Ginkgolide Injection and Preparation Method thereof

Field of the Invention

The invention relates to a preparation method of a traditional Chinese preparation, in particular to a ginkgolide injection and a preparation method thereof, and belongs to the field of drugs.

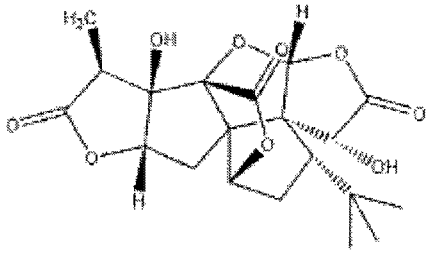
Description of the Related Art

Since 1960s, a lot of countries have adopted the modern separation technology to study chemical components of ginkgo leaves. Pharmacological experiments and clinical verification reveal that multiple biological activities of the ginkgo leaves are related to specific chemical components contained therein. Dr. Willar Schwabe from Germany first registered a simple extract of ginkgo leaves, and applied for patents (W Schwabe DE 176708 and DE 2117429) with name of EGb761 in 1972. The extract is indicated for cardiovascular and cerebrovascular diseases and nervous system diseases, showing significant effect and zero side effect. Ginkgolids can strengthen antagonism of platelet-activating factor. Ginkgo preparations are listed as therapeutic drugs in Germany, France and China, used as health food or over-the-counter drugs in other countries. Ginkgo health food developed in USA has been approved by Food and Drug Administration (FDA).

Ginkgolide is a terpenoid and called as terpene lactone, and consists of sesquiterpene lactone and diterpenoid. Furthermore, ginkgolide is an important active component in ginkgo leaves. Bilobalide is sesquiterpene lactone, was obtained by R.T.Major in 1967 and K.Weinges in 1969 through separation. At present, bilobalide is the only sesquiterpene lactone compound found in ginkgo leaves. Ginkgolids A, B, C, M and J are diterpenoid compounds, were separated from ginkgo leaves by S.Furukawa for the first time in 1932, and were further separated by K.Nakanish, M.Maruyama, K.Okabe et al in 1967, and chemical structures thereof were also determined at that time. Structurally, molecular skeleton of bilobalide is composed of 15 carbon atoms and has 4 mutually fused five-membered rings, including one five-membered carbon ring and three five-membered lactone carbon rings. Tertiary butyl rare in a natural product is connected on the five-membered rings. Bilobalide has very strong biological activity, and plays the role of promoting nerve growth. Bilobalide can prevent functional change caused by oxidative stress of brain cell mitochondria, improve memory function of the older people, prevent dementia and demyelination of brain and spinal nerves. The neurotrophic and neuroprotective effect of bilobalide are stronger than that of ginkgolide. Ginkgolide B can prevent inflammation and shock, protect heart and cerebral vessels, treat acute pancreatitis, etc. The molecular skeleton of the ginkgolid compounds is composed of 20 carbon atoms and has 6 five-membered rings, including two five-membered carbon rings, three five-membered lactone rings and one tetrahydrofuran ring. Two five-membered carbon rings are connected together in the form of a threaded ring, while the remaining rings are fused together to form a special 3D cage-shaped chemical structure. All ginkgolide molecules have tertiary butyl rare in natural products. Ginkgolide includes diterpenoid and sesquiterpene lactone. The diterpene lactone mainly comprises ginkgolides A, B, C, J, M, while the sesquiterpene lactone comprises bilobalide.

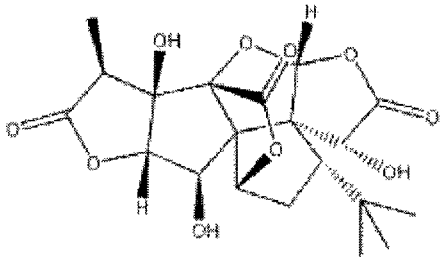
Since the platelet-activating factor was found in 1970s, pharmacologists have studied ginkgolide and acknowledged that ginkgolide is a platelet-activating factor antagonist, can protect immune system, central nervous system and ischemia injury; and can prevent shock,

irritability and inflammation. The structural differences among ginkgolides A, B, C, M, and J lie in different numbers of hydroxyls therein and different connecting positions of hydroxyls. Ginkgolide is platelet-activating factor antagonist, and is a key component with special biological activity in ginkgo leaves.



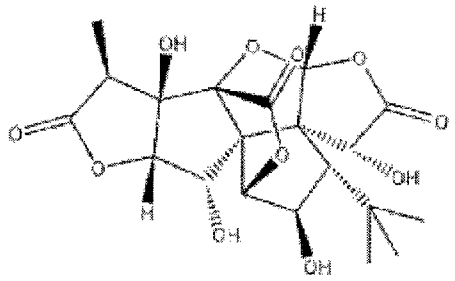
Structural formula of ginkgolide A

Molecular formula: $C_{20}H_{24}O_9$; molecular weight: 408.4



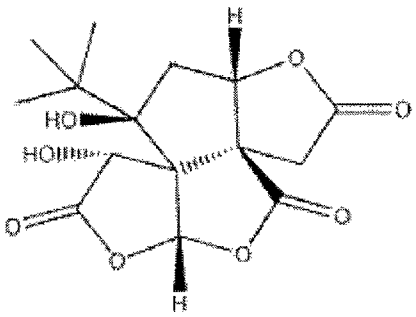
Structural formula of ginkgolide B

Molecular formula: $C_{20}H_{24}O_{10}$; molecular weight: 424.4



Structural formula of ginkgolide C

Molecular formula: $C_{20}H_{24}O_{11}$; molecular weight: 440.4



Structural formula of bilobalide

Molecular formula: $C_{15}H_{18}O_8$; molecular weight: 326.3

Ginkgolide has strong specific inhibitory action on PAF receptors, and has the highest activity against PAF. PAF is an endogenous phospholipid produced by secretion of platelets and multiple inflammatory tissues, and is the most effective platelet aggregation inducer discovered to the date, and closely linked to occurrence and development of a lot of diseases. Ginkgolide is currently considered as the most clinically promising natural PAF receptor antagonist, with antagonistic activity closely linked with chemical structure thereof. When R3 in lactone structure is hydroxyl or the number of hydroxyls increases, the antagonistic activity toward PAF reduces. When R2 is hydroxyl and R3 is H, the activity increases largely, and ginkgolide B has the strongest antagonism against PAF.

Many methods are available for extracting and purifying ginkgolide, mainly comprising solvent extraction method, column extraction method, solvent extraction-column extraction method, supercritical extraction method, purification method by chromatograph or column chromatography. As high-content ginkgolide composition can not be effectively separated by the methods and component ratio of ginkgolide is not clear, the clinical effect is different. Furthermore, ginkgolide has certain safety risks due to low content. Complete

pharmacological, toxicological and clinical trial data are also absent, so the methods are at test stage and not implemented in pharmaceutical manufacture. Although ginkgolide injection patents are reported in China, the components thereof are different from those of the invention. Retrieval on multiple official websites of ICH member states shows that other commercially available ginkgolide injection product is unavailable so far. At present, HPLC-UV method, HPLC-MS method and HPLC-ELSD method are often used for determining ginkgolide components. The methods can be only used to determine the content of each component of ginkgolide, and cannot truly reflect quality characteristics of drugs due to shortage of strict control over harmful substances in ginkgolide products. Therefore, a complete drug quality control system is not available.

In other invention patents, meglumine or sodium hydroxide is used as a cosolvent, and ginkgolide has a unique five-membered ring structure, which may lead to ginkgolide's ring opening, affecting drug efficacy, thus bringing great safety risk to clinical application unavoidably. The invention is to provide a ginkgolide injection with good manufacturing process and strict quality control method.

Summary of the Invention

As ginkgolide is a liposoluble substance and practically insoluble in water, highly safe pharmaceutical excipients (ethanol and glycerol) are used as cosolvents of the invention, and ratio of the cosolvents is strictly controlled at 1:1 to 5:1 with structure of ginkgolide unchanged, which effectively solves the solubility problem of drugs. Using citric acid or hydrochloric acid as a pH regulator can further stabilize drugs, thus avoiding recrystallization during drug placement from causing unacceptable clarity and insoluble particles, and solving the safety hazard of clinical application.

A technical problem to be solved by the invention is to provide a ginkgolide injection with advantages of clear components and fixed ratio. Furthermore, the injection is effectively free of macromolecule and protein, which avoids adverse reaction. Ginkgolide with high purity, clear components and fixed ratio is used as the active component of the injection, and contents of effective parts of the ginkgolide injection are more than 95%, comprising four components namely 25.0% to 50.0% of bilobalide ($C_{15}H_{18}O_8$), 20.0% to 45.0% of ginkgolide A ($C_{20}H_{24}O_9$), 10.0% to 30.0% of ginkgolide B ($C_{20}H_{24}O_{10}$) and 5.0% to 15.0% of ginkgolide C ($C_{20}H_{24}O_{11}$).

The ginkgolide injection of the invention comprises the following components:

1 to 10mg/ml (preferably 5mg/ml) of ginkgolide (counted based on terpene lactone);

0.2 to 0.5ml/ml (preferably 0.4ml/ml) of glycerol;

0.4 to 0.7ml/ml (preferably0.6ml/ml) of ethanol; and

0 to 0.5ml/ml (preferably 0ml/ml) of water for injection.

Two circumstances are essentially present in the formula:

1 When amount of the water for injection is 0, glycerol and ethanol are used as cosolvents.

The volume ratio of the cosolvents is strictly controlled at 1:1 to 5:1, that is, the volume ratio of the ethanol and the glycerol is 1 - 5:1 by parts.

The formula is preferably as follows:

10g of ginkgolide (counted based on terpene lactone);

800ml glycerol;

ethanol added to 2000ml

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to prepare into 1000 tubes.

That is, the ginkgolide injection of the invention comprises the following components:

5mg/ml of ginkgolide (counted based on terpene lactone);

0.4ml/ml of glycerol; and

0.6ml/ml of ethanol.

2 When amount of the water for injection is not 0, glycerol and ethanol are used as the cosolvents.

The volume ratio of the cosolvents is strictly controlled at 1:1 to 5:1, that is, the volume ratio of the ethanol and the glycerol is 1 - 5:1 by parts.

The formula is preferably as follows:

10g ginkgolide (counted based on terpene lactone);

400ml glycerol;

1200ml ethanol; and

water for injection added to 2000ml

to prepare into 1000 tubes.

That is, the ginkgolide injection of the invention contains the following components per milliliter:

5mg/ml of ginkgolide (counted based on terpene lactone);

0.2ml/ml of glycerol;

0.6ml/ml of ethanol; and

0.2ml/ml of water for injection.

Ethanol, glycerol and water for injection are properly intersoluble. Mixed solution thereof has good stability, can keep the original chemical structures of drugs as a solvent, and facilitate absorption by human body to play the curative effects of drugs. Ginkgolide

is well soluble in ethanol, but only using ethanol as the solvent for injection largely irritates human bodies, therefore, glycerol and a small amount of water for injection are used to dilute ethanol, which can effectively alleviate pains during intravenous injection and tolerance of human bodies against ethanol without changing solubility of the solution toward drugs and stability of the solution.

A preparation method of the ginkgolide injection comprises the following steps:

a) Preparation: mixing ethanol and glycerol (ratio of cosolvents is required to be 1:1 to 5:1); adding ginkgolide, stirring for dissolving, supplementing ethanol or water for injection to full volume, and adjusting pH value to 2.5 to 6.5 (preferably 3.2 to 3.8) with 5 to 10% citric acid solution or 1 to 10% hydrochloric acid solution.

b) Filtering for sterilization;

c) Filling and sealing; and

d) Sterilization.

A quality control method of the injection of the invention comprises:

character: colorless clear liquid.

pH value: 3.0 to 4.5, preferably 3.2 to 3.8

solution color: lighter than No. 4 yellow standard colorimetric solution (Chinese Pharmacopoeia).

tannin, resin, oxalate, potassium ion: not detected;

undue toxicity: the injection is prepared into a solution containing 0.2mg per 1ml, and the solution conforms to administration by intravenous injection;

pyrogen: acceptable result is obtained by injecting 5ml per 1kg of body weight of a rabbit (Chinese Pharmacopoeia).

hemolysis and coagulation: acceptable test results are obtained by adding 6ml injection of the invention to 100ml 0.9% sodium chloride injection for diluting (Chinese Pharmacopoeia).

Allergic test: 0.9% sodium chloride injection is added to the injection of the invention to dilute to 0.4mg/ml counted based on terpene lactone, and allergic reaction should be not present within 30min after intravenous injection of the test solution.

Irritation test: 0.9% sodium chloride injection is added to the injection of the invention to dilute to 0.5mg/ml solution; 3 rabbits regardless of gender are weighed; 10ml/kg test solution is intravenously dripped into the right ear edge of each rabbit every day, and equal volume of 0.9% sodium chloride is intravenously dripped into the left ear edge of each rabbit for three times totally. The vessels at the injection sites are observed for congestion, bleeding, necrosis and other irritant symptoms with eyes before administration, 1 and 6 hours after administration and 48 hours after the final administration. Rabbits are killed by bleeding of carotid arteries 48 hours after the final injection, and 2cm ear edge is cut at the place 1cm from the proximal part at the dripping site; the ear edge is fixed by FAA liquid and conventionally dehydrated, sections thereof are embedded by paraffin and HE stained, and placed under an optical microscope for pathological examination of vascular tissues to judge irritability. The intravenous

dripping test solution should not present irritant reaction of vessels.

Total ginkgolic acid: the total content of the ginkgolic acid is not more than 1ppm by external standard method for ginkgoneolic acid control when chromatographic column is C18, mobile phase is methanol:1% glacial acetic acid (90:10), detection wavelength is 310nm.

Fingerprint: the similarity of four common peaks and the reference fingerprint is more than 0.95 based on the similarity evaluation system for chromatographic fingerprints of traditional Chinese medicines using bilobalide control, ginkgolide A control, ginkgolide B control and ginkgolide C control as references when chromatographic column is C18, mobile phase is methanol-tetrahydrofuran-water (25:10:65), temperature of a drift tube of an evaporative light-scattering detector is 105℃, flow rate of a carrier gas is 3.00L/min, and column temperature is 40℃.

Sterility: 0.9% sterile sodium chloride solution is added to dilute by the membrane filtration method, and a filter membrane is flushed with 0.9% sterile sodium chloride solution for three times, achieving acceptable sterility (Chinese Pharmacopoeia).

Content: when chromatographic column is C18, mobile phase is methanol-tetrahydrofuran-water (25:10:65), temperature of a drift tube of an evaporative light-scattering detector is 105℃; flow rate of a carrier gas is 3.00L/min, and column temperature is 40℃, the content is determined after absorbing the sample with an Extrelut-20 column, and the content of terpene lactones per 1ml is determined to be 4.5 - 5.5mg based on total amount of the bilobalide ($C_{15}H_{18}O_8$), the ginkgolide A ($C_{20}H_{24}O_9$), the ginkgolide B ($C_{20}H_{24}O_{10}$) and the ginkgolide C ($C_{20}H_{24}O_{11}$).

The ginkgolide injection has effects of promoting blood circulation to remove blood stasis and clearing and activating the channels and collaterals. The ginkgolide injection is indicated for syndrome of static blood blocking collaterals occurring in recovery period of apoplexy involving the channels and collaterals (mild-to-moderate cerebral infarction) manifested by such symptoms as hemiplegia, distortion of mouth and tongue, stiff tongue and numbness of limb.

Ginkgolide with high purity, clear components and fixed ratio is used for the injection of the invention, and the contents of effective parts of the ginkgolide are more than 95%, four components thereof comprises 25.0% to 50.0% of bilobalide ($C_{15}H_{18}O_8$), 20.0% to 45.0% of ginkgolide A ($C_{20}H_{24}O_9$), 10.0% to 30.0% of ginkgolide B ($C_{20}H_{24}O_{10}$) and 5.0% to 15.0% of ginkgolide C ($C_{20}H_{24}O_{11}$), and the content of the total ginkgolic acid is less than 5ppm, meeting the requirements of European Union. The whole production process thereof ensures stable ratio and clinical effect of terpene lactone, and the gel chromatography and HPLC-MS technology are applied to the detection field, thus solving the residue problem of macromolecular substances, polymers and protein in the conventional production process of traditional Chinese medicine injections, largely reducing occurrence of adverse reaction, and the similarity of four common peaks in the fingerprint is more than 0.95. As terpene lactone is insoluble in water, effective cosolvents (mixed solvent of ethanol and glycerol) are screened, which solves the technical difficulty of the conventional process, prevents use of other acidic and alkaline excipients from causing ring opening of ginkgolides and damage of the structure of the major components, thus further stabilizing the effective components and meeting sterility

requirements for the injection. For quality control indicators, product quality is controlled from multiple perspectives and in all-round manner, and a complete impurity control method is effectively established. Product quality is controlled by the fingerprint, undue toxicity, irrigation test, hemolysis and coagulation, allergic test and other most strict detection technologies for traditional Chinese medicine injections, which largely increases the safety of clinical application. These quality control technologies are far superior to the internationally known technologies for ginkgo leaf extract injections, and achieve the international advanced level.

The invention is a traditional Chinese medicine injection for effective parts, which effectively removes macromolecules and protein, and avoids occurrence of adverse reaction. Furthermore, the injection is characterized by low energy consumption, definite effect, simple operation, easy quality control and low production cost, and is safer than other ginkgo injections.

Brief Description of the Drawings

Figure 1 is the LC-MS graph of lot 1 ginkgolide injection (molecular weight: 400 to 1000).

Figure 2 is the LC-MS graph of lot 1 ginkgolide injection (molecular weight: 400 to 3000).

Figure 3 is the LC-MS graph of lot 2 ginkgolide injection (molecular weight: 400 to 1000).

Figure 4 is the LC-MS graph of lot 2 ginkgolide injection (molecular weight: 400 to 3000).

Figure 5 is the LC-MS graph of lot 3 ginkgolide injection (molecular weight: 400 to 1000).

Figure 6 is the LC-MS graph of lot 3 ginkgolide injection (molecular weight: 400 to 3000).

Figure 7 is the linear relation graph of ginkgoneolic acid.

Figure 8 is the reference fingerprint of ginkgolide injection; including peak 2 for ginkgolide C, peak 3 for bilobalide, peak 4 for ginkgolide A and peak 5 for ginkgolide B in common peaks.

Description of the Preferred Embodiments

Ginkgolide is raw material of the invention and contains 25.0% to 50.0% of bilobalide ($C_{15}H_{18}O_8$), 20.0% to 45.0% of ginkgolide A ($C_{20}H_{24}O_9$), 10.0% to 30.0% of ginkgolide B ($C_{20}H_{24}O_{10}$) and 5.0% to 15.0% of ginkgolide C ($C_{20}H_{24}O_{11}$), and the total amount of the bilobalide, the ginkgolide A, the ginkgolide B and the ginkgolide C is more than 95%.

The basis for selecting cosolvent and pH regulator comprises the following steps.

I Selection of the cosolvent: dosage ratio of the solvent and the cosolvent was selected according to properties of raw materials and auxiliary materials and preparation process requirements of the injection.

Test 1: To 1,200ml ethanol and 800ml glycerol evenly mixed, 10g ginkgolide was added and stirred for dissolving, and pH of the resulting mixture was adjusted to 3.2 to 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Test 2: To 400ml ethanol and 1,600ml glycerol evenly mixed, 10g ginkgolide was added and stirred for dissolving, and pH of the resulting mixture was

adjusted to 3.2 to 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Test 3: To 300ml ethanol and 1,700ml glycerol evenly mixed, 10g ginkgolide was added and stirred for dissolving, and pH of the resulting mixture was adjusted to 3.2 to 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Test 4: To 1,200ml ethanol and 400ml glycerol evenly mixed, 10g ginkgolide was added and stirred for dissolving, water for injection was added to 2,000ml, and pH of the resulting mixture was adjusted to 3.2 to 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Test 5: To 400ml ethanol and 1,000ml glycerol evenly mixed, 10g ginkgolide was added and stirred for dissolving, water for injection was added to 2,000ml, and pH of the resulting mixture was adjusted to 3.2 to 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

The samples experienced cryogenic freezing-thawing test cycles for three times to observe character, insoluble particles and pH change of the solution and observe for crystal precipitation after the test. See Table 1 for test results.

Table 1 Screening test results of the cosolvent

Test name	Test 1	Test 2	Test 3	Test 4	Test 5
State of solution at preparation	Quickly dissolving after stirring	Quickly dissolving after stirring	Slowly dissolving after stirring	Quickly dissolving after stirring	Slowly dissolving after stirring
Appearance character after test	Colorless clear liquid, no crystal precipitation.	Colorless clear liquid, no crystal precipitation.	Colorless clear liquid, certain crystal precipitation.	Colorless clear liquid, no crystal precipitation.	Colorless clear liquid, certain crystal precipitation.
Insoluble particles	Compliant	Compliant	Non-compliant	Compliant	Non-compliant
pH of the solution	Basically unchanged	Slightly changed	An increase of 0.5	Basically unchanged	An increase of 0.6

The volume ratio of the ethanol to the glycerol in the injection was 1:1 to 5:1 when water for injection was absent, and the optimal ratio was 3:2. When water for injection was present, the optimal ratio was 4:1. The greater the ratio of the ethanol was, the faster the ginkgolide dissolved. After the injection experienced the freezing-thawing test, pH, insoluble particles and appearance character thereof conformed to the requirements of Chinese Pharmacopeia.

II Selection of pH regulator: As the ginkgolide is relatively stable under weak acidic conditions, a pH regulator was screened by 5% to 10% citric acid solution or 1% to 10% hydrochloric acid solution respectively.

Test 1: To 1,200ml ethanol and 800ml glycerol evenly mixed, 10g ginkgolide was

added and stirred for dissolving, and pH of the resulting mixture was adjusted to 3.2 to 3.8 with 5% hydrochloric acid. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Test 2: To 400ml ethanol and 1,600ml glycerol evenly mixed, 10g ginkgolide was added and stirred for dissolving, and pH of the resulting mixture was adjusted to 3.2 to 3.8 with 10% hydrochloric acid. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Test 3: To 1,200ml ethanol and 400ml glycerol evenly mixed, 10g ginkgolide was added and stirred for dissolving, water for injection was added to 2,000ml, and pH of the resulting mixture was adjusted to 3.2 to 3.8 with 10% citric acid solution. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Test 4: To 1,200ml ethanol and 800ml glycerol evenly mixed, 10g ginkgolide was added and stirred for dissolving, and pH of the resulting mixture was adjusted to 3.2 to 3.8 with 10% citric acid solution. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

In order to observe the character, insoluble particles and pH change of the solution before and after sterilization, the sample was stored at low temperature (0 to 6 □) for 3 days to observe for crystal precipitation under cryogenic conditions. See Table 2 for test results.

Table 2 Screening test results of the pH regulator

Test name	Test 1	Test 2	Test 3	Test 4
Appearance character	Colorless clear liquid, no crystal precipitation.	Colorless clear liquid, no crystal precipitation.	Colorless clear liquid, no crystal precipitation.	Colorless clear liquid, no crystal precipitation.
Insoluble particles	Compliant	Compliant	Compliant	Compliant
pH of the solution after sterilization	An increase of 0.2	An increase of 0.3	Basically unchanged	Basically unchanged

The screening tests of the pH regulator show 5% to 10% citric acid solution or 1% to 10% hydrochloric acid solution can be used as the pH regulator of the product. After pH adjustment with the hydrochloric acid solution, pH increased slightly but showed a little change after sterilization. Citric acid is weak acid and more stable to adjust pH. Hydrochloric acid and citric acid can be used as the pH regulator of the solution.

The inventor studied and described the contents of the invention to demonstrate technical effects of the invention. The following tests are for further describing and explaining the invention but not limitation thereto.

Test example 1 Ginkgolide quality control - assay

Chromatographic condition and system suitability test: octadecylsilane

chemically bonded silica was used as a filler, methanol-tetrahydrofuran-water (25:10:65) was used as mobile phase, and an evaporative light-scattering detector was used, a drift tube was 105□, flow rate of a carrier gas was 3.00L/mim, and column temperature was 40□. Under the conditions, the number of theoretical plates should be not less than 2,500 counted based on bilobalide peak. The resolution of the bilobalide peak and the ginkgolide C peak should be more than 1.5.

Preparation of control solution: appropriate amount of bilobalide control, ginkgolide A control, ginkgolide B control and ginkgolide C control were precisely weighed, to which methanol was added for dissolving; the resulting mixtures were quantitatively diluted to mixed solutions containing 0.15mg, 0.12mg, 0.1mg and 0.lmg respectively per 1ml, and shaken up.

Preparation of test solution: 6mg ginkgolide was precisely weighed and placed in a 10ml volumetric flask, to which lml methanol was added for dissolving; and mobile phase was added to dilute the resulting mixture to volume, and shaken up.

Assay method: 10μl control solution, 20μl control solution and 10 to 20μl test solution were precisely pipetted and injected into high performance liquid chromatograph to record the chromatograms and calculate contents of bilobalide, ginkgolide A, ginkgolide B and ginkgolide C by two-point external standard method logarithmic equation.

Counted on a dry basis, the contents of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$) and ginkgolide C ($C_{20}H_{24}O_{11}$) were 25.0% to 50.0%, 20.0% to 45.0%, 10.0% to 30.0% and 5.0% to 15.0% respectively; and the total amount of the bilobalide, ginkgolide A, ginkgolide B and ginkgolide C was not less than 95.0%.

Test example 2 Quality control of ginkgolide injection - test of related substances

(1) Protein: 2ml injection of the invention was prepared into 50ml with water which was taken as test solution. About 50mg coomassie brilliant blue G-250 was weighed and dissolved in 25ml ethanol, to which 50ml 85% (w/v) phosphoric acid was added and diluted with water to 500ml, shaken up and filtered. And 5ml filtrate was precisely measured and transferred to a test tube, to which 1ml test stock soultion was added, shaken up and placed for 3min. A blank solution was prepared by the same method, and absorbance thereof was determined at wavelength of 595nm. The absorbance of the test solution was less than 0.05.

(2) Tannin: after one drop of dilute acetic acid and five drops of gelatin sodium chloride test solution were added to 1ml injection of the invention, shaken up and placed for 10min, turbidity or precipitation was not observed.

(3) Resin: after one drop of hydrochloric acid was added to 5ml injection of the invention and placed for 30min, resinous substance was not precipitated.

(4) Oxalate: pH value of 2ml injection of the invention was adjusted to 1 to 2 with dilute hydrochloric acid, the resulting solution was filtered, and pH value of the filtrate was adjusted to 5 to 6 with ammonia, and 3 drops of calcium chloride solution were added to the filtrate and placed for 10min. Then, turbidity or precipitation was not observed.

(5) Potassium ion: 2ml injection of the invention was placed in a 10ml Nessler tube and diluted to 10ml with 0.6ml alkaline formaldehyde solution, and 2 drops of 3% EDTA solution, 0.5ml 3% sodium tetraphenylborate solution and water. Additionally, 0.8ml standard potassium chloride solution was taken and tested by the same method. Turbidity of the test solution was less than that of the control solution.

Test example 3 Quality control of ginkgolide injection - undue toxicity test

Preparation of test solution: injection of the invention and sodium chloride injection were prepared into a solution containing 0.2mg per lml.

Test method: 0.5ml test solution was injected into caudal veins of 5 mice weighing 17 to 20g respectively, and no mice died within 48h.

Test example 4 Quality control of ginkgolide injection - pyrogen test

Preparation of test solution: 2ml injection of the invention was added to 50ml 0.9% sodium chloride injection, and shaken up.

Test method: within 15min after normothermia of 3 rabbits was determined, 5ml test solution was slowly injected into ear veins per 1kg body weight. Body temperature was determined every 30min and for 6 times in total. Body temperature increase was less than 0.6□, and the sum of body temperature increases of 3 rabbits was less than 1.3□.

Test example 5 Quality control of ginkgolide injection - hemolysis and coagulation test

Preparation of test solution: 6ml injection of the invention was added to 100ml 0.9% sodium chloride injection, and shaken up.

Test method: 5 clean glass tubes were numbered. No. 1 and No. 2 tubes were taken as test tubes, No. 3 tube was taken as negative control tube, No. 4 tube was taken as positive control tube, and No. 5 tube was taken as test control tube. As shown in Table 3, 2% red cell suspension, 0.9% sodium chloride solution and distilled water were successively added to the tubes, shaken up and immediately placed in a thermostat at 37□ ± 0.5□ for incubation.

See Table 3 for addition amounts of test solutions.

Table 3 Addition amounts of hemolysis and coagulation test solutions

Tube No.	1	2	3	4	5
2% red cell suspension/ml	2.5	2.5	2.5	2.5	
0.9% sodium chloride solution/ml	2.2	2.2	2.5		4.7
Distilled water/ml				2.5	
Test solution/ml	0.3	0.3			0.3

If the solution in the tubes appeared bright red and no cell or only a small amount of red blood cells remained at the bottom, it indicated that hemolysis may occur.

If red blood cells sank without exception and supernatant was colorless and clear, or supernatant was colored and clear, but obvious difference was not observed in No. 1, No. 2 and No. 5 tubes, it indicated that no hemolysis occurred. If the solution had red brown or umber floc which can not disperse after being inverted gently for 3 times, it indicated that red blood cell coagulation may occur.

Hemolysis and condensation reaction was not observed after 3h.

Test example 6 Quality control of ginkgolide injection – test of macromolecular substances and polymers

a. Test apparatus and reagent

Agilent 1200 HPLC, ultraviolet detector, differential refraction detector and Phenomenex BioSep-SEC-S2000 gel chromatographic column.

Dextran control D2000 (Dextran blue 2000), from National Institute for the Control of Pharmaceutical and Biological Products, lot No.: 140646-2000-01

Glucose control (DO), content: 99.5%; lot No.: 086K0166, SIGMA, and ultrapure water prepared by Millipore-Q ultrapure water system.

Other reagents were analytically pure.

Sample source: ginkgolide injection; lot No.: 100501, 100502 and 100503; specification: 2ml.

b. Selection of mobile phase

0.71% (containing 0.02% sodium azide) sodium sulfate solution was selected as the mobile phase.

c. Selection of the detector

Common differential refraction detector was selected as it could respond to substances with different refraction coefficients well.

d. Proposed chromatographic conditions

Chromatographic column: Phenomenex BioSep-SEC-S2000, 300x7.8mm, 5um

Mobile phase: 0.71% (containing 0.02% sodium azide) sodium sulfate solution

Column temperature: 35□; detector temperature: 35□; flow rate: 0.5ml/min

e. Molecular weight of ginkgolide

Ginkgolide	Ginkgolide	Ginkgolide B	Ginkgolide C	Bilobalide	Ginkgolide J

	A				
Molecular formula	$C_{20}H_{24}O_9$	$C_{20}H_{24}O_{10}$	$C_{20}H_{24}O_{11}$	$C_{15}H_{18}O_8$	$C_{20}H_{24}O_{10}$
Molecular weight	408.4	424.4	440.4	326.3	424.4

f. Methodological study

□ The mobile phase was added to dextran control and glucose control to prepare into 10mg/ml solution respectively, and 20μl control solution was precisely pipetted respectively and injected into a chromatograph to record the chromatogram. The results indicated that peak of dextran appeared at the retention time of 9.816' and peak of glucose appeared at the retention time of 18.712'. This means that peak of a substance with large molecular weight appear earlier than that of a substance with small molecular weight based on gel chromatography.

□ 2ml ginkgolide injection was added to 1ml dextran control solution (10mg/ml) and shaken up, and 10μl resulting solution was precisely pipetted and injected into a chromatograph to record the chromatogram. The results indicated that dextran was detected at the retention time of 9.816', component peaks of ginkgolide injection appeared after 18min, peak appeared at 18min for molecular weight of 180 to 450 and appeared at 9min for molecular weight of 5000 to 2000000. This means that it is feasible to detect macromolecular substances by gel chromatography.

In order to verify that the product does not contain macromolecules and polymers, LC-MS test was performed again.

a. Chromatographic conditions

Mobile phase: methanol-water (90:10), chromatographic column: Agilent RX-C_{18} (2.1x50mm)

Column temperature: 25□, flow rate: 0.3ml/min

b. Preparation of test solution

2ml ginkgolide injection of the invention was precisely measured and transferred to a 10ml volumetric flask, diluted with mobile phase to volume, and shaken up to prepare the test solution.

c LC-MS test

Based on the established test method, three batches of 10μl test solution were injected into a chromatograph to test molecular weight at the molecular weight of 400 to 1000 and 400 to 3000. See Table 4 for test results.

Table 4 Determination results of molecular weight by LC-MS

$[M+Na]^+$	419.1, 431.5, 447.4, 463.3, 475.7, 532.2, 588.8, 701.8
M	396.1, 408.5, 424.4, 440.3, 452.7, 509.2, 678.8

Based on determination results of molecular weight by LC-MS, molecular weights of effective components of the ginkgolide A (molecular weight of

408.5), ginkgolide B (molecular weight of 424.4) and ginkgolide C (molecular weight of 440.4) were completely consistent. As test molecular weight range was 400 - 1000 and 400 - 3000, molecular weight of bilobalide (326.3) was not tested. The injection of the invention did not contain substances with molecular weight more than 700, and other trace substances with low molecular weight had no impact on quality of the injection of the invention. Based on molecular weight test of different components of ginkgolide, the product does not contain macromolecules or polymers.

The molecular weight of three batches of ginkgolide injection was determined to be 400 to 1000 and 400 to 3000. See Figure 1 to 6 for LC-MS spectra.

Test example 7 Quality control of ginkgolide injection - test of total ginkgolic acid

Chromatographic conditions and system suitability test: octadecylsilane chemically bonded silica was used as a filler, and methanol:1% glacial acetic acid (90:10) was used as the mobile phase at flow rate of 1.0ml/min and detection wavelength of 310nm. The number of theoretical plates should be not less than 4000 counted based on ginkgoneolic acid peak.

Preparation of control solution: a proper amount of ginkgoneolic acid control was precisely weighed, and methanol was added to prepare a solution containing 5μg per 1ml as the control solution, and a proper amount of total ginkgolic acid control was precisely weighed, and methanol was added to prepare a solution containing 100μg per 1ml as the control solution for fixing.

Preparation of test solution: 1ml injection of the invention was diluted with water to 10ml, then the resulting mixture was shaken up, and extracted with n-hexane for 3 times (20ml, 15ml, 15ml), and n-hexane extracts were combined to obtain a mixture, the mixture was put in a water bath for drying by distillation, the resulting residue was dissolved in methanol and diluted to 2ml, then the resulting mixture was shaken up and used as the test solution.

Determination method: 20μl test solution, 20μl control solution and 20μl control solution for fixing were precisely pipetted to a liquid chromatograph to calculate the total peak area of corresponding chromatographic peak of the total ginkgolic acid control in the test solution, and the content of total ginkgolic acid was calculated by ginkgoneolic acid control external standard method. The content of total ginkgolic acid was lower than 1ppm.

Methodological study

a. Linear test

Preparation of control solution: methanol was added to ginkgoneolic acid control to prepare a solution containing 0.104mg ginkgoneolic acid per 1ml as control solution (1); and 0.5ml control solution (1) was precisely measured and transferred to a 100ml volumetric flask, diluted

with methanol to volume and shaken up, and the resulting product was used as control solution (2).

Determination method: 2.5μl, 5μl, 10μl and 20μl control solution (1) and 10μl control solution (2) were precisely measured respectively and injected into a liquid chromatograph to record the chromatograms under the chromatographic conditions. See Table 5 for test results.

Table 5 Linear test results of ginkgoneolic acid

Sample size (ng)	5.2	260	520	1040	2080
Peak area	2545	102162	213093	426512	843249

The sample size was used as the ordinate, and the peak area was used as the abscissa for linear regression to obtain the following regression equation: Y=0.0025X-0.6448r=0.9999.

The test results show that the sample size of ginkgoneolic acid is 5.2 - 2080ng, and the sample size and peak area are in a good linear relationship.

See Figure 7 for linear relation graph of ginkgoneolic acid.

b. Quantification and detection limit test

Determination method: 5μl control solution (2) and 2μl control solution (2) under linear test were precisely measured and injected into a liquid chromatograph to record the chromatograms and calculate quantification limit and detection limit of ginkgoneolic acid which were 2.24ng and 0.69ng respectively.

c. Precision test

Determination method: 20μl control solution (2) of ginkgoneolic acid was precisely measured and continuously injected for 5 times to record the chromatograms and calculate RSD value. See Table 6 for test results.

Table 6 Precision test results

No.	1	2	3	4	5
Peak area	4850	4874	4845	4873	4837

The RSD of peak area of the control solution was 0.35%, and the test results showed good precision.

d. Stability test

Determination method: 20μl control solution (2) of ginkgoneolic acid was precisely measured, injected into a liquid chromatograph respectively at 0h, 2h, 4h, 8h and 12h to record the chromatograms and calculate RSD value. See Table 7 for test results.

Table 7 Stability test results of control solution

Time (h)	0	2	4	8	12
Peak area	4861	4853	4865	4843	4870

The test results show that the control solution is stable within 12h.

e. Recovery test

Preparation of control solution: 5.03mg ginkgoneolic acid control was precisely weighed, transferred to a 10ml volumetric flask, diluted with methanol to volume and shaken up, then the resulting mixture was used as the control solution.

Preparation of test solution: 9 parts of injection of the invention were taken, 1.6ml control solution, 2.0ml control solution and 2.4ml control solution were added to every 3 parts respectively, then the resulting mixtures were shaken up, and extracted with n-hexane for 3 times (20ml, 15ml, 15ml), and n-hexane extracts were combined to obtain a mixture, the mixture was placed in a water bath for drying by distillation, then the resulting residue was dissolved with chloroform and quantitatively diluted to 2ml, then the diluent was shaken up and used as the test solution.

Determination method: 20μl control solution and 20μl test solution were precisely measured to record the chromatograms under the chromatographic conditions and calculate the recovery. See Table 8 for test results.

Table 8 Recovery test results

No.	Addition amount (μg)	Measured amount (μg)	Recovery (%)	Mean recovery (%)	RSD (%)
1	0.8046	0.780	96.95	98.04	1.41
2		0.797	98.97		
3		0.778	96.67		
4	1.006	1.007	100.05		
5		0.997	99.06		
6		1.001	99.46		
7	1.272	1.161	96.18		
8		1.173	97.16		
9		1.181	97.85		

The mean recovery is 98.04%, RSD is 1.41%, and the test results show that the method for determining ginkgolic acid limit is accurate and reliable.

Test example 8 Quality control of ginkgolide injection - fingerprint test

Chromatographic conditions and system suitability test: octadecylsilane chemically bonded silica was used as a filler and methanol-tetrahydrofuran-water (25:10:65) was used as mobile phase; an evaporative light-scattering detector was used with the drift tube at 105℃, the flow rate of a carrier gas was 3.00L/mim, and column temperature was 40℃. The number of theoretical plates should be not less than 2500 counted based on bilobalide peak under the conditions. The resolution of the bilobalide peak and the ginkgolide C peak should be more than 1.5.

Preparation of reference solutions: proper amounts of bilobalide control, ginkgolide A control, ginkgolide B control and ginkgolide C control were precisely weighed, to which methanol was added to prepare mixed solutions containing 0.15mg, 0.12mg, 0.1mg and 0.lmg per 1ml respectively, then the mixed solutions were shaken up and used as the reference solutions.

Preparation of test solution: 1ml injection of the invention was precisely measured, to which 14ml phosphate buffer solution (pH 6.5) was added to obtain a mixture, then the mixture was shaken up, injected into an Extrelut-20 column for adsorption for 15min, and eluted with 100ml ethyl acetate, then the resulting eluent was collected and placed in a water bath for drying by distillation, the resulting residue was dissolved with the mobile phase and transferred to a 10ml volumetric flask, the resulting mixture was diluted with the mobile phase to volume, shaken up, and filtered with 0.45μm microporous membrane, and the resulting filtrate was used as the test solution.

Determination method: 20μl reference solutions and 20μl test solution were precisely pipetted and injected into a liquid chromatograph to record the chromatograms at 60min.

The similarity of the test sample fingerprint and the reference fingerprint was calculated more than 0.95 based on *Similarity Evaluation System for Chromatographic Fingerprint of TCM.*

The inventor studied and described the contents of the invention to demonstrate technical effects of the invention. The following tests are for further describing and explaining the invention but not limitation thereto.

In the ginkgolide fingerprint, peak 2 was ginkgolide C, peak 3 was bilobalide, peak 4 was ginkgolide A and peak 5 was ginkgolide B. Four characteristic peaks of the injection of the invention were in one-to-one correspondence in the fingerprint. See Figure 8 for reference fingerprint of the ginkgolide injection.

The 2004 fingerprint software *Similarity Evaluation System for Chromatographic Fingerprint of TCM* (version A) designated by Chinese Pharmacopoeia Commission was used to generate reference fingerprint of 10 lots of ginkgolide injection, and then *Similarity Evaluation System for Chromatographic Fingerprint of TCM* (version B) was used to calculate the similarity of test sample fingerprint of 10 lots of ginkgolide and the reference fingerprint. See Table 9 for test results.

Table 9 Results of similarity of 10 lots of ginkgolide

Lot No.	Similarity
100501	0.991
100502	0.987
100503	0.995
100504	0.996
110201	0.993
110202	0.989

110203	0.992
110701	0.989
110702	0.993
110703	0.991

The similarity of four common peaks in the fingerprint of 10 lots of ginkgolide injection of the invention and the reference fingerprint was more than 0.95.

Test example 9 Quality control of ginkgolide injection - assay

Chromatographic conditions and system suitability test: octadecylsilane chemically bonded silica was used as a filler and methanol-tetrahydrofuran-water (25:10:65) was used as mobile phase; an evaporative light-scattering detector was used with the drift tube at 105℃, the flow rate of a carrier gas was 3.00L/mim, and column temperature was 40℃. The number of theoretical plates should be not less than 2500 counted based on bilobalide peak under the conditions. The resolution of the bilobalide peak and the ginkgolide C peak should be more than 1.5.

Preparation of control solutions: proper amounts of bilobalide control, ginkgolide A control, ginkgolide B control and ginkgolide C control were precisely weighed, to which methanol was added to prepare mixed solutions containing 0.15mg, 0.12mg, 0.1mg and 0.1mg per 1ml respectively, then the mixed solutions were shaken up and used as the control solutions.

Preparation of test solution: 1ml injection of the invention was precisely measured, to which 14ml phosphate buffer solution (pH 6.5) was added to obtain a mixture, then the mixture was shaken up, injected into an Extrelut-20 column for adsorption for 15min, and eluted with 100ml ethyl acetate at different times, then the resulting eluent was collected and placed in a water bath for drying by distillation, the resulting residue was dissolved with the mobile phase to obtain a mixture, then the mixture was transferred to a 10ml volumetric flask, diluted with the mobile phase to volume, shaken up, and filtered with 0.45μm microporous membrane, and the resulting filtrate was used as the test solution.

Assay method: 10μl control solution, 20μl control solution and 15μl test solution were precisely pipetted and injected into a high performance liquid chromatograph to record the chromatograms and calculate the contents of the bilobalide, the ginkgolide A, the ginkgolide B and the ginkgolide C by two-point external standard method logarithmic equation.

Every 1ml injection of the invention contains 1 - 10mg/ml ginkgo terpene lactone counted based on total amount of the bilobalide ($C_{15}H_{18}O_8$), the ginkgolide A ($C_{20}H_{24}O_9$), the ginkgolide B ($C_{20}H_{24}O_{10}$) and the ginkgolide C ($C_{20}H_{24}O_{11}$), preferably 4.25 - 5.75mg.

Examples for preparation of the injection of the invention are as follows.

Example 1

Formula:

10g ginkgolide (counted based on terpene lactone);

600ml of glycerol;

ethanol added to 2000ml

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to prepare into1000 tubes.

Glycerol and ethanol with the total amount of 80% were taken as per the formula and evenly mixed, then to the resulting mixture ginkgolide was added and stirred for dissolving, and ethanol was added to full volume and evenly stirred, then 1 - 10% hydrochloric acid was used to adjust pH to 3.2 - 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Example 2

Formula:

10g ginkgolide (counted based on terpene lactone);

800ml glycerol;

ethanol added to 2000ml

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to prepare into 1000 tubes.

Glycerol and ethanol with the total amount of 80% were taken as per the formula and evenly mixed, then to the resulting mixture ginkgolide was added and stirred for dissolving, ethanol was added to full volume and evenly stirred, then 5 - 10% citric acid was used to adjust pH to 3.2 - 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Example 3

Formula:

10g ginkgolide (counted based on terpene lactone);

1000ml glycerol;

ethanol added to 2000ml

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to prepare into 1000 tubes.

Glycerol and ethanol with the total amount of 80% were taken as per the formula and evenly mixed, then to the resulting mixture ginkgolide was added and stirred for dissolving, ethanol was added to full volume and evenly stirred, then 5 - 10% citric acid was used to adjust pH to 3.2 - 3.8. After satisfactory intermediate examination, the mixture was filtered to

obtain a clear solution which was hermetically packaged and sterilized.

Example 4

Formula:

10g ginkgolide (counted based on terpene lactone);

400ml glycerol;

1200ml ethanol;

water for injection added to 2000ml

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to prepare into 1000 tubes.

Ethanol and glycerol were taken as per the formula and evenly mixed, then to the resulting mixture ginkgolide was added and stirred for dissolving, water for injection was added to full volume and evenly stirred, then 5 - 10% citric acid was used to adjust pH to 3.2 - 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Example 5

Formula:

25g ginkgolide (counted based on terpene lactone);

1000ml glycerol;

3000ml ethanol;

water for injection added to 5000ml

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to prepare into 1000 tubes;

Ethanol and glycerol were taken as per the formula and evenly mixed, then to the resulting mixture ginkgolide was added and stirred for dissolving, water for injection was added to full volume and evenly stirred, then 1 - 10% hydrochloric acid was used to adjust pH to 3.2 - 3.8. After satisfactory intermediate examination, the mixture was filtered to obtain a clear solution which was hermetically packaged and sterilized.

Both the active component content and injection quality of the examples 1 to 5 conform to quality requirements for the ginkgolide injection of the invention.

Claims

1. A ginkgolide injection, characterized in that the injection comprises the following components:

1 - 10mg/ml of ginkgolide (counted based on terpene lactone);

0.2 - 0.5ml/ml of glycerol;

0.4 - 0.7ml/ml of ethanol; and

0 - 0.5ml/ml of water for injection.

2. The ginkgolide injection of claim 1, characterized in that the injection comprises the following components:

5mg/ml of ginkgolide (counted based on terpene lactone);

0.4ml/ml of glycerol; and

0.6ml/ml of ethanol.

3. The ginkgolide injection of claim 1, characterized in that the injection comprises the following components:

5mg/ml of ginkgolide (counted based on terpene lactone);

0.2ml/ml of glycerol;

0.6ml/ml of ethanol; and

0.2ml/ml of water for injection.

4. The ginkgolide injection of claim 1, characterized in that the volume ratio of the ethanol and the glycerol is 1 - 5:1 by parts.

5. The ginkgolide injection of any of claims 1 to 4, characterized in that the ginkgolide contains 25.0 - 50.0% of bilobalide, 20.0 - 45.0% of ginkgolide A, 10.0 - 30.0% of ginkgolide B and 5.0 - 15.0% of ginkgolide C counted on a dry basis; and the total amount of the bilobalide, the ginkgolide A, the ginkgolide B and the ginkgolide C is not less than 95.0%.

6. A preparation method of the ginkgolide injection of claim 1, characterized in that the injection is prepared from the following components:

1 - 10mg/ml of ginkgolide (counted based on terpene lactone);

0.2 - 0.5ml/ml of glycerol;

0.4 - 0.7ml/ml of ethanol; and

0 - 0.5ml/ml of water for injection;

by the following steps:

a) preparation: mixing the ethanol and the glycerol, adding the ginkgolide for dissolving, adding the ethanol or the water for injection to full volume, and adjusting pH value to 2.5 - 6.5 with 5 - 10% citric acid solution or 1 - 10% hydrochloric acid solution;

b) filtration for sterilization;

c) filling and sealing; and

d) sterilization.

7. The preparation method of the ginkgolide injection of claim 6, characterized in that the pH value is adjusted to 3.2 – 3.8 in the step a) preparation.

8. The preparation method of the ginkgolide injection of claim 6 or 7, characterized in that the volume ratio of the ethanol and the glycerol is 1 - 5:1 by parts in the step a) preparation.