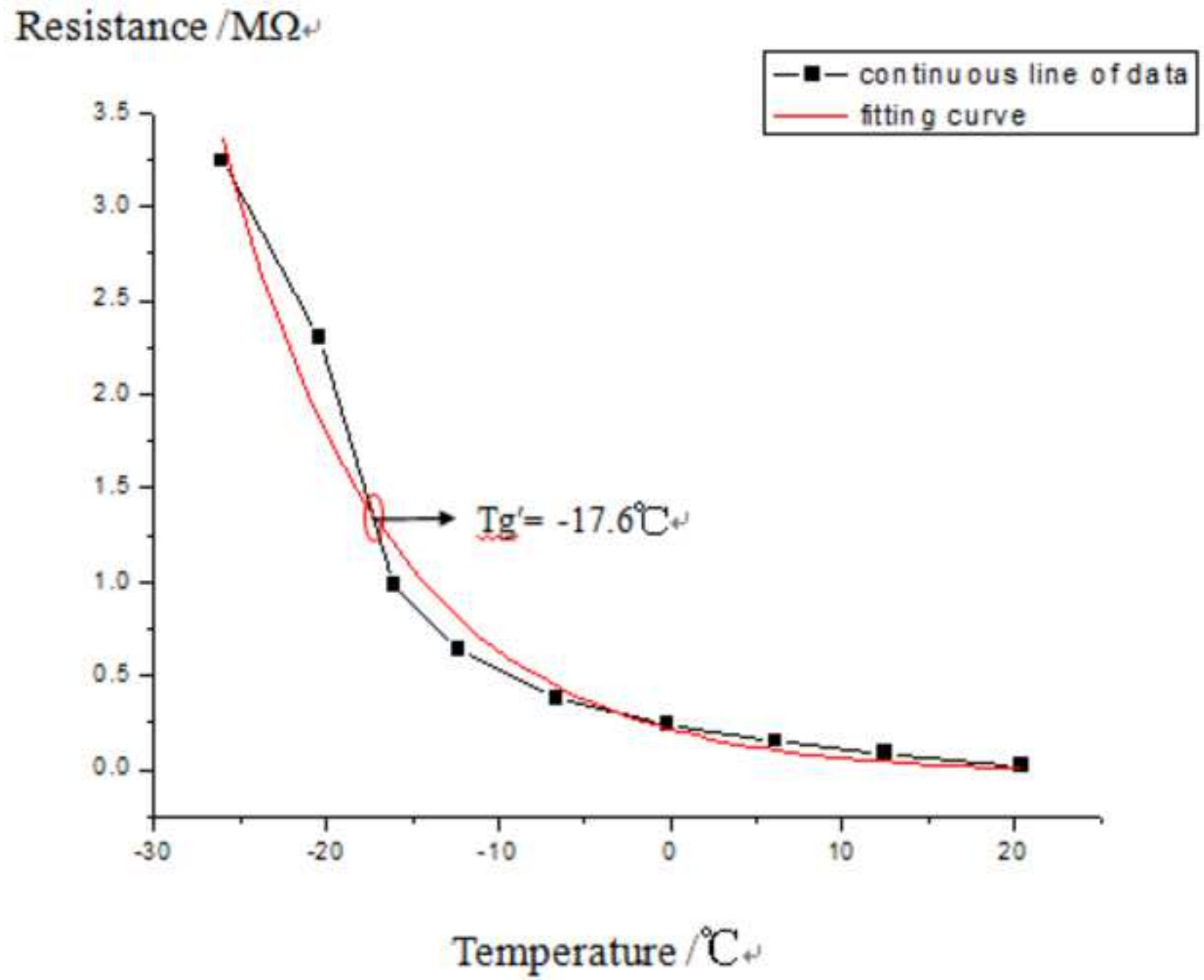

Figure 5-a

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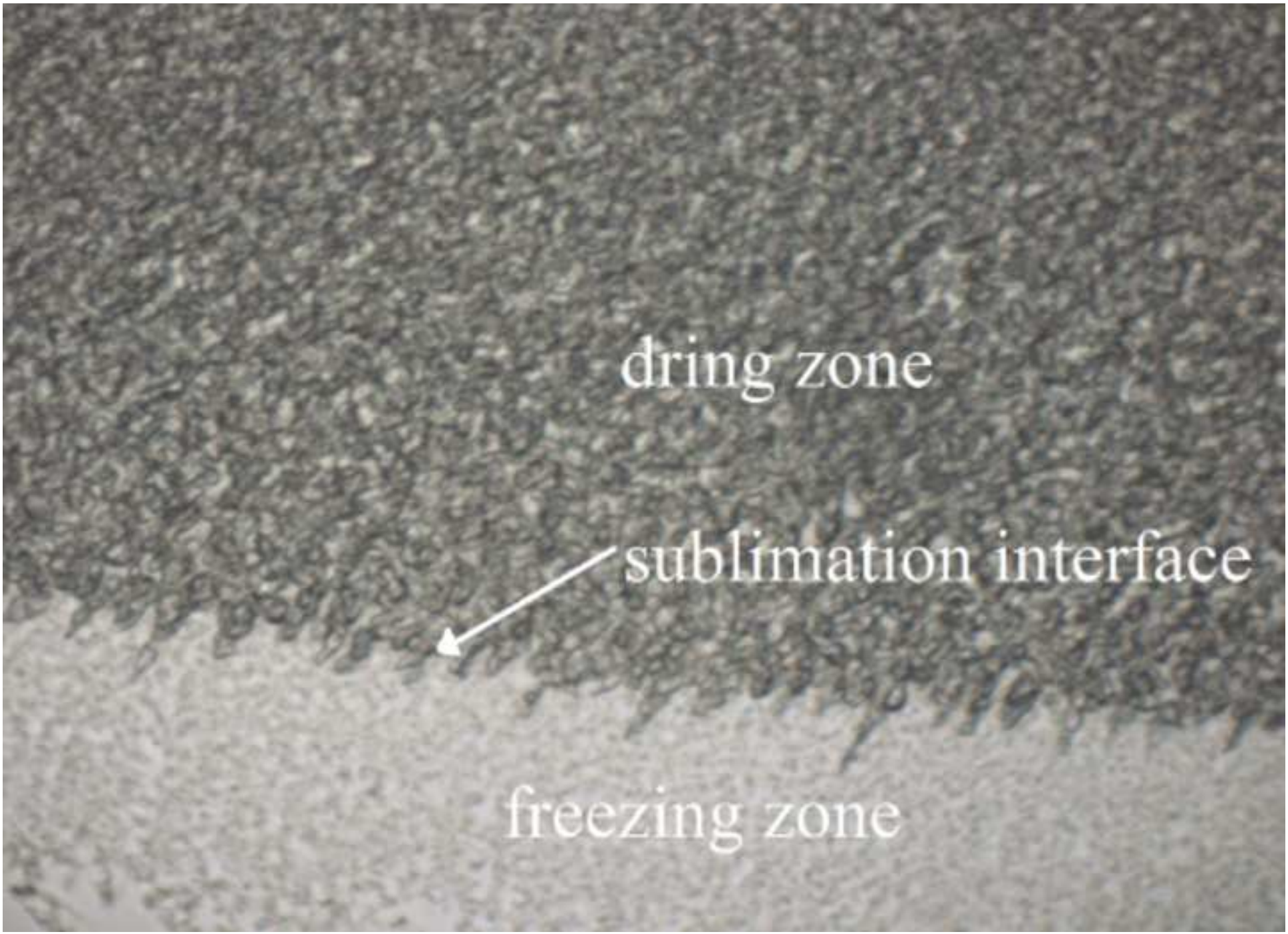

Figure 5-b

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