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(54) **COMPOSITION FOR USE IN PREVENTION OR TREATMENT OF VASCULAR-RELATED DISEASES**

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(57) **ABSTRACT**

The present invention relates to a composition for use in prevention or treatment of a vascular-related disease, particularly used for angiogenesis inhibition, tumor growth inhibition or tumor metastasis inhibition, or immunostimulation, which comprises glutamic acid or derivatives thereof, preferably glutamic acid is anhydrous glutamic acid represented by Formula (1) or pyroglutamic acid, and a pharmaceutically acceptable carrier or an edible carrier, and a method of preventing or treating a vascular-related disease.

COMPOSITION FOR USE IN PREVENTION OR TREATMENT OF VASCULAR-RELATED DISEASES

[0001] The present application is based on Japanese Patent Application No. 2002-177774, Japanese Patent Application filed Apr. 7, 2003, and Japanese Patent Application No. 2003-131997, the entire contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The present invention relates to a composition for prevention and treatment of vascular-related diseases, and more particularly to a composition for prevention and treatment of vascular-related diseases used for angiogenesis inhibition, neoplasm depression or immunostimulation etc. which includes glutamic acid or derivative thereof, in particular anhydrous glutamic acid, pyroglutamic acid, or derivative thereof as active ingredient.

[0004] 2. Prior Art

[0005] A vascular-related disease includes, for example, propagation and transition of neoplasm, inflammation, rheumatoid arthritis, diabetic retinopathy, and psoriasis, and it is greatly related with angiogenesis and immune function. Angiogenesis is phenomenon of generating new capillary blood vessels in animal tissues or organs, in the process of which, vascular basement membrane is disassembled and attacked by protease, vascular endothelial cell is grown by migration and bonded to extra cellular matrix, vascular endothelial cell is differentiated, and vascular cavity is formed. In general, new blood vessels are formed and extended in childhood and growth period. However, when growth period is past, the occasion of angiogenesis in the body is limited. Angiogenesis is observed under normal physiology condition such as luteinization, ovulation, embryogenesis, and placentation, and also occurred in cure process of lesion and resealing process of inflammation. As described above, angiogenesis occurs in normal state and has an important role in restoration of tissues, but it is known that capillary increases in a lot of chronic diseases such as diabetes mellitus and that angiogenesis causes grave lesion to tissues.

[0006] Angiogenesis is involved in etiology and aggravation of case of various diseases. The diseases include enhancement and transition of malignant, diabetic retinopathy, neovascular glaucoma, inflammatory dermatosis, joint fluid rheumatism, osteoarthritis, atherosclerosis, and obstructive affection such as myocardial infarction.

[0007] For example, when malignant tumor multiplies, tumor cell induces vascular neogenesis for itself by angiogenesis promoter to get nourishment and oxygen which are necessary for propagation of tumor cell, and tumor cell further grows while getting nutrient through new blood vessels. Transition of tumor cell to other organ and site also induces angiogenesis, and tumor cell is carried by the bloodstream. In the case of diabetic retinopathy, capillary is clogged up because of the viscosity blood set up by diabetes mellitus and is affected, and bleeding and edema are produced in retina. When bleeding and edema are chronic, the retina falls short of oxygen and nourishment, and then newborn blood vessel originates on the retina or nervous system mammilla so that fiber tissue is formed circumferentially. The retina is pulled by means of the fiber tissue (retinal detachment) or the retinal blood vessel is occurred bleeding (vitreous hemorrhage), and then serious visual handicap or blindness is occurred before long.

[0008] As described above, because angiogenesis is deeply involved in the onset and development of various diseases, a lot of searches of materials inhibiting angiogenesis have been done hitherto and investigation is pushed forward at the present zealously as an aim in a treatment or prevention of these diseases. As material and drug with action inhibiting angiogenesis, sulfation polysaccharide (Japanese Patent Laid-Open No. S63-119500 bulletin), trafermin, heparin and steroid (U.S. Pat. No. 4,994,443 specification; or U.S. Pat. No. 5,001,116 specification), ascorbic acid ether and this related-compound (Japanese Patent Laid-Open No. S58-131978 bulletin), interferon alpha or interferon beta (Sidky et al., "Cancer Research", 47:5155-5161, (1987)), thiazole derivative (Japanese Patent Publication No. H6-62413 bulletin), shark cartilage extract (chondroitin and mucopolysaccharide) (Japanese Patent Laid-Open No. H10-147534 bulletin), polysaccharide derived from streptococcus bacteria (Japanese Patent Publication No. H6-62426 bulletin), O-displacement fumagillol derivative (Japanese Patent No. 3120187 bulletin), neo agarose oligosaccharide (Japanese Patent No. 3071068th bulletin) etc. are proposed.

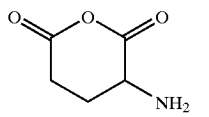
[0009] However, considering practical side, all of the materials having action of angiogenesis inhibition proposed or examined till now as a material did not show the sufficiently satisfied effect. The materials were used based on experimental findings under the dosage condition that is not practical, the materials had worries about adverse reactions, or the materials must be administered in high doses in use. Therefore, the development of materials by which angiogenesis is inhibited more effectively and materials to be used without any worry in a point of safety is demanded.

SUMMARY OF THE INVENTION

[0010] Accordingly, it is an object of the invention to provide a composition for prevention and treatment of vascular-related diseases, more particularly, composition for angiogenesis inhibition that inhibits angiogenesis strongly, composition for neoplasm inhibition, or composition for immunostimulation. It is a further object of the invention to provide a method of preventing or treating a vascular-related disease.

[0011] According to the first feature of the invention, a composition for use in prevention or treatment of a vascular-related disease comprises glutamic acid or derivative thereof and a pharmaceutically acceptable carrier or an edible carrier.

[0012] In accordance with a preferred embodiment of the invention, the glutamic acid has the circular structure that is intramolecular-dehydrated. More preferably, the glutamic acid is anhydrous glutamic acid represented by Formula (1) or pyroglutamic acid represented by Formula (2).



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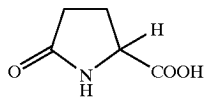


(2)

[0013] The glutamic acid is L-glutamic acid or DL-glutamic acid. The derivative is then a salt or an amide thereof. The glutamic acid and said derivatives are used in form of fruit body of Basidiomycetes or mycelium, a dry powder thereof, or an extract or a purified material thereof. Preferably, the Basidiomycetes is one or more kinds selected from the group consisting of *Lentinus edodes, Flammulina velutipes, Lyophyllum aggregatum, Pleurotus ostreatus,* Agaricus fungus, *Phellinus linteus* fungus, *Ganoderma lucidum, Hericium Erinaceum* fungus, *Coriolus versicolor, Agaricus campestris, Grifola frondosa, Sparassis crispa, Schizophyllum commune, Tremella fuciformis* Berkeley, and *Cordyceps sinensis* (tochukaso). The extract is extracted by using "water and/or a hydrophilic organic solvent", or "water and/or a hydrophilic organic solvent and a hydrophobic organic solvent". The hydrophilic organic solvent is methanol, ethanol, acetone or propanol, and the hydrophobic organic solvent is hexane or chloroform. The composition is used for angiogenesis inhibition, tumor growth inhibition or tumor metastasis inhibition, or immunostimulation.

[0014] According to the second feature of the invention, a method of preventing or treating a vascular-related disease comprises administering to a person or an animal an amount of anhydrous glutamic acid represented by Formula (1), pyroglutamic acid, or derivative thereof effective in preventing or treating said vascular-related disease.

[0015] According to the above present invention, it provides the composition which can be used as drug or eating and drinking product for treatment and prevention of various vascular-related diseases.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0016] A composition of the present invention and a method of preparation thereof will be explained in detail in the following.

[0017] In an anhydrous glutamic acid or derivatives thereof contained as preferred essential component in a composition of the present invention, anhydrous glutamic acid have the structure that glutamic acid is intramolecular-dehydrated and become circular as shown in above-mentioned structural formula.

[0018] Similarly, in pyroglutamic acid (2-pyrrolidone-5-carbonxylic acid) or derivatives thereof contained as preferred essential component in a composition of the present invention, pyroglutamic acid also have the structure that glutamic acid is intramolecular-dehydrated and become circular.

[0019] Both anhydrous glutamic acid and pyroglutamic acid concerning the present invention can be got by means of chemical synthesis, enzymatic method, or hydrolysis process, extraction process or the like from natural product. In all of these methods, L-glutamic acid, D-glutamic acid or DL-glutamic acid of optical isomer can be used as glutamic acid, but L-glutamic acid or DL-glutamic acid is preferable in view of effect of the invention, and, besides, L-glutamic acid is the most desirable.

[0020] Salt or amide is desirable for derivative of anhydrous glutamic acid. Hydrochloride, nitrate, sulfate, or phosphate can be illustrated for the salt. Hydrochloride is preferable. Amides with lower carboxylic acid such as acetic acid, lactic acid, or butyric acid, organic acid such as succinic acid, malic acid, or fumaric acid, medium chain fatty acid of from 6 to 22 carbon atoms (caproic acid, caprylic acid, nonanoic acid, capric acid, lauric acid, etc.) and higher fatty acid (myristic acid, palmitic acid, stearic acid, oleic acid, stearidonic acid, linoleic acid, conjugated linoleic acid, alpha-linolenic acid, γ-linolenic acid, di-homo-γ-linolenic acid, behenic acid, eicosapentaenoic acid, docosapentaenoic acid, docosahexaenoic acid, etc.) can be given as example of amide. Amides with nicotinic acid, glucuronic acid, or salicylic acid are also preferable.

[0021] In addition, amides with various amino acid, particularly neutral amino acid (alanine, glycine, valine, leucine, isoleucine, asparagine, glutamine), acidic amino acid (aspartic acid, glutamic acid), basic amino acid (arginine, lysin), hydroxy amino acid (serine, threonine), cyclic amino acid (histidine, tryptophan, tyrosine, phenylalanine, proline, hydroxyproline), amino acid containing sulfur (cysteine, cystine, methionine) which make proteins of living organisms are also preferable, and amides with peptide comprising combination of above various amino acid or amino sugar comprising above various amino acid and saccharide such as glucose or galactose can be also used.

[0022] Salt or amide is desirable for derivative of pyroglutamic acid. Hydrochloride, nitrate, sulfate, phosphate, sodium salt, potassium salt, calcium salt, or magnesium salt can be illustrated for the salt. Among these salts, sodium and hydrochloride are preferable. In addition, ester can be used as pyroglutamic acid derivative of the present invention. Ester which shows soluble character in water or hydrophilic organic solvent and insoluble character in hydrophobic organic solvent is desirable, and can be illustrated each ester with lower monovalent alcohol class such as methanol, ethanol, n-propanol, isopropanol, or butanol, hydroxy organic acid class such as lactic acid, malic acid, tartaric acid, citric acid, or gluconic acid, polyalcohol class such as ethylene glycol, propylene glycol, glycerin, erythritol, or sorbitol, or saccharide such as sucrose, glucose, galactose, or maltose.

[0023] Amides with various amino acid, particularly neutral amino acid (alanine, glycine, valine, leucine, isoleucine, asparagine, glutamine), acidic amino acid (aspartic acid, glutamic acid), basic amino acid (arginine, lysin), hydroxy amino acid (serine, threonine), cyclic amino acid (histidine, tryptophan, tyrosine, phenylalanine, proline, hydroxyproline), amino acid containing sulfur (cysteine, cystine, methionine) which make proteins of living organisms are also preferable, and amides with peptide comprising combination of above various amino acid or amino sugar com-

prising above various amino acid and saccharide such as glucose or galactose can be also used.

[0024] Anhydrous glutamic acid can be synthesized in accordance with well known method (for example, J. Kollonitsch and A. Rosegay, Chemistry and Industry, 7: 1867, (1964)). For instance, thionyl chloride is added in trifluoroacetic acid solution including L-glutamic acid, and dehydration reaction is caused, and diethyl ether is added there to produce sediment, and then under refrigeration anhydrous L-glutamic acid can be prepared by recrystallization using diethyl ether. In addition, various derivatives can be synthesized chemically or enzymatically by conventional method using this anhydrous L-glutamic acid as starting material.

[0025] Pyroglutamic acid and derivatives thereof can be synthesized chemically in accordance with well known method, for example, L-glutamic acid and water (equal weight each other) is heat-treated at about 130-150 degrees Celsius in autoclave to prepare L-pyroglutamic acid, or heat-treated at about 190-200 degrees Celsius to prepare DL-pyroglutamic acid by racemization. In addition, various derivative can be synthesized chemically or enzymatically by conventional method using this L-pyroglutamic acid or DL-pyroglutamic acid as starting material.

[0026] The following method can be used to prepare anhydrous glutamic acid of the present invention from natural product. Protein or peptide derived from animals, plants, fish and shellfish are hydrolyzed by hydrochloric acid or protease etc. and further refined to prepare anhydrous glutamic acid. Fruit body of Basidiomycetes or mycelium is used preferably as raw materials. These are dried to give powder thing, or extract-processed using extracting solvent to give extract solution or concentrated solution, which are dried to give extract, and further separated or fractionated using organic solvent or adsorbent to prepare purified material of high concentration.

[0027] In the present invention, these are used as preferred embodiment of anhydrous glutamic acid and derivatives thereof.

[0028] When pyroglutamic acid is prepared from natural product, purified material of high concentration can be prepare by the method same as anhydrous glutamic acid, these are used as preferred embodiment of pyroglutamic acid and derivatives thereof.

[0029] In here, it is preferable to use one or more materials selected from group comprising of *Lentinus edodes, Flammulina velutipes, Lyophyllum aggregatum, Pleurotus ostreatus,* Agaricus fungus, *Phellinus linteus* fungus, *Ganoderma lucidum, Hericium Erinaceum* fungus, *Coriolus versicolor, Agaricus campestris, Grifola frondosa, Sparassis crispa, Schizophyllum commune, Tremella fuciformis* Berkeley, and *Cordyceps sinensis* (tochukaso) as Basidiomycetes. All of these fruit bodies of mushroom are cultivated artificially or harvested abundantly, and are in the marketplace, therefore there are available easily. These are eaten raw, or as dried material, the powder, or extract etc . . . . In addition, there are materials that polysaccharides included in extract like *Lentinus edodes, Coriolus versicolor,* or *Schizophyllum commune* are used as drug. In the present invention, judging from desired effect, one kind or more kinds chosen among group comprising of Agaricus mushroom, *Phellinus linteus, Ganoderma lucidum, Hericium Erinaceum* fungus and *Cordyceps sinensis* (tochukaso) are more preferable, and Agaricus fungus is the most desirable.

[0030] Agaricus mushroom is fungus of Agaricaceae and belongs to Agaricus genus, and *Agaricus blazei* Murill or *Agaricus bisporus* can be illustrated. It is known that the former includes polysaccharide (beta-D-glucan) and polysaccharide protein conjugate and have antitumor action and hypoglycemic action. *Phellinus linteus* is fungus of Hymenochaetaceae, and it is said that polysaccharide of the hot water-extract shows anticancer action. *Ganoderma lucidum* is fungus of Polyporaceae s. l. and also referred to as *Ganoderma lucidum.* Antiallergic action, antitumor action, and blood pressure stabilization action by terpenoid and polysaccharide, and hypoglycemic action etc. by proteoglycan are known. A*Hericium erinaceum* mushroom belongs to *Hericium ramosum* (Merat) Banker, and then anticancer action by heterozygous beta-D-glucan component and active oxygen-erasing action are known.

[0031] In the present invention, plain or dried fruit body of the Basidiomycetes can be used as raw materials. However, dried fruit body is preferable in view of handling, keeping quality and extraction efficiency. Plain or dried mycelium, which is prepared by incubation of inoculm using culture medium including suitable carbon source and nitrogen source, can be used. And then, dried mycelium is convenient same as case of the fruit body.

[0032] In addition, according to the present invention, mycelium can be used as raw materials, and culture broth generating in mycelium production can be also used.

[0033] The culture broth is concentrated appropriately, and purified using solvents as described in the following.

[0034] In the present invention, it is characterized that anhydrous glutamic acid and derivatives thereof and pyroglutamic acid and derivatives thereof of the present invention are extracted by "water and/or hydrophilic organic solvent", or "water and/or hydrophilic organic solvent and hydrophobic organic solvent" from fruit body of the Basidiomycetes or mycelium. Desired effect of the present invention does not almost appear in extract that is extracted by only hydrophobic organic solvent. Methanol, ethanol, n-propanol, isopropanol or acetone is desirable for hydrophilic organic solvent, and hexane or chloroform is desirable for hydrophobic organic solvent. Hydrophilic organic solvent can be used in mixture with water, and each of hydrophilic organic solvent and hydrophobic organic solvent can be used in single or mix. The important thing on extraction of active ingredient of composition of the present invention is following point. More specifically, extract including water-soluble component mainly is given by using "water and/or hydrophilic organic solvent", or mix solvent with hydrophobic organic solvent, and readily-soluble component such as saccharide or amino acid, which is more water-soluble, is separated and removed by using hydrophilic organic solvent, and then oiliness component such as lipid class is separated and removed by using hydrophobic organic solvent.

[0035] When hydrophilic organic solvent and hydrophobic organic solvent are mixed, preferable mix ratio (volume ratio) is former/latter=9/1-1/9, more preferably, 5/1-1/5, and most preferably, 3/1-1/1. Extraction efficiency of essential component of the present invention deteriorates possibility

and desired Effect is not provided possibility when mix ratio gets out of the range. Extracting solvent is used in weight of about 3-20 times against dried material or extract of fruit body or mycelium. When it is less than 3 times, yield of extract is low. On the contrary, if using a large quantity beyond 20 times, extraction efficiency does not improve more.

[0036] Extract solution is given by extraction process which is carried out, while fruit body of Basidiomycetes or mycelium come in contact with the extract solvent, for about 10 minutes to about 10 hours under normal pressure or pressurized, preferably 1 to 3 air pressure, at room temperature or around 100 degrees Celsius while stirring or reflowing appropriately. The solvent of the extract solution is removed by vacuum-dry, freeze-dry, or spray-dry etc. to prepare extract of Basidiomycetes. In addition, the extract is fractionated by hydrophilic organic solvent and hydrophobic organic solvent to give concentrate which is further raised content of essential component of the present invention. Furthermore, the concentrate is run through column chromatography with adsorbent such as silica gel, activated alumina, magnesium silicate, activated carbon, cellulose, or ion exchange resin to fractionate, and then purified material of high concentration can be prepared.

[0037] As above described, anhydrous glutamic acid, pyroglutamic acid, or derivatives thereof prepared by chemical synthesis or extract-method from Basidiomycetes, and the extract solution, extract, concentrate, or purified material including thereof, can be used for making composition for angiogenesis inhibition of the present invention with or without appropriate carrier, excipient, or additive. As far as, in composition for angiogenesis inhibition of the present invention, it is not against a purpose of the present invention, various kinds of raw materials and component are used together, for example, excipient, desiccant, antiseptic, nutrient, thickener, emulsifier, antioxidant, sweetener, acidulant, flavor enhancer, colorant, or flavor, which is used for conventional food and drug, as pharmaceutically acceptable carrier or edible carrier. In addition, it is one of the preferable aspects of the present invention to use well-known material having action inhibiting angiogenesis together.

[0038] In addition, composition of the present invention is also the thing which functions as composition for use in neoplasm inhibition or immunostimulation, and prominent effect is shown. In other words, composition of the present invention acts as composition for use in prevention or treatment of a vascular-related disease, and shows, besides angiogenesis inhibitory action, antitumor action, namely inhibition action of development and metastasis of tumors, and further immunoenhancement action by oral ingestion etc . . . . In addition, there is effect for rheumatoid arthritis, diabetic retinopathy, psoriasis, neovascular glaucoma, inflammatory dermatosis, joint fluid rheumatism, osteoarthritis, atherosclerosis, obstructive affection such as myocardial infarction. Therefore, the composition can be utilized as composition having above actions, and eating or drinking product, drug, pet food, or fodder for domestic livestock or domestic chickens etc. can be illustrated for a concrete embodiment of the composition. Especially, eating and drinking product and drug are desirable.

[0039] As an embodiment of eating and drinking product, dry powder, extract, or purified material of the Basidiomycetes, or the composition including them can be added in liquid, gel, powder, or solid food. For example, these can be added in fruit beverage, refreshing drinks, tea, soup, jelly, yogurt, pudding, cake mixture, furikake (powdered food to be sprinkled over rice), miso (bean paste), soy sauce, dressing, mayonnaise, flavor enhancer such as dip of grilled beef, noodles, processed food of meat and fish such as ham or sausage, jam, cow milk, cream, powder, solid, or liquid milk-product such as butter or cheese, margarine, bread, cake, or cookie etc . . . .

[0040] In addition, these are processed into powder, granule, pellet, tablet along with dextrin, lactose, starch or elaboration material thereof, excipient such as cellulose, vitamin, mineral, fat and oil of flora and fauna and fish and shellfish, protein, sugar, coloring agent, flavor, other above edible additive, if necessary, and these are covered in gelatine, and these are molded as capsule, and these are made to health drink usable as nutritional supplementary food and health food. Then, the composition that used well-known edible material having angiogenesis inhibition action together is preferred. In addition, eating and drinking product of the present invention extends over extremely various kinds of forms, it is not limited to these illustrations, but above forms of the nutritional supplementary food and health food are desirable.

[0041] In the present invention, compounding dosage of composition of the present invention in eating and drinking product is hard to be prescribed uniformity because of differences in kind, form, or use purpose of the eating and drinking product, and kind or form of composition to combine, but when adding in general processed food, about 0.01-50% by weight, more preferably 0.1-30% by weight on the basis of anhydrous glutamic acid or pyroglutamic acid. When dosage gets out of the range and is under 0.01%, desired effect of the present invention by oral ingestion is small. On the contrary, if dosage is too much, a flavor is harmed potentially by kind of eating and drinking product, and there is the case that it becomes impossible to prepare the eating and drinking product.

[0042] In embodiments as drug of the present invention, above composition is added well-known excipient or additive which is not against a purpose of the present invention, if necessary, and it was processed by conventional method to form preparation such as tablet, capsules, granulation, powder, or injection. By oral administration, enteral administration, blood vessel administration, or intradermal injection, the composition can be used to develop at least one of effect of angiogenesis inhibition, tumor inhibition, or immunostimulation, and the composition can be applied to prevent or treat various diseases accompanied by vascular neogenesis, propagation and transition of tumor, or reduction of immunocompetence. Combination dosage of composition of the present invention is hard to be prescribed uniformity because of differences in kind, form, or use purpose of the preparation for medicine, but about 0.01-70% by weight, on the basis of anhydrous glutamic acid or pyroglutamic acid. In case of oral administration, dosage is not limited in particular, but it is 0.01-20 g, more preferably 0.1-10 g as base in anhydrous glutamic acid or pyroglutamic acid per adult (50 kg in weight)/1 day. When dosage gets out of the range and is under 0.01 g, desired effect of the present invention is small. On the contrary, if dose is too much, prominent effect cannot be expected more.

EXAMPLE 1

[0043] Dry fruit body of an agaricus mushroom (*Agaricus blazei* Murill) was crushed. It was then added with chloroform/methanol=1/1 mixed solution after which the mixture was warmed to 40 degrees Celsius, and extract-processed for one hour to give a chloroform/methanol=1/1 extract. Methanol was added to the extract, and methanol soluble layer was separated and collected. Furthermore, hexane was added in the methanol soluble layer, and hexane insoluble layer (sample 1) was collected. The hexane insoluble layer was then run through a silica gel column chromatography (silanised Silicagel 60 PF 256: Merck 7751, water/methanol=7/3), and ninhydrin reaction positive fractions (fraction No. 5 and 6) were collected. The fractions (fraction No. 5 and 6) were then run through HPLC (ShimpakPREP-ODS (M), column: 20 ϕ×250 mm, Shimadzu, RT, 6 ml/min, water/methanol=5/1), and ninhydrin reaction positive fractions (Rt=6 to 12 min) were collected. The fractions (Rt=6 to 12 min) were further run through HPLC (ShimpakPREP-ODS (M), column: 20 ϕ×250 mm, Shimadzu, RT, 6 ml/min, water), and ninhydrin reaction positive fraction (Rt=8 to 22 min) were collected. These ninhydrin reaction positive fractions were further refined by running through TLC (Silicagel 60 PF 254:Merck7747, water/methanol=1/100), and it was ensured that an anhydrous glutamic acid was included (Rf=0.41). In addition, results of mass spectrum and nuclear magnetic resonance (NMR) analysis showed that the anhydrous glutamic acid was L-optical isomer.

EXAMPLE 2

[0044] By means of culture apparatus "JARFERMENTER" of 10 liters capacity, while aerating (2 vvm), inoculum culture broth of *Phellinus linteus* (1 liter) was incubated in culture medium including glucose (5% by weight), yeast extract (0.5% by weight) and peptone (2.0% by weight) at 28 degrees Celsius for 72 hours, and culture mycelium (165 g) was collected. The mycelium was dried and crushed to give mycelium powder. A hexane/ethanol/water=2/3/1 mixed solvent was then added to the mycelium powder after which the mixture was extract-processed at room temperature for three hours to give an extract (sample 2). The extract was further fractionation-processed by ethanol, and an ethanol soluble layer was separated and collected. The ethanol soluble layer was then fractionation-processed by hexane, and hexane insoluble layer was collected. The hexane insoluble layer was fractionated and purified by silica gel column chromatography, HPLC, and TLC as same as example 1. Existence of anhydrous L-glutamic acid was ensured from results of mass spectrometry and nuclear magnetic resonance assay.

EXAMPLE 3

[0045] In accordance with a method as described in the above documents, an anhydrous L-glutamate was synthesized. More specifically, trifluoroacetic acid 75 ml and L-glutamic acid mol were added to four-mouth flask, and the mixture was stirred to dissolve. While stirring the solution, thionyl chloride 0.28 mol was dripped slowly into the flask. 30 minutes later of end of dripping, diethyl ether 35 ml were added slowly into the flask to produce sediment. After holding to 5 degrees Celsius for one hour, diethyl ether 100 ml were added to repeat recrystallization. An anhydrous L-glutamate acid hydrochloride (sample 3) was then prepared.

EXAMPLE 4

[0046] An anhydrous DL-glutamate acid hydrochloride (sample 4) was prepared using the same way as in example 3 except that DL-glutamic acid substituted for L-glutamic acid of raw materials.

EXAMPLE 5

[0047] Dry fruit body of *Agaricus blazei* Murill was crushed, and water was added there to extract-process using hot water at 80-95 degrees Celsius by conventional method. The extract was then dried under reduced pressure to prepare a hot water-extract of agaricus fungus. Ethanol multiplied by three (weight) was added to 40% by weight aqueous solution of the hot water-extract, and after mixing, ethanol layer was collected. The ethanol layer was then dried under reduced pressure to prepare ethanol soluble material (sample 5).

COMPARATIVE EXAMPLE 1

[0048] Dry fruit body of *Agaricus blazei* Murill, and water was added to extract-process using hot water at 80-95 degrees Celsius by conventional method. The extract was then dried under reduced pressure to prepare a hot water-extract of agaricus fungus corresponding to marketing product (reference sample 1).

TEST EXAMPLE 1

[0049] Angiogenesis inhibition action of anhydrous glutamic acid, its derivatives, and various processed materials containing them concerning the present invention was examined by degree of angiogenesis induced by means of MATRIGEL™ matrix (made by Becton Dikinson Labware Company, it is "cell culture backing material", following "MATRIGEL".) according to a method of Passaniti et al (Laboratory Invest., Vol. 67, Page 519-528, 1992).

[0050] More specifically, normal mice (5 mice/1 group) were used after breeding C57BL/6 female mice (5 week old, purchased from Charles River JAPAN, Inc.) preliminarily for one week. While cooling test materials as shown in the following, each of the test materials 0.5 ml were transplanted to subcutaneous of abdomen of the mice. MATRIGEL was taken out on the sixth day after transplantation, and state of angiogenesis was observed. In addition, the MATRIGEL was freeze-dried, and it was estimated the weight. Furthermore, pure water 1 ml was added to the MATRIGEL. After homogenizing by Polytron and centrifuging at 2000 rpm for five minutes, supernatant was filtrated through filter with 0.2 μm and hemoglobin dosage was measured by using Hemoglobin-TestWako™ (made by Wako Pure Chemical Industries, Ltd.).

[0051] Group 0 of nothing addition: MATRIGEL

[0052] Control group: MATRIGEL, heparin (64 units), Acidic Fibroblast Growth Factor (it is abbreviated to a-FGF as follows.) (1 ng/ml)

[0053] Group 1 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 3 (800 μg/ml)

[0054] Group 2 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 3 (400 μg/ml)

[0055] Group 3 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 3 (200 μg/ml)

[0056] Group 4 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 4 (800 μg/ml)

[0057] Group 5 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 1 (600 μg/ml)

[0058] Group 6 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 2 (800 μg/ml)

[0059] Group 7 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample1+sample 3 (for each 200 μg/ml)

[0060] Group 8 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 5 (600 μg/ml)

[0061] Group 9 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), reference sample 1 (800 μg/ml)

[0062] A result of the test was shown in Table 1 and Table 2. The numerical values of each table were displayed in n=5, average value±standard error. As is apparent from each table, in control group, angiogenesis was promoted remarkably and weight of MATRIGEL and hemoglobin dosage were increased compared with a group 0 of nothing addition. On the other hand, in groups of test material addition, augmentation of weight of MATRIGEL and hemoglobin dosage were concentration-dependent controlled at the group using sample 3 (anhydrous L-glutamate acid hydrochloride), and these results showed excellent angiogenesis inhibition effect. The group using sample 4 (anhydrous DL-glutamate acid hydrochloride) was also recognized an effect, which is slightly low, but almost equal to sample 3, of angiogenesis inhibition. In addition, sample 1 (purification material of agaricus mushroom extract) and sample 2 (extract of *Phellinus linteus*) also showed strong angiogenesis inhibition effect. However, the angiogenesis inhibition effect was small with reference sample 1 (hot water extract of an agaricus mushroom).

TABLE 1

No.	Group	material (μg/mL)	MATRIGEL Matrix weight (mg) n = 5
1	Group 0 (Normal)		77.7 ± 10.1
2	Control		413.3 ± 42.5
3	Group 1	sample 3 (800)	110.9 ± 11.4
4	Group 2	sample 3 (400)	232.2 ± 52.7
5	Group 3	sample 3 (200)	276.6 ± 27.6
6	Group 4	sample 4 (800)	237.3 ± 30.1
7	Group 5	sample 1 (600)	105.0 ± 12.3
8	Group 6	sample 2 (800)	149.4 ± 24.7
9	Group 7	sample 1/sample 3 = 1/1 (400)	167.3 ± 33.2
10	Group 8	sample 5 (600)	181.1 ± 20.2
11	Group 9	reference sample 1 (800)	357.5 ± 36.2

[0063]

TABLE 2

No.	Group	material (μg/mL)	Quantity of hemoglobin (mg) n = 5
1	Group 0 (Normal)		4.6 ± 1.0
2	Control		30.4 ± 2.6

TABLE 2-continued

No.	Group	material (μg/mL)	Quantity of hemoglobin (mg) n = 5
3	Group 1	sample 3 (800)	3.9 ± 0.5
4	Group 2	sample 3 (400)	15.5 ± 4.6
5	Group 3	sample 3 (200)	21.5 ± 4.0
6	Group 4	sample 4 (800)	13.0 ± 2.9
7	Group 5	sample 1 (600)	4.0 ± 0.8
8	Group 6	sample 2 (800)	5.4 ± 0.7
9	Group 7	sample 1/sample 3 = 1/1 (400)	9.3 ± 2.2
10	Group 8	sample 5 (600)	7.6 ± 2.4
11	Group 9	reference sample 1 (800)	27.3 ± 3.7

TEST EXAMPLE 2

[0064] Antiproliferative action of tumor and metastasis inhibition action of tumor were examined and evaluated by the following method about samples concerning the present invention. The Lewis lung cancer (it is abbreviated to LLC as follows) cell which was sold in lots from Institute of Physical and Chemical Research, was suspended to phosphoric acid/physiology salt buffer solution (pH 7.4). On the other hand, normal mice (7 mice/1 group) were used after breeding C57BL/6J female mice (6 week old, purchased from Kurea Japan, Inc.) preliminarily for one week. Under nembutal anesthesia the mice were exposed spleen through a small incision, and after injecting the LLC cell suspension (number of the LLC cell: 1.0×10^5) into the exposed spleen, the small incision was sewed up promptly. After 12 hours of LLC cell transplantation, the mice were orally administered agaricus mushroom extract (sample 1) 100 mg/kg (body weight) or 300 mg/kg (body weight) once a day for 20 days in succession. Distilled water was administered to a normal group and control group (LLC tumor-bearing mice) instead of sample 1. During this test period, the propagation degree of cancer cell was measured every two or three days by measuring quantity of carcinoma tissue volume (it was calculated with (major axis)×(minor axis)^2/2). On the 21st day after cancer cell transplantation, under ether anesthesia, mice of each group were taken venous blood sample into heparin tube, and white blood cell count, red blood cell count and hemoglobin dosage in the blood were measured by a call counter of blood cell. In addition, mice were slaughtered after collection of blood, and carcinoma tissue, liver, lung, spleen and thymus gland were removed and weighed, and then the number of cancer cell colony which spread to pulmonary tissue was measured under stereoscopic microscope.

[0065] Quantity of volume of tumor tissue in the LLC cell transplantation mice are shown in Table 3, weight of carcinoma tissue and each organ are shown in Table 4, and white blood cell count, red blood cell count, hemoglobin dosage and the number of transition colony to lung are shown in Table 5. Numerical values in each table are shown by average value±standard error. Fisher's Protect LSD Test was executed for significant difference assay, using a significant difference ($P<0.05$).

TABLE 3

Group (n = 7) Material (mg/kg)	control	test group1 sample 1 (100)	test group2 sample 1 (300)
quantity of neoplasm volume (mm^3) after transplantation			
5 days	362 ± 33	290 ± 40	164 ± 30 (*)
8 days	461 ± 94	307 ± 64	247 ± 52 (*)
10 days	538 ± 108	322 ± 57 (*)	301 ± 73 (*)
14 days	745 ± 142	493 ± 115	323 ± 101 (*)
17 days	872 ± 173	517 ± 121 (*)	346 ± 108 (*)
20days	1505 ± 362	608 ± 133 (*)	410 ± 89 (*)

(*): There was a significant difference in comparison with control group ($P < 0.05$)

[0066]

TABLE 4

Group (n = 7) Material (mg/kg)	normal	control	test group1 sample 1 (100)	test group2 sample 1 (300)
First body weight (g)	17.9 ± 0.30	18.2 ± 0.21	18.0 ± 0.23	17.8 ± 0.25
Last body weight (g)	21.0 ± 0.41	19.9 ± 0.54	20.3 ± 0.72	20.0 ± 0.24
Neoplasm weight (mg)	—	1872 ± 604	531 ± 267*	460 ± 115*
Spleen (g)	0.07 ± 0.01*	0.71 ± 0.03	0.35 ± 0.02*	0.22 ± 0.02*
Liver (g)	1.18 ± 0.05	1.28 ± 0.04	1.22 ± 0.03	1.24 ± 0.06
Lung (mg)	152.8 ± 4.51	166.8 ± 9.20	165.0 ± 6.76	155.0 ± 5.14
Thymus gland (mg)	47.0 ± 5.3	41.6 ± 10.3	45.2 ± 4.2	50.5 ± 3.0

*There was a significant difference in comparison with control group ($P < 0.05$)

[0067]

TABLE 5

Group (n = 7) Material (mg/kg)	normal	control	test group1 sample 1 (100)	test group2 sample 1 (300)
White blood cell count ($\times 10^3/\mu L$)	3.65 ± 0.34 (*)	4.85 ± 0.30	4.08 ± 0.54 (*)	4.30 ± 0.79 (*)
Red blood cell count ($\times 10^4/\mu L$)	782.0 ± 21.0 (*)	494.2 ± 30.4	631.5 ± 26.2 (*)	699.2 ± 51.5 (*)
Quantity of hemoglobin (g/100 mL)	11.70 ± 1.09	6.93 ± 0.72	9.62 ± 0.43 (*)	10.51 ± 0.54 (*)
Number of transition colony in lung	—	21.5 ± 2.5	10.0 ± 1.5	7.0 ± 1.0

(*): There was a significant difference in comparison with control group ($P < 0.05$)

[0068] From data of table 3, it was showed that volume of neoplasm increased with time in control group (tumor-bearing mice) by transplantation of the LLC cell, and that increase of volume of neoplasm was inhibited in the group administered orally the test material (sample 1: agaricus mushroom extract including anhydrous glutamic acid) and the LLC cellular propagation was controlled.

[0069] From data of table 4, it was showed that increase of weight of neoplasm was inhibited clearly by intake of the test material, and that there was no significant difference in weight of each organ aside from spleen and final body weight between normal group, LLC cell transplantation group (control group) and test material administrated group.

[0070] Spleen weight increased in control group, but depression of increase was recognized in test material administrated group (there was significance difference with $P<0.05$). Therefore, it was proved that propagation of LLC cell was depressed by oral ingestion of test material (sample 1).

[0071] From data of table 5, it was showed that white blood cell count increased in control group compared with normal group, and that there was no significant difference between test material administrated group and normal group. Moreover, it was showed that red blood cell count and quantity of hemoglobin were significantly reduced and anemia state was shown in control group, but in test material administrated group, they were increased in significance ($P<0.05$) and anemia state recovered to close to normal by means of oral administration of agaricus fungus extract (sample 1). In addition, the number of transition colony of LLC cell in lung decreased in significance in test material administrated group compared with control group, and then it was proved that transition of cancer cell was inhibited by oral ingestion of the agaricus fungus extract which contained anhydrous glutamic acid (sample 1).

TEST EXAMPLE 3

[0072] Effect on immune function was examined and evaluated by the following method about samples concerning the present invention. Splenocyte was separated from spleen removed with test example 2. The splenocyte was layered on lymphocyte separate solution ("Lymphocyte Segregation Solution", made by Dainippon Pharmaceutical Co., Ltd.) and centrifuged for 30 minutes at 2000 rpm to separate lymphocyte. The coexisting erythrocyte was then hypotonic-solutionized and removed. The number of the lymphocyte was measured and adjusted to 1×10^6 cell count/100 μL. Antibody of various cell surface antigen ("CD 4, antimouse, FITC label", "CD 8, antimouse, FITC label" and "NK1.1, antimouse, R-PE label", made by Dainippon Pharmaceutical

Co., Ltd.) 10 μL were added to the lymphocyte and reacted at 4 degrees Celsius for 30 minutes, after which it was washed twice in phosphate buffer (reagent for biochemical analysis made by Wako Pure Chemical Industries, LTD). The phosphate buffer was added there so that total volume was 1 mL, and then the numbers of CD 4^+, CD $8^+$and $NK1.1^+$T cell were measured using a flow cytometry. The results are shown in Table 6.

TABLE 6

Group (n = 7) Material (mg/kg)	normal	control	test group1 sample 1 (100)	test group2 sample 1 (300)
Number of the lymphocyte ($\times 10^7$/spleen)	(*) 3.68 ± 0.34	1.23 ± 0.20	(*) 3.15 ± 0.41	(*) 2.98 ± 0.90
Number of $CD4^+$T CELL ($\times 10^6$/spleen)	(*) 6.0 ± 1.5	2.0 ± 0.5	3.5 ± 1.0	(*) 5.5 ± 1.0
Number of $CD8^+$T CELL ($\times 10^6$/spleen)	(*) 8.1 ± 1.0	3.5 ± 0.6	(*) 6.7 ± 0.7	(*) 6.5 ± 1.0
Number of $NK1.1^+$T CELL ($\times 10^5$/spleen)	2.0 ± 0.5	2.0 ± 0.3	(*) 4.5 ± 0.5	(*) 3.5 ± 0.5

(*): There was a significant difference in comparison with control group ($P < 0.05$)

[0073] From data of table 6, it was showed that the number of the lymphocyte in spleen decreased in significance in control group (tumor-bearing mice) compared with normal group, and that this decrease was controlled in test material administrated group. In addition, it was proved that the numbers of CD 4^+T cell and CD 8^+T cell in spleen decreased in significance in control group (tumor-bearing mice) compared with normal group, and that decrease of the numbers of both cell was inhibited and the number of $NK1.1^+$T cell was increased by intake of test material (sample 1). From these finding, it became clear that immune function was reinforced by oral ingestion of agaricus mushroom extract including anhydrous glutamic acid.

EXAMPLE 6

[0074] Angiogenesis inhibition composition of the present invention comprising sample 1 and oolong tea leaf powder (3:2 (a weight ratio)) 5.0 kg, modified starch (a product made by Matsutani Chemical Industry Co, .Ltd, brand name: pine flow) 3.5 kg, tricalcium phosphate 0.3 kg, vitamin B_1 0.3 kg, vitamin B_2 0.2 kg, vitamin B_6 0.2 kg, and vitamin C 0.5 kg were tucked into blender and mixed for 10 minutes. After the mixture was supplied into a tablet machine of the direct compression formulation type and made a tablet (diameter of 7 mm, height of 4 mm, 150 mg in weight), food in tablet form was produced experimentally by coating with shellac thin-film using coating machine. This tablet can be used for purpose of enhancement of internal immune strength, and prevention of lifestyle-related diseases such as diabetes mellitus or carcinoma.

EXAMPLE 7

[0075] Butter 110 g, shortening 110 g, very-refined sugar 90 g and milk 100 mL were put in bowl for families, and one egg was more added there while whipping together. After mixing enough, a mixture of sample 2 and sample 3 (3:1, weight ratio) of the present invention 10 g was added there along with soft flour 190 g and baking powder 2 g, and then the mixture was kneaded enough together. After setting dough for 30 minutes, the mixed compound was split into 50 using mould and burnt in oven, and then a butter cookie was produced experimentally.

EXAMPLE 8

[0076] Composition of the present invention comprising of sample 1, sample 2, and grape seed extract (made by Interhealthl company, brand name: Activin) (1:2:1 (a weight ratio)) 5 g were added to a commercial vegetable juice 1 L and mixed together, and then angiogenesis inhibition vegetable juice was produced experimentally for a person worried about malignant growth or rheumatoid arthritis. There was no inferiority in this in comparison with original vegetable juice at all.

EXAMPLE 9

[0077] A mixture of sample 3/sample 5=1/1 (a weight ratio) 130 kg, propolis 90 kg, yellow beeswax 15 kg and corn oil 150 kg were mixed enough to become homogeneous liquid thing while warming to 40 degrees Celsius. This was supplied to the capsule filling up machine, and then gelatine covered-capsule formulation, of which quantity of one grain was 250 mg, was produced experimentally. This formulation can be used as edible composition (food and drink) or composition for medicine (drug) that oral ingestion is possible.

EXAMPLE 10

[0078] Dry fruit body of an agaricus mushroom (*Agaricus blazei* Murill) was crushed. It was then added with chloroform/methanol=1/1 mixed solution after which the mixture was warmed to 50 degrees Celsius, and extract-processed for one hour to give a chloroform/methanol=1/1 extract. Methanol was added to the extract, and then methanol soluble layer was separated and collected except methanol insoluble materials including mannitol. Furthermore, hexane was added in the methanol soluble layer, and then hexane insoluble layer (sample 6) was collected. The hexane insoluble layer was then run through a silica gel column chromatography (silanised Silicagel 60 PF 254:Merck 7751, water/methanol=7/3), and ninhydrin reaction positive fractions (fraction No. 5 and 6) were collected. The fractions (fraction No. 5 and 6) were then run through HPLC (Shimadzu LC-8A system: Shimpak PREP-ODS (M), column: 20 ϕ×250 mm, Shimadzu, RT, 6 ml/min, water/methanol=5/1), and ninhydrin reaction positive fractions (Rt=6 to 12 min) were collected. The fractions (Rt=6 to 12 min) were further run through HPLC (Shimadzu LC-8A system: Shimpak PREP-ODS (M), column:20ϕ×250 mm, Shimadzu, RT, 6 ml/min, water), and ninhydrin reaction positive fraction (Rt=8 to 22 min) were collected. These ninhydrin reaction

positive fractions were further refined by running through TLC (Silicagel 60 PF 254:Merck7747, water/methanol=1/100), and it was ensured that materials shown in the following were included.

[0079] More specifically, as a result of TLC (Silicagel 60, precoat TLC: Merck5715, water/methanol=1/100) assay, it was showed that there were alanine (the neighborhood of Rf (rate of flow)=0.27, ninhydrin positivity), proline (the neighborhood of Rf=0.20, ninhydrin positivity), gamma aminobutyric acid (the neighborhood of Rf=0.15, ninhydrin positivity) and unknown substance (the neighborhood of Rf=0.40, ninhydrin negativity) (49:5:25:21 (a weight ratio)). Subsequently, the unknown material was analyzed by NMR (nuclear magnetic resonance) spectrum analysis (device: Varian Unity Inova 500) and mass spectrum analysis (device: M-4000H, made by Hitachi, Ltd.), the results were 1H-NMR spectra (δ ppm, D_2O): 2.09, 2.39 (each 1H, m, H-3), 2.50 (2H, m, H-4), and 4.22 (1H, dd, J=5.2 and 9.0 Hz), 13C-NMR spectra (δ ppm, D_2O): 184 (COOH or —C═O—), 182 (—C═O—), 57 (—CH—), 32 (—O═C—CH_2—), and 28 (—CH_2—CH_2—), and mass spectrum (m/z): 42, 84 and 129 (M+). In addition, the result of Optical Rotatory Dispersion (ORD) spectrum analysis (made by JASCO Corporation (NIHON BUNKOU), ORD/UV-820) was $[alpha]_D^{23}$ −11.5° (c=2, H_2O). From these assay results, the unknown material was identified as L-pyroglutamic acid.

EXAMPLE 11

[0080] By means of culture apparatus of 10 liters capacity, while aerating (2 vvm) and stirring (150 rpm), inoculum culture broth of *Phellinus linteus* (1 liter) was incubated in culture medium including glucose (5% by weight), yeast extract (0.5% by weight) and poly peptone (1.5% by weight) at 26 degrees Celsius for seven days, and culture mycelium (200 g) was collected. The culture mycelium was dried and crushed to give mycelium powder. A hexane/ethanol/water=3/4/1 mixed solvent was then added to the mycelium powder after which the mixture was warmed to 40 degrees Celsius, and extract-processed for 30 minutes to give an soluble material (sample 7). The soluble material was further fractionation-processed by ethanol, and an ethanol soluble layer was separated and collected. The ethanol soluble layer was then fractionation-processed by hexane, and hexane insoluble layer was collected. The hexane insoluble layer was fractionated and purified by silica gel column chromatography, HPLC, and TLC as same as example 10. Existence of L-pyroglutamic acid was ensured from results of mass spectrometry and nuclear magnetic resonance assay etc.

EXAMPLE 12

[0081] L-pyroglutamic acid sodium was prepared in accordance with conventional method. More specifically, L-pyroglutamic acid aqueous solution (30% by weight) was added to a flask with stirrer, and while stirring slowly at room temperature, sodium hydroxide aqueous solution (0.5N) was added there untill no pH fluctuation. After salting out, the solution was dried to give L-pyroglutamic acid sodium (sample 8).

EXAMPLE 13

[0082] DL-pyroglutamic acid sodium (sample 9) was prepared using the same way as in example 12 except that DL-glutamic acid substituted for L-glutamic acid of raw materials.

EXAMPLE 14

[0083] Dry fruit body of *Agaricus blazei* Murill was crushed, and water was added there to extract-process using hot water at 80-95 degrees Celsius by conventional method. The extract was then dried under reduced pressure to prepare a hot water-extract of agaricus fungus. Ethanol multiplied by three (weight) was added to 40% by weight aqueous solution of the hot water-extract, and after mixing, ethanol layer was collected. The ethanol layer was then dried under reduced pressure to give ethanol soluble material. The ethanol soluble material was washed by hexane of 5 times (weight), and then dried under reduced pressure to prepare hexane insoluble material (sample 10).

TEST EXAMPLE 4

[0084] Angiogenesis inhibition action of pyroglutamic acid, its derivatives, and various processed materials containing them concerning the present invention was examined by degree of angiogenesis induced by means of MATRIGEL™ matrix (Becton Dikinson Labware company, following "MATRIGEL") according to the same method as test example 1.

[0085] More specifically, normal mice (5 mice/1 group) were used after breeding C57BL/6 female mice (5 week old, purchased from Charles River JAPAN, Inc.) preliminarily for one week. While cooling test materials as shown in the following, each of the test materials 0.5 ml were transplanted to subcutaneous of abdomen of the mice. MATRIGEL was taken out on the sixth day after transplantation, and state of angiogenesis was observed. In addition, the MATRIGEL was freeze-dried, and it was weighed. Furthermore, pure water 1 ml was added to the MATRIGEL. After homogenizing by Polytron and centrifuging at 2000 rpm for five minutes, supernatant was filtrated through filter with 0.2 μm and hemoglobin dosage was measured by using Hemoglobin-TestWako™ (product made in Wako Pure Chemical Industries, Ltd.).

[0086] Group 0 of nothing addition: MATRIGEL

[0087] Control group: MATRIGEL, heparin (64 units), Acidic Fibroblast Growth Factor (it is abbreviated to a-FGF as follows.) (1 ng/ml)

[0088] Group 1 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 8 (800 μg/ml)

[0089] Group 2 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 8 (400 μg/ml)

[0090] Group 3 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 8 (200 μg/ml)

[0091] Group 4 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 9 (800 μg/ml)

[0092] Group 5 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 6 (600 μg/ml)

[0093] Group 6 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 7 (800 μg/ml)

[0094] Group 7 of test material addition: MATRIGEL, heparin (64 unit), a-FGF (1 ng/ml), sample 6+sample 8 (for each 200 μg/ml)

[0095] Group 8 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), sample 10 (600 μg/ml)

[0096] Group 9 of test material addition: MATRIGEL, heparin (64 units), a-FGF (1 ng/ml), reference sample 1 (800 μg/ml)

[0097] A test result was shown in Table 7.

[0098] The numerical values of Table 7 were displayed in n=5, average value±standard error.

[0099] As is apparent from Table 7, in control group, angiogenesis was promoted remarkably and weight of MATRIGEL and hemoglobin dosage were increased compared with a group 0 of nothing addition.

[0100] On the other hand, in groups of test material addition, augmentation of weight of MATRIGEL and hemoglobin dosage was concentration-dependent controlled at the group using sample 8 (L-pyroglutamic acid salt), and these results showed excellent angiogenesis inhibition effect. The group using sample 9 (DL-pyroglutamic acid salt) was also recognized an effect, which is slightly low, but almost equal to sample 8, of angiogenesis inhibition. In addition, sample 6 (purification material of agaricus mushroom extract) and sample 7 (extract of *Phellinus linteus*) also showed strong angiogenesis inhibition effect. However, the angiogenesis inhibition effect was small with reference sample 1 (hot water extract of an agaricus mushroom). Furthermore, presence of angiogenesis inhibitory effect was also examined about alanine, proline and gamma aminobutyric acid similarly, but the angiogenesis inhibitory effect was not recognized in these materials.

TABLE 7

Group n = 5	material (μg/mL)	MATRIGEL weight (mg)	Quantity of hemoglobin (mg/MATRIGEL)
Group 0 (Normal)		100.0 ± 12.1	5.3 ± 1.7
Control		393.6 ± 22.7	33.0 ± 3.4
Group 1	sample 8 (800)	121.5 ± 28.1	4.8 ± 1.2
Group 2	sample 8 (400)	190.2 ± 30.4	17.5 ± 3.6
Group 3	sample 8 (200)	265.3 ± 18.6	23.4 ± 2.0
Group 4	sample 9 (800)	209.0 ± 30.8	15.8 ± 2.7
Group 5	sample 6 (600)	110.7 ± 10.3	4.0 ± 0.5
Group 6	sample 7 (800)	136.4 ± 24.5	6.1 ± 2.4
Group 7	sample 6/sample 8 = 1/1 (400)	150.5 ± 23.1	8.3 ± 1.8
Group 8	sample 10 (600)	154.1 ± 27.2	9.0 ± 4.3
Group 9	reference sample 1 (800)	320.4 ± 35.2	30.5 ± 4.5

MATRIGEL ™ (Becton Dikinson Labware company) matrix

TEST EXAMPLE 5

[0101] Antiproliferative action of tumor and metastasis control action of tumor were examined and evaluated according to the same method as Test example 2 except the following condition. 7 mice/1 group was replaced with 8 mice/1 group. Sample 1 was replaced with sample 6. The period of administration was 30 days instead of 20 days. The propagation degree of cancer cell was measured every 3 to 5 days instead of every 2 to 3 days. Slaughter of mice was conducted on the 31st day after cancer cell transplantation, instead of the 21st day.

[0102] Quantity of volume of tumor tissue in the LLC cell transplantation mice are shown in Table 8, weight of carcinoma tissue and each organ are shown in Table 9, and white blood cell count, red blood cell count, hemoglobin dosage and the number of transition colony to lung are shown in Table 10. Numerical values in each table are shown by average value±standard error. Fisher's Protect LSD Test was executed for significant difference assay, using a significant difference ($P<0.05$).

TABLE 8

Group (n = 8) Material (mg/kg)	control	test group1 sample 6 (100)	test group2 sample 6 (300)
quantity of neoplasm volume (mm^3) after transplantation			
7 days	405 ± 40	285 ± 72	182 ± 31 (*)
12 days	617 ± 84	310 ± 94 (*)	244 ± 83 (*)
17 days	806 ± 137	552 ± 108	317 ± 105 (*)
21 days	1430 ± 280	503 ± 131 (*)	416 ± 122 (*)
24 days	1916 ± 325	745 ± 186 (*)	503 ± 169 (*)
30 days	2190 ± 382	1067 ± 292 (*)	670 ± 213 (*)

(*): There was a significant difference in comparison with control group ($P < 0.05$)

[0103]

TABLE 9

Group (n = 8) Material (mg/kg)	normal	control	test group1 sample 6 (100)	test group2 sample 6 (300)
First body weight (g)	18.5 ± 0.25	18.3 ± 0.34	18.5 ± 0.26	18.2 ± 0.22
Last body weight (g)	21.4 ± 0.36	23.1 ± 0.54	21.6 ± 0.87	22.1 ± 0.43
Neoplasm weight (mg)	—	3061 ± 785	745 ± 403	480 ± 130
Spleen (g)	0.08 ± 0.02*	1.43 ± 0.07	0.81 ± 0.10*	0.45 ± 0.03*
Liver (g)	1.21 ± 0.05	1.39 ± 0.10	1.25 ± 0.05	1.22 ± 0.04
Lung (mg)	164.8 ± 7.1	187.7 ± 15.2	184.4 ± 13.1	175.0 ± 5.2
Thymus gland (mg)	58.5 ± 3.9	46.8 ± 6.6	47.9 ± 4.9	51.5 ± 5.0

(*): There was a significant difference in comparison with control group ($P < 0.05$)

[0104]

TABLE 10

Group (n = 8) Material (mg/kg)	normal	control	test group1 sample 6 (100)	test group2 sample 6 (300)
White blood cell count		(*1)		
($\times 10^3/\mu L$)	3.32 ± 0.18	5.73 ± 1.40	4.72 ± 1.13	4.12 ± 0.59
Red blood cell count	(*)		(*)	(*)
($\times 10^4/\mu L$)	799.0 ± 7.4	506.3 ± 78.4	692.6 ± 37.2	743.2 ± 22.0
Quantity of hemoglobin	(*)		(*)	(*)
(g/100 mL)	12.4 ± 0.09	7.80 ± 1.25	10.6 ± 0.59	11.3 ± 0.39
Number of transition			(*)	(*)
colony in lung	—	30.0 ± 2.8	15.8 ± 2.5	13.6 ± 1.4

(*): There was a significant difference in comparison with control group ($P < 0.05$)
(*1): There was a significant difference in comparison with normal group ($P < 0.05$)

[0105] From data of table 8, it was showed that volume of neoplasm increased with time in control group (tumor-bearing mice) because of transplantation of the LLC cell, and that increase of volume of neoplasm was inhibited in the group administered orally the test material (sample 6: agaricus mushroom extract including pyroglutamic acid) and the LLC cellular propagation was controlled.

[0106] From data of table 9, it was showed that increase of weight of neoplasm was inhibited clearly by intake of the test material, and that there was no significant difference in weight of each organ aside from spleen and final body weight between normal group, LLC cell transplantation group (control group) and test material administrated group. Spleen weight increased in control group, but depression of increase was recognized in test material administrated group (there was significance difference with $P<0.05$). Therefore, it was proved that propagation of LLC cell was depressed by oral ingestion of test material (sample 6).

[0107] From data of table 10, it was showed that white blood cell count increased in significance in control group compared with normal group, and that there was no significant difference between test material administrated group and normal group. Moreover, it was showed that red blood cell count and quantity of hemoglobin were significantly reduced and anemia state was shown in control group, but in test material administrated group, they were increased in significance ($P<0.05$) and anemia state recovered to close to normal by means of oral administration of agaricus fungus extract (sample 6). In addition, the number of transition colony of LLC cell in lung decreased in significance in test material administrated group compared with control group, and then it was proved that transition of cancer cell was inhibited by oral ingestion of the agaricus fungus extract which contained pyroglutamic acid (sample 6).

TEST EXAMPLE 6

[0108] Effect on immune function was examined and evaluated by the following method about samples concerning the present invention. Splenocyte was separated from spleen removed with test example 5. The splenocyte was processed according to the same method as test example 3, and then the numbers of CD 4^+, CD 8^+ and NK1.1^+T cell were measured using a flow cytometry. The results are shown in Table 11.

TABLE 11

Group (n = 8) Material (mg/kg)	normal	control	test group1 sample 6 (100)	test group2 sample 6 (300)
Number of the lymphocyte	(*)		(*)	(*)
($\times 10^7$/spleen)	3.52 ± 0.48	1.47 ± 0.22	2.84 ± 0.36	3.01 ± 0.45
Number of CD4$^+$T CELL	(*)			(*)
($\times 10^6$/spleen)	6.5 ± 0.8	2.1 ± 0.4	4.6 ± 0.5	6.0 ± 1.1
Number of CD8$^+$T CELL	(*)		(*)	(*)
($\times 10^6$/spleen)	7.7 ± 1.2	4.5 ± 0.7	6.6 ± 0.6	8.9 ± 0.8
Number of NK1.1$^+$T CELL	(*)		(*)	(*)
($\times 10^5$/spleen)	1.7 ± 0.2	2.6 ± 0.3	2.8 ± 0.3	4.0 ± 0.5

(*): There was a significant difference in comparison with control group ($P < 0.05$)

[0109] From data of table 11, it was showed that the number of the lymphocyte in spleen decreased in significance in control group (tumor-bearing mice) compared with normal group, and that this decrease was controlled in test material administrated group. In addition, it was also proved that the numbers of CD 4^+T cell and CD 8^+T cell in spleen decreased in significance in control group (tumor-bearing mice) compared with normal group, and that decrease of the numbers of both cell was inhibited and the number of NK1.1^+T cell was increased by intake of test material (sample 6). From these finding, it became clear that immune function was reinforced by oral ingestion of agaricus mushroom extract including anhydrous glutamic acid.

EXAMPLE 15

[0110] Angiogenesis inhibition composition of the present invention comprising sample 10, oolong tea leaf powder, and Guava leaf hot water extract (3:2:1 (a weight ratio)) 10.0

kg, modified starch (made by Matsutani Chemical Industry Co, .Ltd, brand name: pine flow) 7.0 kg, tricalcium phosphate 0.5 kg, vitamin B_1 0.4 kg, vitamin B_2 0.4 kg, vitamin B_6 0.5 kg, and vitamin C 1.2 kg were tucked into blender and mixed for 10 minutes. After the mixture was supplied into a tablet machine of the direct compression formulation type and made a tablet (diameter of 7 mm, a height of 4 mm, 150 mg in weight), food in tablet form was produced experimentally by coating with shellac thin-film using coating machine. This tablet can be used for purpose of enhancement of internal immune strength, and prevention of lifestyle-related diseases such as diabetes mellitus or carcinoma.

EXAMPLE 16

[0111] Butter 120 g, shortening 100 g, very-refined sugar 100 g and milk 100 mL were put in bowl for families, and one egg was more added there while whipping together. After mixing enough, a mixture of sample 6, sample 7, and sample 8 (2:2:1, weight ratio) of the present invention 30 g was added there along with soft flour 200 g and baking powder 2 g, and then the mixture was kneaded enough together. After setting dough for 30 minutes, the mixed compound was split into 50 using mould and burnt in oven, and then butter cookies were produced experimentally.

EXAMPLE 17

[0112] Composition of the present invention comprising of sample 8, sample 10, and grape seed extract (made by Interhealthl company, brand name: Activin) (1:2:1 (a weight ratio)) 20 g were added to a commercial vegetable juice 1 L and mixed together, and then angiogenesis inhibition vegetable juice was produced experimentally for use in antioxidation of tissue or prevention of diseases such as malignant, rheumatoid arthritis, and diabetes mellitus. There was no inferiority in this in comparison with original vegetable juice at all.

EXAMPLE 18

[0113] A mixture of sample 6/sample 10=1/1 (a weight ratio) 100 kg, Ginkgo biloba extract 20 kg, shark cartilage extract 30 kg, yellow beeswax 10 kg and corn oil 140 kg were mixed enough to give homogeneous liquid thing while warming to 80 degrees Celsius. This was supplied to the capsule filling up machine, and then gelatine covered-capsule formulation, of which quantity of one grain was 250 mg, was produced experimentally. This formulation can be used as edible composition (food and drink) or composition for medicine (drug) that oral ingestion is possible.

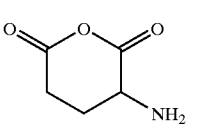
[0114] This invention is not limited to the above embodiments and explanation thereof, and variations and modifications can be effected within the scope which does not depart from the description in the claims and can be easily conceived by a person having ordinary skill in the art.

What is claimed is:

1. A composition for use in prevention or treatment of a vascular-related disease, which comprises glutamic acid or derivative thereof and a pharmaceutically acceptable carrier or an edible carrier.

2. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said glutamic acid has the circular structure that is intramolecular-dehydrated.

3. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said glutamic acid is anhydrous glutamic acid represented by Formula (1).



(1)

4. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said glutamic acid is pyroglutamic acid.

5. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said glutamic acid is L-glutamic acid or DL-glutamic acid.

6. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said derivative is a salt or an amide thereof.

7. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said glutamic acid and said derivative are used in form of fruit body of Basidiomycetes or mycelium, a dry powder thereof, or an extract or a purified material thereof.

8. The composition for use in prevention or treatment of a vascular-related disease according to claim 7, wherein said Basidiomycetes is one or more kinds selected from the group consisting of *Lentinus edodes, Flammulina velutipes, Lyophyllum aggregatum, Pleurotus ostreatus,* Agaricus fungus, *Phellinus linteus* fungus, *Ganoderma lucidum, Hericium erinaceum* fungus, *Coriolus versicolor, Agaricus campestris, Grifola frondosa, Sparassis crispa, Schizophyllum commune, Tremella fuciformis* Berkeley, and *Cordyceps sinensis*(tochukaso).

9. The composition for use in prevention or treatment of a vascular-related disease according to claim 7, wherein said extract is extracted by using "water and/or a hydrophilic organic solvent", or "water and/or a hydrophilic organic solvent and a hydrophobic organic solvent".

10. The composition for use in prevention or treatment of a vascular-related disease according to claim 9, wherein said hydrophilic organic solvent is methanol, ethanol, acetone or propanol, and said hydrophobic organic solvent is hexane or chloroform.

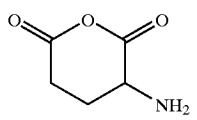
11. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said composition is used for angiogenesis inhibition.

12. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said composition is used for tumor growth inhibition or tumor metastasis inhibition.

13. The composition for use in prevention or treatment of a vascular-related disease according to claim 1, wherein said composition is used for immunostimulation.

14. A method of preventing or treating a vascular-related disease, which comprises administering to a person or an animal an amount of anhydrous glutamic acid represented

by Formula (1) or derivatives thereof effective in preventing or treating said vascular-related disease.



(1)

15. A method of preventing or treating a vascular-related disease, which comprises administering to a person or an animal an amount of pyroglutamic acid or derivatives thereof effective in preventing or treating said vascular-related disease.

* * * * *

CAPÍTULO REIVINDICATORIO

1. Un compuesto caracterizado porque es Piroglutamato de vortioxetina.

2. El compuesto de acuerdo con la Reivindicación 1, caracterizado porque se selecciona de (L)-piroglutamato de vortioxetina, (D)-piroglutamato de vortioxetina y (DL)-piroglutamato de vortioxetina.

3. El compuesto de acuerdo con la Reivindicación 2, caracterizado porque el (L)-piroglutamato de vortioxetina o el (D)-piroglutamato de vortioxetina en forma cristalina tienen reflexiones de XRPD a 7.02, 10.72, 12.14, 14.45, 14.61, 15.56, 16.05, 16.22, 17.53, 17.70, 18.45 y 18.59 (°2θ) (±0.1 °2θ).

4. El Compuesto de acuerdo con la Reivindicación 2, caracterizado por que el (DL)-piroglutamato de vortioxetina monohidratado en forma cristalina tiene reflexiones de XRPD a 6.16, 9.25, 9.38, 12.10, 14.03, 14.61, 15.02, 15.88, 16.33, 16.91, 17.68 y 18.12 (°2θ) (±0.1 °2θ).

5. El Compuesto de acuerdo con la Reivindicación 2, caracterizado por que la forma α de (DL)-piroglutamato de vortioxetina en forma cristalina tiene reflexiones de XRPD a 14.27, 15.75, 17.06 y 18.59 (°2θ) (±0.1 °2θ).

6. Una composición farmacéutica que comprende un compuesto de acuerdo con cualquiera de las Reivindicaciones 1-5 junto con uno o más portadores o diluyentes farmacéuticamente aceptables.

7. Una Composición farmacéutica sólida para administración oral que comprende un compuesto de acuerdo con cualquiera de las Reivindicaciones 1-5 y un recubrimiento entérico.

8. Una composición farmacéutica gelificable que comprende un compuesto de acuerdo con cualquiera de las Reivindicaciones 1-5 y una sal.

9. Un gel que comprende un compuesto de acuerdo con cualquiera de las Reivindicaciones 1-5, una sal y agua.

10. El Método para preparar un gel, comprendiendo dicho método mezclar un compuesto de acuerdo con cualquiera de las Reivindicaciones 1-5, una sal y una disolución acuosa.

⟨941⟩ CHARACTERIZATION OF CRYSTALLINE AND PARTIALLY CRYSTALLINE SOLIDS BY X-RAY POWDER DIFFRACTION (XRPD)

INTRODUCTION

Every crystalline phase of a given substance produces a characteristic X-ray diffraction pattern. Diffraction patterns can be obtained from a randomly oriented crystalline powder composed of crystallites or crystal fragments of finite size. Essentially three types of information can be derived from a powder diffraction pattern: the angular position of diffraction lines (depending on geometry and size of the unit cell), the intensities of diffraction lines (depending mainly on atom type and arrangement, and particle orientation within the sample), and diffraction line profiles (depending on instrumental resolution, crystallite size, strain, and specimen thickness).

Experiments giving angular positions and intensities of lines can be used for applications such as qualitative phase analysis (e.g., identification of crystalline phases) and quantitative phase analysis of crystalline materials. An estimate of the amorphous and crystalline fractions[1] can also be made.

The X-ray powder diffraction (XRPD) method provides an advantage over other means of analysis in that it is usually nondestructive in nature (to ensure a randomly oriented sample, specimen preparation is usually limited to grinding). XRPD investigations can also be carried out under *in situ* conditions on specimens exposed to nonambient conditions such as low or high temperature and humidity.

PRINCIPLES

X-ray diffraction results from the interaction between X-rays and electron clouds of atoms. Depending on atomic arrangement, interferences arise from the scattered X-rays. These interferences are constructive when the path difference between two diffracted X-ray waves differs by an integral number of wavelengths. This selective condition is described by the Bragg equation, also called Bragg's law (see *Figure 1*).

$$2d_{hkl}\sin\theta_{hkl} = n\lambda$$

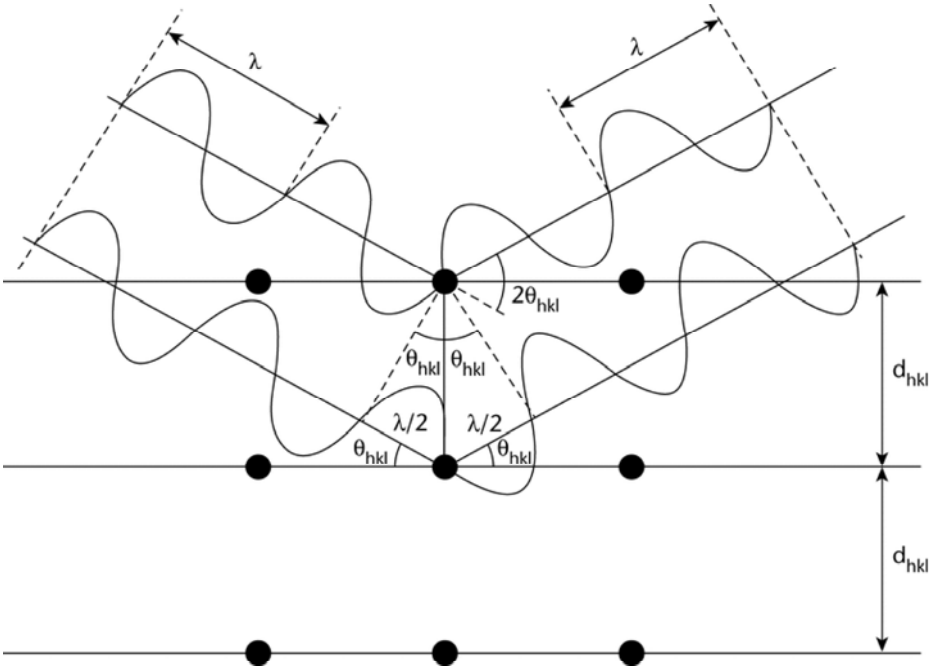


Figure 1. Diffraction of X-rays by a crystal according to Bragg's Law.

The wavelength, λ, of the X-rays is of the same order of magnitude as the distance between successive crystal lattice planes, or d_{hkl} (also called d-spacings). θ_{hkl} is the angle between the incident ray and the family of lattice planes, and sin θ_{hkl} is inversely proportional to the distance between successive crystal planes or d-spacings.

The direction and spacing of the planes with reference to the unit cell axes are defined by the Miller indices {hkl}. These indices are the reciprocals, reduced to the next-lower integer, of the intercepts that a plane makes with the unit cell axes. The unit cell dimensions are given by the spacings a, b, and c, and the angles between them α, β, and γ.

The interplanar spacing for a specified set of parallel hkl planes is denoted by d_{hkl}. Each such family of planes may show higher orders of diffraction where the d values for the related families of planes nh, nk, nl are diminished by the factor 1/n (n being an integer: 2, 3, 4, etc.).

Every set of planes throughout a crystal has a corresponding Bragg diffraction angle, θ_{hkl}, associated with it (for a specific λ).

A powder specimen is assumed to be polycrystalline so that at any angle θ_{hkl} there are always crystallites in an orientation allowing diffraction according to Bragg's law.[2] For a given X-ray wavelength, the positions of the diffraction peaks (also referred to as “lines”, “reflections”, or “Bragg reflections”) are characteristic of the crystal lattice (d-spacings), their theoretical intensities depend on the crystallographic unit cell content (nature and positions of atoms), and

the line profiles depend on the perfection and extent of the crystal lattice. Under these conditions, the diffraction peak has a finite intensity arising from atomic arrangement, type of atoms, thermal motion, and structural imperfections, as well as from instrument characteristics.

The intensity is dependent upon many factors such as structure factor, temperature factor, crystallinity, polarization factor, multiplicity, and Lorentz factor.

The main characteristics of diffraction line profiles are 2θ position, peak height, peak area, and shape (characterized by, e.g., peak width, or asymmetry, analytical function, and empirical representation). An example of the type of powder patterns obtained for five different solid phases of a substance are shown in *Figure 2.*

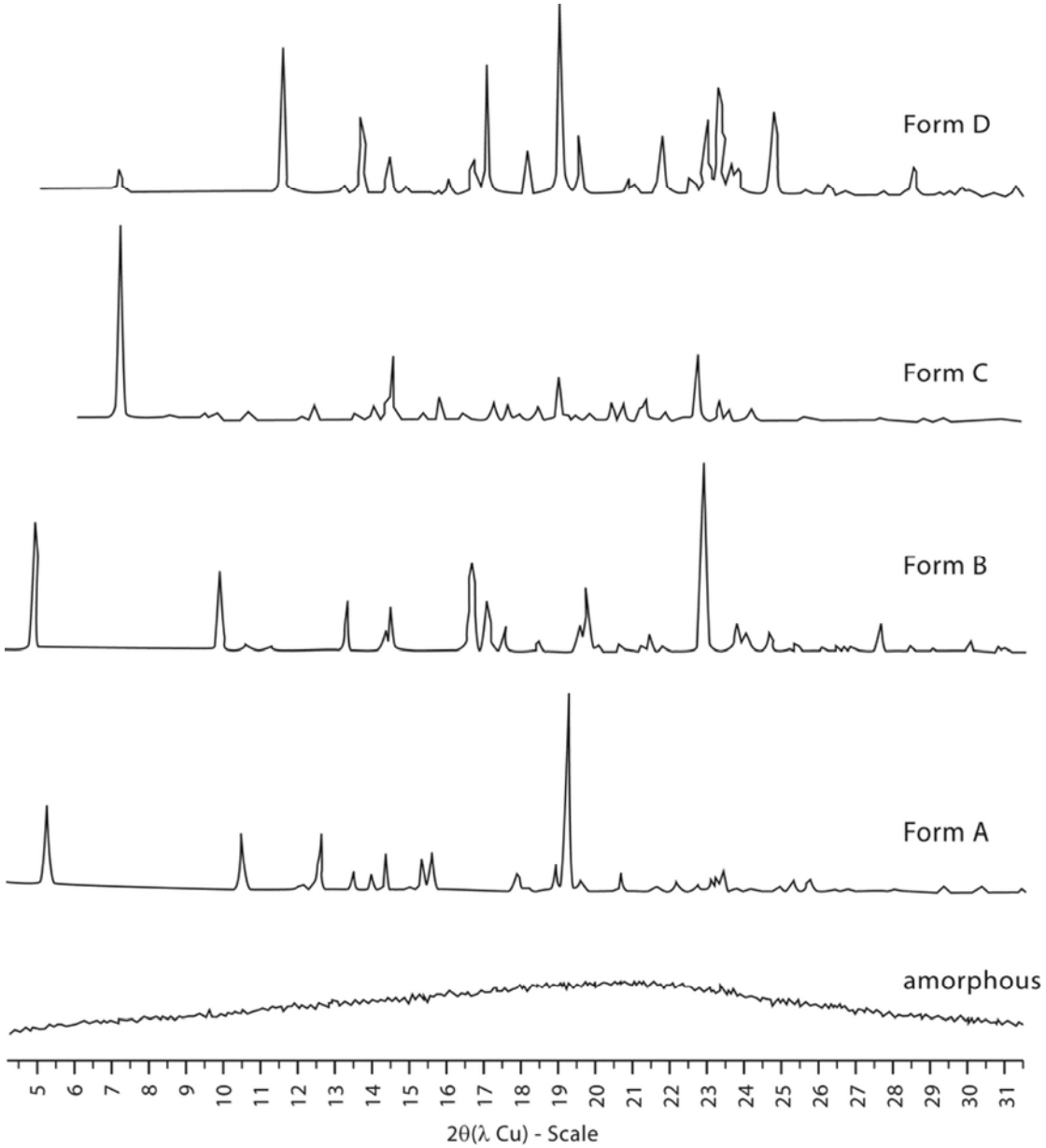


Figure 2. X-ray powder diffraction patterns collected for five different solid phases of a substance (the intensities are normalized).

In addition to the diffraction peaks, an X-ray diffraction experiment also generates a more or less uniform background, upon which the peaks are superimposed. Besides specimen preparation, other factors contribute to the background—for example, sample holder, diffuse scattering from air and equipment, and other instrumental parameters such as detector noise and general radiation from the X-ray tube. The peak-to-background ratio can be increased by minimizing background and by choosing prolonged exposure times.

INSTRUMENT

Instrument Setup

X-ray diffraction experiments are usually performed using powder diffractometers or powder cameras.

A powder diffractometer generally comprises five main parts: an X-ray source; the incident beam optics, which may perform monochromatization, filtering, collimation, and/or focusing of the beam; a goniometer; the diffraction beam optics, which may include monochromatization, filtering, collimation, and focusing or parallelizing of beam; and a detector. Data collection and data processing systems are also required and are generally included in current diffraction measurement equipment.

Depending on the type of analysis to be performed (phase identification, quantitative analysis, lattice parameters determination, etc.), different XRPD instrument configurations and performance levels are required. The simplest instruments used to measure powder patterns are powder cameras. Replacement

of photographic film as the detection method by photon detectors has led to the design of diffractometers in which the geometric arrangement of the optics is not truly focusing, but parafocusing, such as in Bragg-Brentano geometry. The Bragg-Brentano parafocusing configuration is currently the most widely used and is therefore briefly described here.

A given instrument may provide a horizontal or vertical $\theta/2\theta$ geometry or a vertical θ/θ geometry. For both geometries, the incident X-ray beam forms an angle θ with the specimen surface plane, and the diffracted X-ray beam forms an angle 2θ with the direction of the incident X-ray beam (an angle θ with the specimen surface plane). The basic geometric arrangement is represented in *Figure 3.* The divergent beam of radiation from the X-ray tube (the so-called primary beam) passes through the parallel plate collimators and a divergence slit assembly and illuminates the flat surface of the specimen. All the rays diffracted by suitably oriented crystallites in the specimen at an angle 2θ converge to a line at the receiving slit. A second set of parallel plate collimators and a scatter slit may be placed either behind or before the receiving slit. The axes of the line focus and of the receiving slit are at equal distances from the axis of the goniometer. The X-ray quanta are counted by a radiation detector, usually a scintillation counter, a sealed-gas proportional counter, or a position-sensitive solid-state detector such as an imaging plate or CCD detector. The receiving slit assembly and the detector are coupled together and move tangentially to the focusing circle. For $\theta/2\theta$ scans, the goniometer rotates the specimen around the same axis as that of the detector, but at half the rotational speed, in a $\theta/2\theta$ motion. The surface of the specimen thus remains tangential to the focusing circle. The parallel plate collimator limits the axial divergence of the beam and hence partially controls the shape of the diffracted line profile.

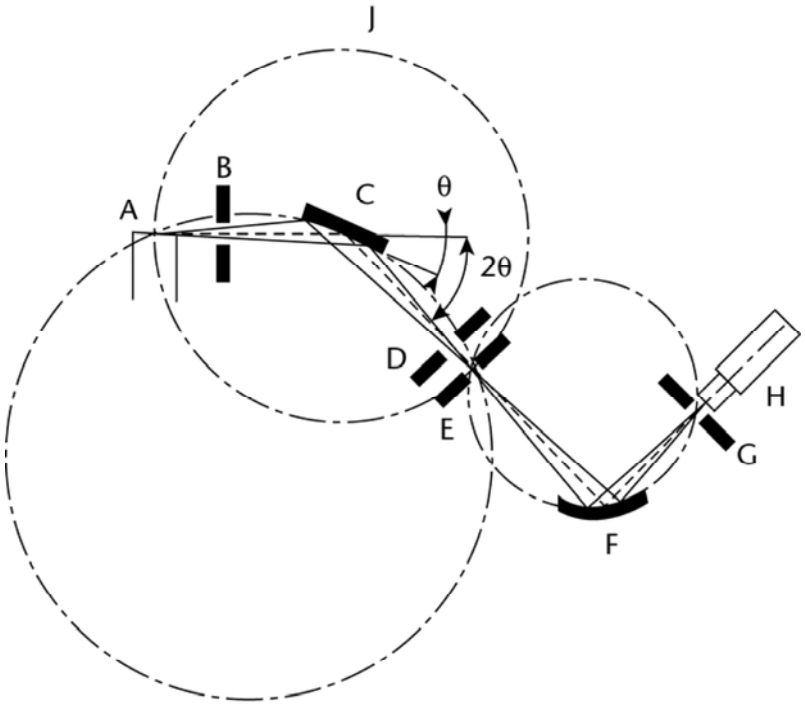


Figure 3. Geometric arrangement of the Bragg-Brentano parafocusing geometry.

A diffractometer may also be used in transmission mode. The advantage with this technology is to lessen the effects due to preferred orientation. A capillary of about 0.5- to 2-mm thickness can also be used for small sample amounts.

X-Ray Radiation

In the laboratory, X-rays are obtained by bombarding a metal anode with electrons emitted by the thermionic effect and accelerated in a strong electric field (using a high-voltage generator). Most of the kinetic energy of the electrons is converted to heat, which limits the power of the tubes and requires efficient anode cooling. A 20- to 30-fold increase in brilliance can be obtained by using rotating anodes and by using X-ray optics. Alternatively, X-ray photons may be produced in a large-scale facility (synchrotron).

The spectrum emitted by an X-ray tube operating at sufficient voltage consists of a continuous background of polychromatic radiation and additional characteristic radiation that depends on the type of anode. Only this characteristic radiation is used in X-ray diffraction experiments. The principal radiation sources used for X-ray diffraction are vacuum tubes using copper, molybdenum, iron, cobalt, or chromium as anodes; copper, molybdenum, or cobalt X-rays are employed most commonly for organic substances (the use of a cobalt anode can especially be preferred to separate distinct X-ray lines). The choice of radiation to be used depends on the absorption characteristics of the specimen and possible fluorescence by atoms present in the specimen. The wavelengths used in powder diffraction generally correspond to the K_α radiation from the anode. Consequently, it is advantageous to make the X-ray beam "monochromatic" by eliminating all the other components of the emission spectrum. This can be partly achieved using K_β filters—that is, metal filters selected as having an absorption edge between the K_α and K_β wavelengths emitted by the tube. Such a filter is usually inserted between the X-ray tube and the specimen. Another more commonly used way to obtain a monochromatic X-ray beam is via a large monochromator crystal (usually referred to as a "monochromator"). This crystal is placed before or behind the specimen and diffracts the different characteristic peaks of the X-ray beam (i.e., K_α and K_β) at different angles so that only one of them may be selected to enter into the detector. It is even possible to separate $K_{\alpha 1}$ and $K_{\alpha 2}$ radiations by using a specialized monochromator. Unfortunately, the

gain in getting a monochromatic beam by using a filter or a monochromator is counteracted by a loss in intensity. Another way of separating K_α and K_β wavelengths is by using curved X-ray mirrors that can simultaneously monochromate and focus or parallelize the X-ray beam.

RADIATION PROTECTION

Exposure of any part of the human body to X-rays can be injurious to health. It is therefore essential that whenever X-ray equipment is used, adequate precautions be taken to protect the operator and any other person in the vicinity. Recommended practice for radiation protection as well as limits for the levels of X-radiation exposure are those established by national legislation in each country. If there are no official regulations or recommendations in a country, the latest recommendations of the International Commission on Radiological Protection should be applied.

SPECIMEN PREPARATION AND MOUNTING

The preparation of the powdered material and the mounting of the specimen in a suitable holder are critical steps in many analytical methods, particularly for X-ray powder diffraction analysis, since they can greatly affect the quality of the data to be collected.[3] The main sources of errors due to specimen preparation and mounting are briefly discussed in the following section for instruments in Bragg-Brentano parafocusing geometry.

Specimen Preparation

In general, the morphology of many crystalline particles tends to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is particularly evident for needle-like or platelike crystals when size reduction yields finer needles or platelets. Preferred orientation in the specimen influences the intensities of various reflections so that some are more intense and others less intense, compared to what would be expected from a completely random specimen. Several techniques can be employed to improve randomness in the orientation of crystallites (and therefore to minimize preferred orientation), but further reduction of particle size is often the best and simplest approach. The optimum number of crystallites depends on the diffractometer geometry, the required resolution, and the specimen attenuation of the X-ray beam. In some cases, particle sizes as large as 50 μm will provide satisfactory results in phase identification. However, excessive milling (crystallite sizes less than approximately 0.5 μm) may cause line broadening and significant changes to the sample itself, such as

- specimen contamination by particles abraded from the milling instruments (mortar, pestle, balls, etc.),
- reduced degree of crystallinity,
- solid-state transition to another polymorph,
- chemical decomposition,
- introduction of internal stress, and
- solid-state reactions.

Therefore, it is advisable to compare the diffraction pattern of the nonground specimen with that corresponding to a specimen of smaller particle size (e.g., a milled specimen). If the X-ray powder diffraction pattern obtained is of adequate quality considering its intended use, then grinding may not be required.

It should be noted that if a sample contains more than one phase and if sieving is used to isolate particles to a specific size, the initial composition may be altered.

Specimen Mounting

EFFECT OF SPECIMEN DISPLACEMENT

A specimen surface that is offset by D with reference to the diffractometer rotation axis causes systematic errors that are very difficult to avoid entirely; for the reflection mode, this results in absolute $D \cdot \cos\theta$ shifts[4] in 2θ positions (typically of the order of 0.01° in 2θ at low angles

$$[\cos\theta \simeq 1]$$

for a displacement D = 15 μm) and asymmetric broadening of the profile toward low 2θ values. Use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen transparency. This effect is by far the largest source of errors in data collected on well-aligned diffractometers.

EFFECT OF SPECIMEN THICKNESS AND TRANSPARENCY

When the XRPD method in reflection mode is applied, it is often preferable to work with specimens of “infinite thickness”. To minimize the transparency effect, it is advisable to use a nondiffracting substrate (zero background holder)—for example, a plate of single crystalline silicon cut parallel to the 510 lattice planes.[5] One advantage of the transmission mode is that problems with sample height and specimen transparency are less important.

The use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen displacement.

CONTROL OF THE INSTRUMENT PERFORMANCE

The goniometer and the corresponding incident and diffracted X-ray beam optics have many mechanical parts that need adjustment. The degree of alignment or misalignment directly influences the quality of the results of an XRPD investigation. Therefore, the different components of the diffractometer must be carefully adjusted (optical and mechanical systems, etc.) to adequately minimize systematic errors, while optimizing the intensities received by the detector. The search for maximum intensity and maximum resolution is always antagonistic when aligning a diffractometer. Hence, the best compromise must be sought while performing the alignment procedure. There are many different configurations, and each supplier's equipment requires specific alignment procedures. The overall diffractometer performance must be tested and monitored periodically, using suitable certified reference materials. Depending on the type of analysis, other well-defined reference materials may also be employed, although the use of certified reference materials is preferred.

QUALITATIVE PHASE ANALYSIS (IDENTIFICATION OF PHASES)

The identification of the phase composition of an unknown sample by XRPD is usually based on the visual or computer-assisted comparison of a portion of its X-ray powder pattern to the experimental or calculated pattern of a reference material. Ideally, these reference patterns are collected on well-characterized single-phase specimens. This approach makes it possible in most cases to identify a crystalline substance by its 2θ-diffraction angles or d-spacings and by its relative intensities. The computer-aided comparison of the diffraction pattern of the unknown sample to the comparison data can be based on either a more or less extended 2θ range of the whole diffraction pattern or on a set of reduced data derived from the pattern. For example, the list of d-spacings and normalized intensities, I_{norm}, a so-called (d, I_{norm}) list extracted from the pattern, is the crystallographic fingerprint of the material and can be compared to (d, I_{norm}) lists of single-phase samples compiled in databases.

For most organic crystals, when using Cu K_α radiation, it is appropriate to record the diffraction pattern in a 2θ-range from as near 0° as possible to at least 40°. The agreement in the 2θ-diffraction angles between specimen and reference is within 0.2° for the same crystal form, while relative intensities between specimen and reference may vary considerably due to preferred orientation effects. By their very nature, variable hydrates and solvates are recognized to have varying unit cell dimensions, and as such, shifting occurs in peak positions of the measured XRPD patterns for these materials. In these unique materials, variance in 2-θ positions of greater than 0.2° is not unexpected. As such, peak position variances such as 0.2° are not applicable to these materials. For other

types of samples (e.g., inorganic salts), it may be necessary to extend the 2θ region scanned to well beyond 40°. It is generally sufficient to scan past the 10 strongest reflections identified in single-phase X-ray powder diffraction database files.

It is sometimes difficult or even impossible to identify phases in the following cases:

- noncrystallized or amorphous substances,
- the components to be identified are present in low mass fractions of the analyte amounts (generally less than 10% m/m),
- pronounced preferred orientation effects,
- the phase has not been filed in the database used,
- the formation of solid solutions,
- the presence of disordered structures that alter the unit cell,
- the specimen comprises too many phases,
- the presence of lattice deformations,
- the structural similarity of different phases.

QUANTITATIVE PHASE ANALYSIS

If the sample under investigation is a mixture of two or more known phases, of which not more than one is amorphous, the percentage (by volume or by mass) of each crystalline phase and of the amorphous phase can in many cases be determined. Quantitative phase analysis can be based on the integrated intensities, on the peak heights of several individual diffraction lines,[6] or on the full pattern. These integrated intensities, peak heights, or full-pattern data points are compared to the corresponding values of reference materials. These reference materials must be single phase or a mixture of known phases. The difficulties encountered during quantitative analysis are due to specimen preparation (the accuracy and precision of the results require, in particular, homogeneity of all phases and a suitable particle size distribution in each phase) and to matrix effects.

In favorable cases, amounts of crystalline phases as small as 10% may be determined in solid matrices.

Polymorphic Samples

For a sample composed of two polymorphic phases *a* and *b*, the following expression may be used to quantify the fraction F_α of phase *a*:

$$F_\alpha = 1/[1 + K(I_b/I_\alpha)]$$

The fraction is derived by measuring the intensity ratio between the two phases, knowing the value of the constant K. K is the ratio of the absolute intensities of the two pure polymorphic phases I_{oa}/I_{ob}. Its value can be determined by measuring standard samples.

Methods Using a Standard

The most commonly used methods for quantitative analysis are

- the external standard method,
- the internal standard method, and
- the spiking method (also often called the standard addition method).

The external standard method is the most general method and consists of comparing the X-ray diffraction pattern of the mixture, or the respective line intensities, with those measured in a reference mixture or with the theoretical intensities of a structural model, if it is fully known.

To limit errors due to matrix effects, an internal reference material can be used that has a crystallite size and X-ray absorption coefficient comparable to those of the components of the sample and with a diffraction pattern that does not overlap at all that of the sample to be analyzed. A known quantity of this reference material is added to the sample to be analyzed and to each of the reference mixtures. Under these conditions, a linear relationship between line intensity and concentration exists. This application, called the internal standard method, requires precise measurement of diffraction intensities.

In the spiking method (or standard addition method), some of the pure phase a is added to the mixture containing the unknown concentration of a. Multiple additions are made to prepare an intensity-versus-concentration plot in which the negative x-intercept is the concentration of the phase a in the original sample.

ESTIMATE OF THE AMORPHOUS AND CRYSTALLINE FRACTIONS

In a mixture of crystalline and amorphous phases, the crystalline and amorphous fractions can be estimated in several ways. The choice of the method used depends on the nature of the sample:

- If the sample consists of crystalline fractions and an amorphous fraction of different chemical compositions, the amounts of each of the individual crystalline phases may be estimated using appropriate standard substances, as described above. The amorphous fraction is then deduced indirectly by subtraction.
- If the sample consists of one amorphous and one crystalline fraction, either as a 1-phase or a 2-phase mixture, with the same elemental composition, the amount of the crystalline phase (the “degree of crystallinity”) can be estimated by measuring three areas of the diffractogram:

  A = total area of the peaks arising from diffraction from the crystalline fraction of the sample,
  B = total area below area A,
  C = background area (due to air scattering, fluorescence, equipment, etc).

When these areas have been measured, the degree of crystallinity can be roughly estimated as:

$$\% \text{ crystallinity} = 100A/(A + B - C)$$

It is noteworthy that this method does not yield an absolute degree of crystallinity values and hence is generally used for comparative purposes only. More sophisticated methods are also available, such as the Ruland method.

SINGLE CRYSTAL STRUCTURE

In general, the determination of crystal structures is performed from X-ray diffraction data obtained using single crystals. However, crystal structure analysis of organic crystals is a challenging task, since the lattice parameters are comparatively large, the symmetry is low, and the scattering properties are normally very low. For any given crystalline form of a substance, the knowledge of the crystal structure allows for calculating the corresponding XRPD pattern, thereby providing a preferred orientation-free reference XRPD pattern, which may be used for phase identification.

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[1] There are many other applications of the X-ray powder diffraction technique that can be applied to crystalline pharmaceutical substances, such as determination of crystal structures, refinement of crystal structures, determination of the crystallographic purity of crystalline phases, and characterization of crystallographic texture. These applications are not described in this chapter.

[2] An ideal powder for diffraction experiments consists of a large number of small, randomly oriented spherical crystallites (coherently diffracting crystalline domains). If this number is sufficiently large, there are always enough crystallites in any diffracting orientation to give reproducible diffraction patterns.

[3] Similarly, changes in the specimen can occur during data collection in the case of a nonequilibrium specimen (temperature, humidity).

[4] Note that a goniometer zero alignment shift would result in a constant shift on all observed 2θ-line positions; in other words, the whole diffraction pattern is, in this case, translated by an offset of Z° in 2θ.

[5] In the case of a thin specimen with low attenuation, accurate measurements of line positions can be made with focusing diffractometer configurations in either transmission or reflection geometry. Accurate measurements of line positions on specimens with low attenuation are preferably made using diffractometers with parallel beam optics. This helps to reduce the effects of specimen thickness.

[6] If the crystal structures of all components are known, the Rietveld method can be used to quantify them with good accuracy. If the crystal structures of the components are not known, the Pawley method or the partial least-squares (PLS) method can be used.

Auxiliary Information- Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
<941> CHARACTERIZATION OF CRYSTALLINE AND PARTIALLY CRYSTALLINE SOLIDS BY X-RAY POWDER DIFFRACTION (XRPD)	Edmond Biba Senior Scientific Liaison +1 (301) 230-3270	GCPA2015 General Chapters-Physical Analysis 2015

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Señora Directora

DIRECCION DE NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D

Referencia:	Expediente No. NC2017/0011295
Asunto:	Solicitud de patente para: PIROGLUTAMATO DE VORTIOXETINA
Solicitante:	H. LUNDBECK A/S
Fecha de solicitud:	02 de noviembre de 2017

RESPUESTA AL PRIMER EXAMEN DE PATENTABILIDAD

ANDRÉS RICNÓN USCÁTEGUI, identificado como aparece al pie de mi firma, en mi condición de apoderado de la sociedad solicitante de la patente de la referencia, doy respuesta al Oficio 2756, notificado por fijación en lista el 17 de abril de 2019.

I. MODIFICACIONES AL CAPÍTULO REIVINDICATORIO

En respuesta al examen de patentabilidad, aporto un Capítulo Reivindicatorio Modificado, conformado por 10 Reivindicaciones, en las que se han incluido las siguientes modificaciones:

- En la Reivindicación 1, se modifica el preámbulo de la reivindicación con el fin de dar mayor claridad al objeto reclamado en la invención. De igual forma se modifican los preámbulos de las Reivindicaciones 2-5, las cuales son dependientes de la Reivindicación 1.
- En la Reivindicación 3 y 4 se incluyen señales adicionales del patrón de rayos X, para el (L)-piroglutamato de vortioxetina o el (D)-piroglutamato de vortioxetina y el (DL)-piroglutamato de vortioxetina monohidratado. Esta modificación tiene sustento en los Ejemplos 11 y 13 del Capítulo Descriptivo de la presente invención.
- En la Reivindicación 6 se remplaza la expresión *"al menos"* por la expresión *"uno o más*".

- El Capitulo Reivindicatorio Modificado es nuevamente reorganizado con el fin de hacer más claro las reivindicaciones dependientes y el alcance de la invención.

Me permito resaltar que estas modificaciones se ajustan completamente a lo establecido por el Artículo 34 de la Decisión 486 de 2000, ya que no constituyen una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial

II. EN CUANTO A LA SUPUESTA FALTA DE CLARIDAD

El Examinador afirma que la Reivindicación 1 no es clara debido a que el objeto de la invención consiste en reclamar la forma cristalina (L), (D) y (DL) del compuesto piroglutamato de vortioxetina; así las cosas es necesario definir adecuadamente cada una de las tres formas cristalinas reclamadas.

En este sentido difiero de la opinión del Examinador, toda vez que dentro de campo de la Química es conocido que un compuesto puede o no presentar formas cristalinas de su estructura. En este sentido el solicitante tiene derecho a reclamar tanto las formas cristalinas de la estructura reivindicada como los compuestos amorfos de la misma. Así mismo, un inventor está en todo su derecho de reclamar protección para la totalidad del concepto inventivo desarrollado, el cual en este caso corresponde a todas las posibles formas estructurales del piroglutamato de vortioxetina y no existe obligación alguna de realizar una limitación exclusivamente a las formas cristalinas reclamadas en las modalidades preferidas de la invención.

Adicionalmente, el Examinador parece creer que la invención está limitada únicamente a las formas cristalinas del piroglutamato de vortioxetina. En cuanto a este particular, vale la pena resaltar que el alcance de la invención está dirigido a las propiedades de formación de gel del piroglutamato de vortioxetina y no a sus formas cristalinas como el Examinador indica. Esto es más claro a la luz de los ejemplos descritos en el Capítulo Descriptivo, en los cuales se muestra en detalle que el piroglutamato de vortioxetina (D), (L) y (DL) en solución posee las propiedades gelificantes deseadas. En los ejemplos en solución de la invención, todas las propiedades cristalinas han desaparecido, lo que demuestra que las propiedades de la invención no están limitadas a formas cristalinas específicas.

En este orden de ideas, restringir la materia reclamada únicamente a las formas cristalinas (L), (D) y (DL) del compuesto piroglutamato de vortioxetina limitaría injustificadamente el alcance de la protección a la cual el inventor tiene derecho, más aún, si el arte previo citado no anticipa las enseñanzas de la presente invención. De acuerdo con lo anterior, esta objeción de claridad debe darse por superada.

Respecto a la objeción elevada por el Examinador en lo referente a la claridad de las Reivindicaciones 3 a 5, me permito señalar que el mismo Examinador reconoce la novedad del compuesto de piroglutamato de vortioxetina, al no citar arte previo que afecte la patentabilidad de estas reivindicaciones (el Examinador solo cita a D1 como arte previo que anticipa la novedad de la Reivindicación 1), por lo tanto, no hay arte previo que divulgue o comprenda otras sales de piroglutamato de vortioxetina frente al cual sea necesario mayor diferenciación. Es más, como se evidencia en las Reivindicaciones 3, 4 y 5, y en las Figuras 1, 5 y 9, las reflexiones del patrón de difracción de rayos X (XRPD) de los tres polimorfos reclamados son distintos entre sí y pueden ser fácilmente diferenciados con base en la lista de reflexiones incluida en las Reivindicaciones 3 a 5 tal y como se presentaron. Adicionalmente, en cuanto a los valores de intensidad solicitados por el Examinador, vale la pena resaltar que según la Farmacopea de EE.UU para XRPD[1]. (Documento adjunto a este memorial), en la sección resaltada se menciona que se espera que las intensidades de los picos varíen considerablemente y, por lo tanto, no se pueden usar para definir una forma cristalina. Así las cosas, incluir las intensidades en las Reivindicaciones 3 a 5 no dan claridad al alcance de la invención.

Sin embargo, para facilitar el trámite de la presente solicitud, llamo respetuosamente la atención del Examinador en cuanto al Capítulo Reivindicatorio Modificado adjunto a este memorial donde las nuevas Reivindicaciones 3, 4 y 5 incluyen reflexiones adicionales del espectro XPRD. De acuerdo con lo anterior, esta objeción de claridad debe darse por superada.

Tomando en consideración todo lo expuesto anteriormente me permito resaltar que, en virtud de las modificaciones realizadas, el Capítulo Reivindicatorio al igual que el Capítulo Descriptivo presentados junto con este memorial son completamente claros y concisos al reclamar un compuesto definido, ajustándose de esta forma a lo exigido por el artículo 34 de la Decisión Andina 486 de 2000

[1] Pappa, H. (2009). (941) Characterization of crystalline and partially crystalline solids BY X-ray powder diffraction (XRPD). 35. 731-740.

III. ESTADO DE LA TÉCNICA

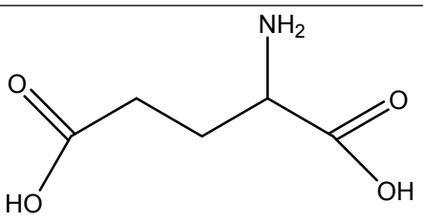
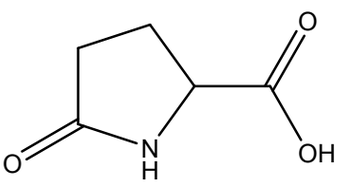
El Examinador indica que los siguientes documentos son relevantes para la patentabilidad de la presente solicitud:

No.	Documento	Título	Fecha de Publicación	Fecha de Presentación	Fecha de Prioridad
D1	15194209	*"Proceso de preparación de vortioxetina"*	N/A	18 de agosto de 2015	22 de marzo de 2013
D2	WO2007144005	*"1- [2- (2, 4-dimethyl phenylsulfanyl) -phenyl] piperazine as a compound with combined serotonin reuptake, 5-ht3 and 5-ht1a activity for the treatment of cognitive impairment"*	12 de diciembre de 2007	N/A	N/A
D3	GB1006728	*"Amine salts of pyrrolidone carboxylic acid"*	06 de octubre de 1965	N/A	N/A

V. EN CUANTO A LA SUPUESTA FALTA DE NOVEDAD

El Examinador establece que la Reivindicación 1 de la presente solicitud carece de novedad de acuerdo con lo divulgado en D1.

Difiero de la opinión del Examinador. En primer lugar, según el Examinador, D1 describe la sal de ácido glutámico de vortioxetina al igual que la Reivindicación 1 de la presente solicitud. En cuanto a este particular, deseo señalar que la sal del ácido glutámico tiene una estructura molecular diferente a las sales del ácido **piro**glutámico; es decir, no son idénticos por lo que el análisis del Examinador es desacertado.

ácido piroglutámico	ácido glutámico
	

Adicionalmente, El Examinador cita a *Mena et al*[2] para demostrar supuestamente un equilibrio existente entre los dos compuestos anteriores. En cuanto a este particular, deseo señalar que en la segunda columna, página 92 de este mismo documento, se analiza la formación de glutamato en una solución de piroglutamato y se concluye que

> *"a pH neutro, la constante de equilibrio favorece mucho más la formación de piroglutamato, y no la formación de glutamato". PP. 92. Col 2*

Lo cual es totalmente coherente con los datos experimentales presentados en la Tabla 2 de dicho documento (Figura 1). Los datos de la Tabla 2 muestran que una solución de L-glutamato **no forma piro**glutamato a lo largo del tiempo. El nivel de piroglutamato es esencialmente constante en todo el experimento, es decir, 1.4-1.2%. Como se explica en la oración que une las páginas 92 y 93, la señal del 1.4% es una contaminación presente desde el inicio del experimento. De manera similar, una solución de **piro**glutamato **no forma** glutamato con el tiempo. El nivel de glutamato permanece constante en el transcurso del experimento (0.3%), y nuevamente este nivel se explica por la contaminación.

El "*equilibrio químico*" al que hace referencia el Examinador y por lo cual compara la sal del ácido glutámico reclamada en D1, se ha demostrado que no existe por el mismo artículo citado por el Examinador como pie de página en condiciones fisiológicamente relevantes. Este equilibrio solo existe en condiciones muy específicas sin relevancia fisiológica. Esto es más claro a la luz de las enseñanzas del documento adjunto a este memorial Patente (US2004/0029955A1) *"Composition for use in prevention or treatment of vascular-related diseases"*, *Kouge et al.* Párrafo [0025]. En este documento se describe que el ácido glutámico y el ácido piroglutámico existen en equilibrio a temperaturas muy por encima de 130°C. De acuerdo con esto, el hecho de que el Compuesto B puede obtenerse en una reacción química a partir del Compuesto A, no anticipa la formación del Compuesto B a cualquier condición del medio de reacción.

También se puede observar en el Capítulo Descriptivo de la presente invención que los termogramas de las formas de piroglutamato obtenidos por Calorimetría Diferencial de Barrido (DSC) muestran claramente picos agudos indicativos de compuestos individuales, es decir, que no están en equilibrio con otros compuestos. El Ejemplo 11 y la Figura 3 de

[2] Mena, F. V., Baab, P. J., Zielke, C. L., Huang, Y., & Zielke, H. R. (2005). Formation of extracellular glutamate from glutamine: Exclusion of pyroglutamate as an intermediate. Brain Research, 1052(1), 88–96.

del Capítulo Descriptivo muestran un solo pico agudo a 138.9°C para el (L)-piroglutamato de vortioxetina. El Ejemplo 13 y la Figura 6 muestran una deshidratación inicial del (DL)-piroglutamato de vortioxetina monohidratado, para generar la forma α de (DL)-piroglutamato de vortioxetina que se funde mostrando un solo pico agudo. De manera similar, el Ejemplo 15 y la Figura 10 muestran que la forma α de (DL)-piroglutamato de vortioxetina se caracteriza por un solo pico agudo de DSC a 178.2°C.s

Table 2
Stability of [U-^{14}C]-L-glutamine, [U-^{14}C]-L-glutamate, and [U-^{14}C]-L-pyroglutamate after 8 h of incubation in aCSF at pH 7.0 and 37 °C

	0 h (%)	2 h (%)	8 h (%)
[U-^{14}C]-L-glutamine			
Glutamine	98.0	97.9	97.0
Pyroglutamate	1.8	2.0	2.8*
Glutamate	0.2	0.1	0.2
[U-^{14}C]-L-glutamate			
Glutamine	0	0.0	0.0
Pyroglutamate	1.4	1.4	1.2
Glutamate	98.6	98.6	98.8
[U-^{14}C]-L-pyroglutamate			
Glutamine	0.0	0.0	0.0
Pyroglutamate	99.7	99.7	99.7
Glutamate	0.3	0.3	0.3

Figura 1: Tabla 2 del Artículo *"Formation of extracellular glutamate from glutamine: Exclusion of pyroglutamate as an intermediate"*

Por lo tanto, los datos experimentales mostrados en la presente solicitud revelan claramente que la posición del Examinador sobre la existencia de un equilibrio relevante entre el piroglutamato y el glutamato de vortioxetina es infundada. Así las cosas, no cabe duda que ni D1 ni el articulo citado por el Examinador como pie de página divulgan el mismo compuesto reclamado en la Reivindicación 1 de la presente invención. En conclusión, la Reivindicación 1 de la presente invención es novedosa respecto a al arte previo citado por el Examinador.

VI. EN CUANTO A LA SUPUESTA FALTA DE NIVEL INVENTIVO

El Examinador considera que Reivindicaciones 2 a 10 carecen de nivel inventivo a la luz de las divulgaciones de D2 y D3. Respetuosamente difiero de la opinión del Examinador por las siguientes razones.

En primer lugar llamo la atención del Examinador en cuanto a que el efecto logrado por la sal de piroglutamato de vortioxetina se relaciona con las propiedades gelificantes demostradas en los Ejemplos del Capítulo Descriptivo. En los Ejemplos 1 a 9 de la presente invención se explica detalladamente la formación de un gel trasparente **estable** de piroglutamato de vortioxetina añadiendo una solución de bromuro de sodio, cloruro de sodio o bromuro de potasio. Las soluciones de piroglutamato de vortioxetina de diferentes tipos forman geles cuando se mezclan con una sal, por lo que es probable que el piroglutamato de vortioxetina sea útil en formulaciones de gel evitando la necesidad de polímeros gelificantes adicionales. El piroglutamato de vortioxetina proporciona, por lo tanto, una formulación de gel alternativa y más simple en comparación con la tecnología existente.

En cuanto al arte previo citado por el Examinador, deseo señalar que D2 divulga formas cristalinas de sales bromuro, cloruro, mesilato, maleato, (D/L)-tartrato, sulfato, fosfato, nitrato, glutamato, entre otras del compuesto vortioxetina (Págs. 5-6 y Pág. 55, Rv. 1-7 de D2). Sin embargo, D2 no menciona nada acerca del comportamiento de una sal de **piro**glutamato, ni mucho menos habla de sus propiedades gelificantes en solución. Por lo cual este documento no es un documento que pueda ser comparado con la presente invención debido a que, en primer lugar no describe el mismo compuesto de la presente solicitud y en segundo lugar busca caracterizar las formas cristalinas más que estudiar sus propiedades en solución.

En cuanto a D3, este documento divulga que las sales de amina del ácido carboxílico pirrolidona aumentan dramáticamente la solubilidad de los compuestos activos en agua, mejorando la dosificación y administración de los compuestos (Pág. 4, Líneas 106-128 de D3). Sin embargo, D3 no menciona específicamente la relación existente entre la solubilidad de la sal y las propiedades gelificantes de la misma. D3 revela una solubilidad aumentada de ciertas sales de piroglutamato. Sin embargo, D3 no extiende esta enseñanza a todas las sales de piroglutamato, y mucho menos a las sales de vortioxetina. Así las cosas, para una persona versada en la materia que busca desarrollar una formulación en gel de vortioxetina, no encontraría útil las enseñanzas de D3 debido a que no establece o menciona ningún vínculo entre las propiedades gelificantes y la solubilidad.

Adicionalmente, deseo hacer hincapié en el hecho del uso del artículo citado por el Examinador como pie de página *Mena et al* para establecer la falta de actividad inventiva.

En cuanto a este particular deseo señalar que esto no es más que una interpretación errónea por parte del Examinador de las enseñanzas de este artículo, como se explicó anteriormente, *Mena et al* no ha establecido un equilibrio relevante entre el glutamato y el piroglutamato de vortioxetina.

Las composiciones de gel para la administración oral dan solución a un problema conocido en la técnica debido a que es bien conocido en la técnica que tragar comprimidos y cápsulas puede ser un problema para un número significativo de pacientes, y esto puede conducir en última instancia a un incumplimiento con la ingesta del medicamento. Así las cosas, la presente invención al reclamar las sales de piroglutamato de vortioxetina y demostrar sus propiedades gelificantes para recubrimientos de fármacos, es no solo novedosa sino también es inventiva respecto a D1, D2 y D3.

En consecuencia, teniendo en cuenta lo anterior, considero pertinente señalar que el Manual para el Examen de Solicitudes de Patente de Invención en las Oficinas de Propiedad Industrial de los Países de la Comunidad Andina establece que se considera que las invenciones tienen nivel inventivo si la materia reclamada tiene una estructura inesperada y efectos sorprendentes o mejorados. En el presente caso, es evidente que las características técnicas esenciales de la invención no se podían prever a partir de las enseñanzas del arte previo citado por el Examinador sin llevar a cabo actividad inventiva.

Quisiera añadir que, acorde al Artículo 18 de la Decisión 486, una invención tiene nivel inventivo cuando no hubiese resultado obvia ni se hubiera derivado de manera evidente del estado de la técnica para la persona medianamente versada en la materia. Así entonces, el análisis de nivel inventivo no debería basarse en si la invención era -**posible de obtener**-, pues esto haría todas las invenciones obvias haría que el sistema de patentes fuera inviable. En otras palabras, lo que debería hacer parte del análisis no es lo que la persona medianamente versada en la materia podría haber hecho en la fecha de prioridad sino lo que habría hecho teniendo en cuenta de sus conocimientos en el área y lo enseñado en el arte previo. Además, no puede basarse el análisis tampoco en si la invención es -**mejor**- que el arte previo o si presenta efectos mejorados, o si existe un documento que invite al científico a llevar a cabo investigación, sino únicamente en si la invención resulta obvia o evidentemente derivable del arte previo, lo cual, como se explicó anteriormente, no aplica para el presente caso.

En conclusión, una persona medianamente versada en la materia reconocería que la presente invención es inventiva, toda vez que el arte previo citado en efecto no es suficiente para brindar indicaciones acerca de la posibilidad de desarrollar la invención de manera específica, por lo que es claro que dicha invención no puede ser considerada obvia a partir de dicho arte previo.

IV. PETICIÓN

1. Puesto que se ha dado respuesta a todas las objeciones formuladas por la Dirección de Nuevas Creaciones, solicito proceder al otorgamiento de la solicitud de patente.

2. De manera subsidiaria, y en caso de que su Despacho considere que la modificación introducida al Capítulo Reivindicatorio amerita revisión adicional, solicito un segundo examen de patentabilidad de conformidad con lo dispuesto en el Artículo 2 de la Resolución 3719 de 02 de febrero de 2016, para lo cual realizo el pago de tasa correspondiente.

V. ANEXOS

1. Capítulo Reivindicatorio modificado conformado por 10 Reivindicaciones;
2. Pago en línea por concepto de examen de patentabilidad adicional.
3. Apartado de Farmacopea EE.UU para XRPD
4. Patente (US2004/0029955A1) *"Composition for use in prevention or treatment of vascular-related diseases",* Kouge et al.

Señora Directora,

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