The present invention relates to lipid microparticles of crystalline appearance called microcrystals. It relates to microcrystals comprising a substance which is insoluble in water having an affinity for phospholipids, and to at least one phospholipid.

The present invention also relates to a process for the preparation of microcrystals comprising a substance which is insoluble in water having an affinity for phospholipids. It relates to the microcrystals obtained, as well as to pharmaceutical compositions containing them.

The process is characterised in that it comprises the following steps:

a) the solvent(s) is(are) evaporated from a solution of phospholipid(s) and the said substance,
b) the film obtained after evaporation of the solvent(s) is resuspended in an aqueous solution after vigorous stirring.

In the present application, 'microcrystals' are understood to mean lipid microparticles of crystalline appearance obtained particularly by the above process or a process derived therefrom, it being understood that the crystalline structure is not necessarily obtained in the strict sense of the word.

In this process, 'substance which is insoluble in water', should be understood to mean a substance which is sparingly soluble or insoluble in water and 'substance having an affinity for phospholipids' to mean a chemical compound more particularly capable of interacting physically with phospholipids. Examples will be given below.

The article in the Journal of Pharmaceutical Sciences, Vol. 73 (1984), No. 6 describes microcrystals of griseofulvin and of dimyristoylphosphatidylcholine which are of low stability. The article, moreover, does not describe any advantageous therapeutic application.

The microcrystals produced according to the invention offer principally the advantage of obtaining a

microsuspension which is stable in aqueous solution of microcrystals of a substance which is otherwise insoluble which permits the said substance to be administered, when it is a medicament, in injectable or pulverizable form. Compared with liposomes, preparations in the form of microcrystals, according to the invention, are firstly simpler and much more economical to prepare to the extent that they involve the presence of a reduced number of constituents. They are much more stable, whereas the instability of liposomes considerably limits their conditions of use.

In addition, apparently by virtue of an increased piloting effect particularly towards macrophages on the one hand, and a gradual release effect of the incorporated active substance, on the other hand, the microcrystal preparations give results of therapeutic activity and of toxicity which are also considerably more advantageous than vesicles of the liposome type.

In general, according to the invention, phosphatidylcholine will preferably be used as phospholipid. If necessary, however, it can be used in combination with other known elements, for example, in the preparation of liposomes, namely sterols such as cholesterol or alternatively stearylamine.

However, since the formation of microcrystals is concerned, the molar ratio (phospholipid(s)/substance) is of determining influence with respect particularly to the molar ratio (phospholipid(s)/sterol(s)) for example, which does not have an influence on the latter.

In certain cases however, it may be advantageous to add one or more sterols to reinforce the activity of the insoluble active substance to be incorporated.

The molar ratio (phospholipid(s)/substance) must be less than that beyond which only liposome-type vesicles are observed.

This is because, if the molar ratio (phospholipid(s)/substance) engaged in the process according to the invention is too high, in particular higher than 10 for example, essentially the formation of lipid vesicles

resembling liposomes is observed.

On the other hand, if the molar ratio is less than or equal to 2, preferably close to 1, a specific interaction is observed, with the homogeneous production of microcrystals whose average size is between 0.1 µm and 1 µm.

For intermediate ratios, mixtures of lipid vesicles and of microcrystals are obtained.

The molar ratio between the phospholipid(s) or one or more sterol(s), as the case may require, like cholesterol, does not have a determining influence on the formation of microcrystals. However, in certain cases, it may be advantageous to add one or more sterol(s) to reinforce the activity of the active substance.

According to a variant of the process, step a) of the process is subdivided as follows:

a1) the solvent(s) is(are) evaporated from the solution of phospholipid(s),

a2) the lipid film obtained after evaporation is redissolved by adding a solution of the said substance, the solvent(s) is(are) evaporated from the solution of phospholipid(s) and the said substance.

In the process, the molar ratio between the phospholipid(s) on the one hand, and the said substance on the other hand, is preferably between 0.1 and 2 and preferably between 0.8 and 1.2, or alternatively close to 1.

When a sterol is associated with the substance to be processed, the molar ratio in the process between the phospholipid(s) and the sterol(s) is preferably between 1 and 2.

To resuspend the film obtained in step b), this may be carried out by various known techniques, but it is advantageous to be able to use treatment by ultrasound.

In a particular embodiment of the invention, the solvents used are the usual organic solvents, such as chloroform or methanol or alternatively solvent mixtures, for example of chloroform and methanol.

For example, the solution of phospholipid is a

solution in chloroform and the solution of the substance concerned is a solution in a chloroform/methanol mixture. The sterol solution, as the case may require, can be a methanol solution.

According to another characteristic of the process, the microcrystals are purified by centrifugation and washing with distilled water or with an aqueous solution.

The present invention relates, for example, to microcrystals comprising as active substance ginkgolides.

The best known of the ginkgolides are ginkgolides A, B, C and M which are used in therapy for the treatment and prevention of disorders caused by the PAF-acether as described in Belgian Patent 901915. Among these compounds, ginkgolide B has proved to be particularly advantageous.

Ginkgolide B is of very low solubility; this constitutes a drawback when it is desired to incorporate it in certain pharmaceutical compositions, in particular injectable compositions. Moreover, it is advantageous to have available pharmaceutical compositions capable of conveying the active substance to the activity sites and thus increasing the specificity of the active substance whilst reducing undesirable effects as much as possible.

It is an object of the present invention to resolve the problems discussed above with the novel crystalline form called "microcrystals".

In the particular case mentioned, the insoluble substances according to the invention will be selected from ginkgolides and their derivatives and more particularly will be ginkgolide B. Although among the ginkgolides, ginkgolide B is preferred, it is also possible to use the derivatives, such as monoacetates, triacetates, and tetrahydro- or acetylated derivatives.

Ginkgolides are substances which are involved in the treatment of diseases caused by the acether of the "platelet activating factor".

The acether of the "platelet activating factor" (PAF-acether) is a phospholipid which can be the cause of

numerous diseases in humans or in animals. The acether of the "platelet activating factor" causes the aggregation of the platelets, as well as the release of their baso-active amine. The release is caused in animals and humans under the effect of different types of shock, such as anaphylactic shock, burns, septic shock, shock through irradiation and trauma. Its release leads subsequently to a suppression of immune reactions following the exhaus-tion of the defence means of the body. In fact, since its identification (1-O-alkyl-2(R)-acetyl-glycero-3-phos-phorylcholine) and its total synthesis, proof of its involvement in certain physiopathological processes has accumulated, principally in pulmonary anaphylaxis and different states of shock.

Numerous specific antagonists of PAF-acether are known. Of these, two main series may be distinguished:

1) substances having a structure similar to that of PAF-acether,

2) natural substances with a specific antagonistic activity.

Among the second category are to be found on the one hand lignans and neolignans such as kadsurenone (Piper futokadsurae), magnosalicine (Magnolia salicifolia), nectandrines A and B (Nectandra rigida) and synthetic dinorlignan L-652.731 and, on the other hand, terpenoides isolated from Ginko biloba (Ginkgolides).

However, these substances which are antagonists of the acether of "the platelet activating factor" have a major drawback, in that they are very insoluble, which makes their use in fact almost non-existent since it is dependent in practice on administration by perfusion.

Quite surprisingly, it has been discovered according to the invention, that the ginkgolides may be rendered injectable by interacting with phospholipids in the form of microcrystals.

The same is true of other substances which are antagonists of the acether of the "platelet activating factor"; these are hydrophobic substances which interact

with phospholipids. The majority of these substances are in fact known, on the one hand, to be hydrophobic and, on the other hand, also show an affinity for phospholipids and hence an aptitude for the formation of microcrystals with phospholipids, these microcrystals constituting a microsuspension which is stable in aqueous solution.

The affinity of substances which are antagonists of the acether of "platelet activating factor" for phospholipids is doubtless the cause of their inhibitory action on PAF-acether which has, as has been seen, also a phospholipid structure. As has been discovered by the applicant, this affinity offers the possibility of injecting these substances by the preparation of microcrystals. However, in addition, it is possible that the similarity of the phospholipid structures on the one hand, of the microcrystals formed and, on the other hand, of the PAF-acether also contributes to greater therapeutic activity of the pharmaceutical compositions containing these microcrystals of substances which are antagonists of PAF-acether.

According to another example of the present invention, the active substance concerned may therefore be chosen from substances which are antagonists of the acether of "platelet activating factor".

In this process, substances which are antagonists of the acether of "platelet activating factor" should be understood to mean a substance enabling the effective treatment of diseases caused by the acether of the "platelet activating factor".

More particularly, according to the present invention, substances which are antagonists of the acether of the "platelet activating factor" can be chosen from synthetic products having a structure similar to that of "platelet activating factor" or natural products, such as terpenoids, lignans and neolignans.

Particularly among lignans and neolignans, the substances which are antagonists of the acether of the "platelet activating factor" are selected from among kadsurenone, magnosalicine, nectandrines A and B and synthetic dinorlignan L-652.731.

Among the synthetic products, the derivatives of canthene 5, 6 may be mentioned.

According to another example of the present invention, the active substance concerned may be chosen from amphotericin B and derivatives thereof which have an antifungal activity. A description of certain of these compounds will be found in the literature [1].

Amphotericin B and its derivatives, particularly aminoacyl derivatives, continue to be the medicaments of choice for the treatment of numerous deep fungal infections, despite their severe toxicity and considerable unpleasant side-effects.

It has already been proposed to encapsulate amphotericin B in liposomes in order to reduce its toxicity and increase its activity. From this point of view however, the results obtained, although positive, remain inadequate.

The microcrystals of amphotericin B or of its derivatives enable these disadvantages of high toxicity to be alleviated, whilst increasing the antifungal activity of the said substances, distinctly more effectively than liposomes, probably inter alia by virtue of an improved piloting effect of the substance towards target cells like macrophages, as well as an improved effect of gradual release of the active substance. In addition, the process for the preparation of microcrystals is generally simpler to perform than encapsulation in liposomes and the preparations obtained are more stable.

The preparation of microcrystals also permits amphotericin B to be injected without the use of the deoxycholate as in fungizone®, the latter being toxic.

Amphotericin has an affinity for sterols, which is why, in one embodiment of the process according to the present invention, amphotericin B or one of its derivatives is supplemented with sterol(s), particularly cholesterol (Ch) or ergosterol.

Advantageously in step B of the process, when the substance concerned is an anti-PAF-acether agent, the film obtained after evaporation of the solvent is resuspended in a solution buffered at a pH of between 5 and 8, preferably at pH 5.9, for example in an acetate or phosphate buffer.

In step b) of the process, when the substance is amphotericin B, particularly in the absence of sterol, the aqueous solution is, for example, an NaCl solution. In the presence of sterol, the aqueous solution of step b) of the process may be distilled water or NaCl. Then, advantageously after step b) of the process, the microcrystals are purified by centrifugation and washing in an aqueous solution from step B.

Advantageously, particularly when the substance concerned is amphotericin B, it is also possible to use saccharide solutions as aqueous solution, for example lactose or alternatively glucose, particularly 0.1M solutions. The microcrystals obtained may, under these conditions, be lyophilized. This makes long-term storage possible.

The present invention also relates to the microcrystals obtained and particularly microcrystal preparations which may be lyophilized.

These are microcrystals of a substance which is insoluble in water, having an affinity for phospholipids

and at least one phospholipid, in a molar ratio (phospholipid(s)/insoluble substance) is less than or equal to 2 and in particular is between 0.8 and 1.4, or alternatively close to 1 [sic].

These microcrystals have a size of between 0.1 µm and 2 µm, or more particularly, close to 500 nm.

The microcrystals according to the invention may include, for example, an insoluble substance chosen from the ginkgolides and derivatives thereof and a phospholipid which may be phosphatidylcholine or one of its derivatives.

For example, microcrystals may be mentioned which are characterized in that they comprise ginkgolide B and phosphatidylcholine in a substantially equimolecular ratio.

More generally, the invention also relates to microcrystals containing an anti-PAF-acether agent and one or more phospholipid(s) preferably in a molar ratio close to 1.

In another example, the present invention relates to microcrystals containing amphotericin B or one of its antifungal derivatives.

In a particular embodiment, these microcrystals comprise amphotericin B or one of its derivatives, with sterol(s) which may or may not be supplemented and phosphatidylcholine.

For example, the microcrystals comprise amphoteracin B and phosphatidylcholine in a molar ratio close to 1.

When a sterol is combined with the active substance, from the point of view of the molar ratio of the components incorporated in the microcrystals, the molar ratio (phospholipid(s)/sterol(s)+substance) is between 0.8 and 1.4, or alternatively close to 1.

Thus there may be mentioned particularly the molar ratio (phosphatidylcholine/cholesterol/amphotericin B) of 4/2/1 (namely an overall molar ratio between the

phospholipid and the substance to be treated supplemented with sterol(s)) of 4/3.

The present invention also relates to pharmaceutical compositions comprising microcrystals according to the present invention by way of active principles.

Preferably, these pharmaceutical compositions are presented in injectable or pulverizable form or alternatively in lyophilized form.

Other characteristics and advantages of the present invention will appear in the light of the detailed description which follows in the form of examples.

EXAMPLE 1

DEVELOPMENT OF ADMINISTRABLE FORMS OF GINKGOLIDE B [sic]

Ginkgolides A, B and C are terpenes isolated from the leaves of Ginkgo biloba and possessing an antagonist activity with respect to the acether of the platelet activating factor, the phospholipid involved in inflammatory reactions and immediate hypersensitivity reactions (for example, platelet aggregation, bronchial spasms, ...).

The most active compound: ginkgolide B has a solubility in water limited to 100 µg/ml. On the other hand, the treatment of prevention of diseases caused by the acether of the platelet activating factor involves the administration of 1 to 50 mg of ginkgolide per kg: namely for 1 man of 70 kg, between 70 and 3500 mg of ginkgolide B.

The problem of producing an administerable form of ginkgolide B hence arises. Since the molecule is very hydrophobic, it was first of all thought to coat the ginkgolide B in lipid vesicles (liposomes) and subsequently, a method for obtaining microcrystals of ginkgolide B combined with phosphatidylcholine was developed which gave the best results.

A) INCORPORATION IN LIPOSOMES

It was first of all decided to incorporate it in multilamellar liposomes composed of phosphatidylcholine (PC), cholesterol (Ch) and stearylamine (SA).

The liposomes were prepared at pH 7.4, then at a slightly acid pH.

1. Preparation of liposomes

Multilamellar liposomes containing ginkgolide B were prepared as follows.

The lipids: 50 µmol (39.25 mg) of phosphatidylcholine (PC), 37.5 µmol (14.51 mg) of cholesterol (Ch) and 12.5 µmol (3.37 mg) of stearylamine (SA) are dissolved in 10 ml of chloroform/methanol (4:1 by volume) in a 500 ml flask. The ginkgolide B dissolved in the methanol is added thereto (0.86 to 4.25 mg namely 2-10 µmol) and the solvent is evaporated under vacuum by means of a rotary evaporator at a temperature of 30°C. The film is taken up in 10 ml of chloroform and reevaporated to obtain a uniform lipid film in the flask.

The multilamellar liposomes (MLV) are formed by adding 4 ml of phosphate buffer pH 7.4, 0.15 M in NaCl and vigorous stirring at the vortex for 5 minutes and are then left under nitrogen for 16 hours.

The degree to which the ginkgolide B is effectively incorporated in the liposomes MLV depends on the amount of ginkgolide B employed and reaches a maximum of 64% at pH 7.4.

A study of the stability of these liposomes containing ginkgolide B indicates that with each washing of the MLVs, up to about 100 µg of ginkgolide B is lost.

2. Liposomes prepared at pH 6.5

Ginkgolide B was incorporated in liposomes MLV composed of phosphatidylcholine, cholesterol and stearylamine (4:3:1) at pH 6.5 (phosphate buffer 0.15 M) following the same procedure as at pH 7.4, but increasing the amount of ginkgolide added to the lipids. The results indicate that the percentage incorporation reaches 87%,

namely 1 molecule of ginkgolide B per 4.4 molecules of phosphatidylcholine.

3. Liposomes prepared at pH 5.9

Ginkgolide B was incorporated in the liposomes MLV at pH 5.9 following the same procedure as at pH 7.4. The results show that the percentage incorporation is improved further, namely 1 molecule of G-B per 2.8 molecules of PC. The improved incorporation at pH 5.9 is doubtless explained by the greater stability of ginkgolide B at this pH, the opening of the lactone rings at neutral or alkaline pH, leading to more hydrophilic products being known. However, the stability of the liposomes is not improved.

B) PREPARATION OF MICROCRYSTALS OF GINKGOLIDE B

In addition to liposomes, the presence of stable microcrystals has been observed in a preparation whose PC/GB molar ratio was 1.54.

Observation of microcrystals in place of liposomes containing ginkgolide B has led to a study of the conditions required for their maximum production. First of all, the influence on their formation of the lipid composition of the liposomes and that of the ratio between the amount of phosphatidylcholine and ginkgolide B, was analysed.

1. Microcrystals obtained from PC, Ch and SA

In a first stage, the proportions of phosphatidylcholine (PC), of cholesterol (Ch) and of stearylamine (SA) were varied. However, the PC/GB molar ratios are not similar and it emerges from the results, shown in Table I below, that the ratio between the phosphatidylcholine and the cholesterol does not have a determining influence on the formation of microcrystals. On the other hand, the PC/GB ratio is essential and hence was studied in a second stage.

Preparations of the "liposome" type composed of

PC:Ch:SA in a ratio (5:4:1) were obtained with a PC/GB ratio of 7.5 and more (preparation E-31). Examination by optical phase-contrast microscopy shows that the preparation after sonication is essentially made up of liposomes with rare microcrystals of the same size as the liposomes.

The influence of the PC/GB ratio on the formation of microcrystals containing ginkgolide B is set out in detail in Table II below for lipid mixtures composed of PC:Ch:SA in a molar ratio 4:3:1. It emerges therefrom that if the PC/GB ratio is higher than 7.5, the formation of lipid vesicles resembling liposomes is essentially observed whilst if the molar ratio is less than or equal to 2, preferably close to 1, microcrystals whose average size is between 0.2 and 0.7 µm are principally observed. For intermediate PC/GB ratios, mixtures of liposomes and of microcrystals are obtained.

These microcrystals are much smaller than those obtained by treating ginkgolide B alone under the same conditions.

2. Toxicity

Aliquot parts of the preparations E-31 (liposomes) and E-34 (microcrystals) were stored for an acute toxicity study in mice.

The preparation E-31 (liposomes) injected i.v. into mice at a dose of 13.1 and 25.9 mg/kg induces a loss in weight of 1.8 and 7.1% respectively after 12 days. On the other hand, the preparation E-34 (microcrystals) is toxic only at a dose of 48.9 mg/kg (mouse dead 2 hours after injection i.v.) whilst at 21.8 mg/kg, no toxicity is observed: on the contrary, a gain in weight of 24% after 12 days is observed.

EXAMPLE 2

PREPARATION OF MICROCRYSTALS OF GINKGOLIDE B BY PREFERENTIAL INTERACTION WITH PHOSPHATIDYLCHOLINE

The production of microcrystals by interaction between ginkgolide B and the constituents customarily used for the preparation of liposomes has lead to the removal of these constituents one at a time and determining the simplest way of preparing them. It appears that, for the production of microcrystals, phosphatidylcholine is essential, whereas the presence of cholesterol and of stearylamine is not, resulting in a great simplification of the process.

If microcrystals having a PC/GB molar ratio of 1.0 are prepared without cholesterol, small microcrystals are obtained of homogeneous size and shape. Microcrystals prepared in the same manner from PC and Ch, without SA and having a PC/GB molar ratio of 1.0 have a tendency to aggregate and their average size is greater than 4 µm.

1. PC:GB microcrystals

Finally, if the cholesterol and stearylamine are removed, only the phosphatidylcholine and the ginkgolide B remain. If the PC/GB molar ratio is less than or equal to 2.0, only microcrystals are obtained.

As indicated in Table III below, if the PC/GB molar ratio is greater than 2.0, the presence of lipid microvesicles ("liposomes") is observed in the preparation, the average size of which is greater than 1 µm. If the PC/GB molar ratio is less than 1.0, a microcrystal population which is heterogeneous from the point of view of size and shape, but whose average size is greater than 1 µm, is observed.

For an equimolar PC/GB ratio, smaller microcrystals, of average size between 0.2 and 0.7 µm are obtained. Their formation results from a specific interaction between ginkgolide B and phosphatidylcholine.

Preparation of microcrystals at pH 5.9 (E-35)

Microcrystals containing ginkgolide B (GB) are prepared from 0.10 mmol of PC and 0.10 mmol of ginkgolide B.

a) Preparation

The lipid film is formed in a 500 ml flask by dissolving 78.5 mg of PC in 10 ml of chloroform-methanol (4:1 in volume) and 42.5 mg of ginkgolide B in 50 ml of methanol and by evaporating the solvent under vacuum by means of a rotary evaporator at a temperature of 30°C.

The microcrystals are formed by adding 50 ml of acetate buffer 0.15 M pH 5.9 and sonication for 5 min. at 100 watts in an ultrasound bath. The GB:PC microcrystals are stored under nitrogen at 4°C.

b) Purification

The preparation of microcrystals is centrifuged at 2000 g for 15 min. and the microcrystals are washed twice with double-distilled water. The microcrystals in the water have the same microscopic appearance as those obtained in an acetate buffer at pH 5.9.

A suspension of microcrystals of Ginkgolide B [sic] having a homogeneous size of the order of 500 nm at a ginkgolide B concentration of 12.8 mg/ml is obtained.

2) Anti-PAF-acether activity

Ginkgolide B was compared with 2 preparations of liposomes containing ginkgolide B (preparation E-30 and E-31) as well as with a microcrystal preparation (preparation E-34) in ex vivo tests on rabbits for percentage inhibition of platelet aggregation (PAF 2.5 nM).

It emerges therefrom that the preparations based on liposomes do not have an inhibitory effect on platelet aggregation essentially for 1h 30 only [sic], whilst the

effect of microcrystals was more prolonged at doses of 1 mg/kg administered i.v.

E-30 = Liposomes (PC/CH/SA) = (7/2/1) at 1.283 mg GB/ml
E-31 = Liposomes (PC/CH/SA) = (5/4/1) 2.695 mg GB/ml
E-34 = microcrystals with 4.250 mg GB/ml
buffer - acetate buffer PH 6.0 ; 0.15 M.

TABLE I : EFFECT OF THE LIPID COMPOSITION ON THE PRODUCTION OF MICROCRYSTALS CONTAINING GINGKOLID B

Composition	Molar Ratio (PC/GB)	Average size (nm)	Appearance in optical phase contrast microscopy
PC:Ch:SA (7:2:1)	3.1*	N.D.	Round and elongated liposomes, no microcrystals
PC:Ch:SA (4:3:1)	1.25	634 nm	Microcrystals, little or no liposomes
PC:Ch:SA (5:4:1)	7.5**	N.D.	Liposomes (>95%) + some microcrystals
PC:Ch:SA (4:6:1)	1.0	>4000 nm	Elongated microcrystals + large crystals of cholesterol

* preparation E-30
** preparation E-31
N.D. = Not determined

TABLE II : EFFECT OF THE PC/GB MOLAR RATIO ON THE PRODUCTION OF MICROCRYSTALS CONTAINING GINGKOLID B

Composition	Molar Ratio (PC/GB)	Average size (nm)	Appearance in optical phase contrast microscopy
PC:Ch:SA (4:3:1)	10.0	N.D.	Essentially small liposomes
PC:Ch:SA (4:3:1)	7.5	N.D.	Round and elongated liposomes + very few microcrystals (1-5%)
PC:Ch:SA (4:3:1)	5.0	N.D.	Liposomes + microcrystals (10-25%)
PC:Ch:SA (4:3:1)	2.5	N.D.	Microcrystals (50-70%) + liposomes
PC:Ch:SA (4:3:1)	1.25	634 nm	Principally small microcrystals + very few liposomes (1-5%)
PC:Ch:SA (4:3:1)	0.60*	242 nm	Essentially microcrystals of ± homogeneous size

* preparation E-34

TABLE III : EFFECT OF THE PC/GB MOLAR RATIO ON THE PRODUCTION OF MICROCRYSTALS CONTAINING GINGKOLID B

Composition	Molar Ratio (PC/GB)	Average size (nm)	Appearance in optical phase contrast microscopy
PC:GB	4.0*	2100	- Liposomes + aggregates of liposomes - Few microcrystals
PC:GB	2.0	2200	Mixture of small and aggregated liposomes and of microcrystals
PC:GB	1.0**	250 to 650	Small microcrystals, no liposomes
PC:GB	0.5	2970	Small microcrystals and a heterogeneous population of larger crystals
PC:GB	0.25***	3390	Larger microcrystals, more elongated and of more irregular shape than for the PC/GB molar ratio of 1.0

* preparation E-39
** preparation E-35
*** preparation E-40

3. Toxicity

The preparation E-35 of microcrystals GB:PC (molar ratio of 1.0) was injected i.v. into Balb/c mice of 22 grams. At a dose of 118.4 mg/kg, the maximum weight loss was 16.2% on day 3 then the mouse recovered its initial weight on day 8.

At a dose of 178.5 mg/kg, the weight loss continued and the mouse died on day 5. At doses of 56.6 and 28.4 mg/kg, no toxicity was observed, and a gain in weight of 6 to 8% was even observed after 2 days at the lowest dose tried.

4. Stability of the GB microcrystals

The preparation E-35 (PC/GB ratio of 1.0) was stored under nitrogen at 4°C for 100 days and examined under optical phase contrast microscopy, the day of their preparation, on days 7, 15, 20 and 100. On day 7, the population was relatively homogeneous with an average size a little greater than on day 1. On day 100, small microcrystals were still observed, but the presence of microcrystal clusters was also observed. Measurement of the average sizes by means of a nanosizer confirmed the results. Between day 1 and day 20 the average size doubled and, on day 100, it was about 2 µm.

The increase in the average size of the microcrystals therefore results from the slow formation of microcrystal aggregates whose individual sizes do not seem to increase over time. This aggregate formation during storage at 4°C may be avoided by the addition of additives such as antioxidants or saccharides.

In conclusion, it is possible to obtain a relatively homogeneous population of ginkgolide B microcrystals, of size between 0.1 and 1 µm by specific and equimolar interaction of this anti-PAF-acether agent with an amphiphilic molecule like phosphatidylcholine. Under examination by optical microscopy, it seems that the microcrystal population is relatively homogeneous from the point of view of size and shape. In electron

microscopy, these microcrystals are in the form of "pebbles" with rounded edges.

Without posing any preparation problems a milky suspension can be obtained containing 12.8 mg of ginkgolide B per ml.

The toxicity of this preparation of microcrystals after i.v. administration in mice is low. The anti-PAF-acether activity of this preparation is confirmed.

If phosphatidylcholine, cholesterol and stearylamine (PC:Ch:SA; 4:3:1) are also added, and provided that the PC/GB molar ratio is less than 2, microcrystals are also obtained.

EXAMPLE 3

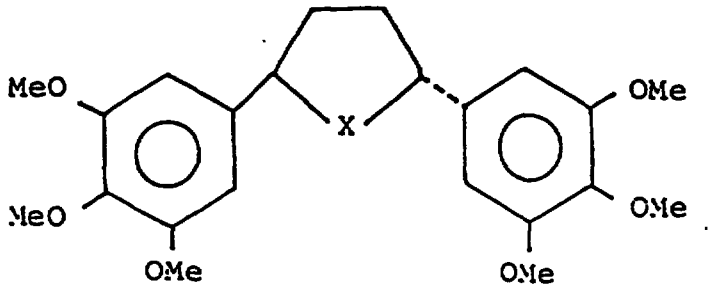
ANTI-PAF-ACETHER AGENTS

Numerous other anti-PAF-acether agents which can be applied to the process according to the present invention will be found in the literature, particularly in "PAF-acether specific binding sites: 2. Design of specific antagonists" of P. Braquet and J.J. Godfroid (TIPS - October 1986 - Elsevier Science Publishers B.V., Amsterdam).

Several series of anti-PAF agents may be distinguished inter alia:

1) antagonists having a molecular structure similar to that of PAF-acether, such as CV-3988 (TAKEDA), RU-45703 (ROUSSEL-UCLAF/PHARMACOCHIMIE MOLECULAIRE), ONO-6240 (ONO), Ro 19-3704 (HOFMANN-LA ROCHE), SRI 63-119 (SANDOZ) and SRI 63-072 (SANDOZ) or alternatively antagonists having a structure modified with respect to that of PAF-acether such as PIPERIDINE SRI 63-073 (SANDOZ) or DIOXANONE (HOFFMANN-LA ROCHE).

2) natural products like lignans and neolignans such as KADSURENONE (PIPER FUTOKADSURAE), MAGNOSALICIDE (MAGNOLIA SALICIFOLIA), NECTANDRINES A and B (NECTANDRA RIGIDA) and SYNTHETIC DINORLIGNAN L-652.731 of formula:



L-652.731 (X=O,M

Besides the terpenoids isolated from GINKGO BILOBA (GINKGOLIDES) to which the present patent application also relates.

EXAMPLE 4

PREPARATION OF KADSURENONE MICROCRYSTALS AT PH 5.9

Microcrystals containing kadsurenone and phosphatidylcholine are prepared according to the following scheme.

a) Preparation

A lipid film is formed in a flask by dissolving phosphatidylcholine in one solvent and kadsurenone in another solvent in equimolar amounts, and after evaporation of the solvent under vacuum by means of a rotary evaporator at a temperature of 30°C.

Thus, to one milligram of kadsurenone (1 mg/ml) in chloroform:methanol (1:1 by volume), in a 50 ml flask is added 4.6 ml of phosphatidylcholine (0.5 mg/ml) in chloroform:methanol (4:1 by volume).

The microcrystals are formed by the addition of 0.5 ml of acetate buffer 0.15 M pH 5.9 and sonication for 5 minutes at 100 W in an ultrasound bath. The microcrystals are stored under nitrogen at 4°C. In this way microcrystals having a substantially homogeneous size of

the order of 500 nm are obtained.

b) Purification

The microcrystal preparation is centrifuged at 2000 g for 15 minutes and the microcrystals are washed twice with double-distilled water. The microcrystals in the water have the same microscopic appearance as those obtained in acetate buffer at pH 5.9.

EXAMPLE 5

PREPARATION OF MICROCRYSTALS AT pH 5.9 CONTAINING THE PRODUCT L-652.731 (MSD)

Microcrystals containing phosphatidylcholine and the product L-652.731 (MSD) were prepared according to the scheme described in the preceding example. To 1 mg of the product L-652.731 are added 3.9 ml of phosphatidylcholine (0.5 mg/ml) in a 50 ml flask and the solvent is evaporated off under vacuum at 30°C.

The film is taken up in 0.5 ml of acetate buffer 0.15 M, PH 5.9 [sic] and subjected to ultrasound for 5 minutes at a power of 100 W.

EXAMPLE 6: Preparation of amphotericin B microcrystals

Microcrystals containing amphotericin B and phosphatidylcholine in an equimolar ratio are prepared according to the following scheme:

a) Preparation: Into a 500 ml flask, are introduced 15.7 ml of phosphatidylcholine (99% pure egg lecithin) with 10 mg/ml of chloroform (0.2 mM). A lipid film is formed by evaporation of the solvent under vacuum by means of a rotary evaporator at a temperature of 30°C. Then 370 ml of amphotericin B are added to 0.5 mg/ml of chloroform-methanol mixture (1:1, by volume), namely 0.2 mM.

After evaporation of the solvent under vacuum by

means of a rotary evaporator, the microcrystals are formed by addition of 10 ml of 9% NaCl and sonication for 15 min. in an ultrasound bath JULABO model USR 6.

b) Purification: The preparation of microcrystals of amphotericin B is centrifuged for 2 minutes at 2000 g in a table centrifuge. The supernatant is applied to the top of a Sepharose 6B column eluted by 9% NaCl. The fraction of the first peak most enriched in amphotericin B contained 8.23 mg of amphotericin B/ml and 7.10 mg of phosphatidylcholine/ml, namely an ampho/PC molar ratio of 0.98.

c) Acute Toxicity in mice: At a dose of amphotericin B of 82.2 mg/kg administered intravenously, the preparation of microcrystals of amphotericin B is toxic with respect to OF1 female mice, the death of the mouse taking place within 3 minutes of the injection. At a dose of 41.1 mg/kg the mouse injected intravenously died on the second day. On the other hand, at a dose of 20.6 mg/kg, the mouse injected intravenously lost about 20% of its weight during the 7 days following the injection and then recovered its weight by day 22.

Amphotericin in the form of fungizone® (amphotericin B deoxycholate) administered intravenously at LD_{50} of 1.2 mg/kg according to LOPEZ-BERESTEIN et al. [2], of 2.3 mg/kg according to SZOKA et al. [3] and of 5 mg/kg according to WRIGHT et al. [1].

The LD_{50} of amphotericin B-liposomes administered intravenously varies from 4 to 19 mg/kg according to the composition of liposomes (SZOKA et al., op. cit.)

The acute toxicity of amphotericin B-phosphatidylcholine microcrystals, administered i.v. to NMRI mice, was compared with that of amphotericin B complexed with deoxycholate (FUNGIZONE®) and with that of liposomes composed of phosphatidylcholine, cholesterol and stearylamine (molar ratios = 4:3:1) containing amphotericin B prepared according to the same technique as that of Example I-A)1. for Ginkgolide B [sic].

The dose inducing death of 50% of the mice, 14 days after the single administration of the products (LD_{50}) is 2.5 mg/kg for free amphotericin B; 18.3 mg/kg for PC:AMPHO B microcrystals.

In the form of microcrystals, AMPHO B is hence 7.3 times less toxic than in the form of a complex with deoxycholate.

d) In vitro cytotoxicity: The in vitro cytotoxicity is determined by the reduction in reducing enzymatic activity with respect to MTT (tetrazolium salt) of murine J774-G8 macrophages incubated overnight in the presence of RPMI medium enriched with 10% of foetal calf serum and containing the products at various doses.

The cytotoxicity is expressed in the form of the micromolar concentration of amphotericin B which reduces by 50% the reducing enzymatic activity of the macrophages (TOX_{50}).

Table I - Cytotoxicity of the different formulations of amphotericin B

Product	TOX_{50} (µM)
FUNGIZONE	4.0
PC:AMPHO microcrystals	132
Lyophilized and reconstituted microcrystals	132
Liposomes: AMPHO	17

The table indicates that, in the form of microcrystals, AMPHO B is 33 times less toxic in vitro with respect to J774-G8 macrophages than free AMPHO B and 7.7 times less toxic than in liposome form.

The lyophilization of the microcrystals in the presence of 0.1 M sucrose and their reconstitution did not modify their cytotoxicity.

e) Direct antifungal activity. (that is to say in vitro on extracellular parasites): The activity of the

microcrystals of amphotericin B was compared with that of the control amphotericin B solubilized in DMSO then diluted in RPMI culture medium containing 10% of lipid-depleted serum. With respect to Candida Albicans and Candida Tropicalis, the MIC (minimum inhibitory concentration of the growth of parasites in vitro) of the two forms of amphotericin B is identical and situated between 0.05 and 0.1 µM (0.04 and 0.08 µg/ml).

The direct antifungal activity of amphotericin B in the form of fungizone, of microcrystals or of liposomes was also measured on the growth of Candida tropicalis in RPMI medium containing 20% of foetal calf serum. Inhibition of the growth after 16 h was observed for increasing concentrations of amphotericin B (14 concentrations ranging from 0.012 to 100 µM). The minimum inhibiting concentration (MIC) is the lowest concentration at which growth of Candida is not observed.

The MIC of amphotericin B in the form of ampho B:PC microcrystals or in the form of fungizone is identical (0.195 µM). On the other hand amphotericin B encapsulated in liposomes composed of phosphatidylcholine, cholesterol and stearylamine in a molar ratio 4:3:1, is 8 times less active than free amphotericin (1.56 µm).

Lyophilizing the microcrystals of amphotericin B:PC in the presence of 0.1M sucrose does not affect their antifungal activity with respect to extracellular fungi.

f) In vitro activity with respect to macrophages infected by Candida Tropicalis (with respect to intracellular parasites):

The antifungal activity of amphotericin B with respect to intracellular fungi is determined in vitro in the model of the line J-774-G8 of macrophages infected in a proportion of 1 Candida tropicalis per 10 macrophages. The macrophages are cultivated in RPMI medium containing 10 mM MES pH 7.2 supplemented with 10% decomplemented human plasma. The action of the medicaments is measured

in LIMBRO dishes with 24 wells containing 10^5 infected macrophages, adhering to the glass slides, and the range of concentrations tested varies from 0.048 µM to 100 µM.

The percentage of infected cells is determined after 16 h of coculture. On microscope slides, after MAY-GRUNWALT-GIEMSA dyeing, the total number of macrophages (NT) is counted as well as the number of macrophages infected with Candida (Ni). The percentage of infection is the ratio Ni/NT (expressed in %).

The concentration, expressed in µM, which reduces by 50% the intracellular infection (IC_{50}) is then determined.

The IC_{50} of FUNGIZONE is 0.2 µM whilst for the microcrystals it is 0.1 µM, whereas the IC_{50} of liposomes containing AMPHO B is 0.8 µM.

The microcrystals are hence at least as active as amphotericin B, both with respect to extracellular fungi and with respect to intracellular fungi.

g) Therapeutic index: The therapeutic index (TI), defined on the intracellular forms of Candida, is the ratio between the toxicity of the product for the macrophages, expressed by the TOX_{50} and the therapeutic activity expressed by the IC_{50}, the dose which reduces intracellular infection by 50%.

As illustrated in the table below, the therapeutic index of the microcrystals PC:AMPHO B is 66 times higher than that of free AMPHO B (in the form of FUNGIZONE) and 60 times higher than that of the liposomes containing AMPHO B.

Table 2: Therapeutic index of the various formulations of amphotericin B

Product	TOX_{50} [a] (µM)	IC_{50} [b] (µM)	TI [c]	Product TI / FUNGIZONE TI
FUNGIZONE	4.0	0.2	20	1.0
Microcrystals PC:AMPHO B	132	0.1	1,320	66.0
Lyophilized microcrystals	132	0.6	220	11.0
Liposomes:AMPHO	17.0	0.8	21.3	1.1

a. TOX_{50}: Concentration, expressed in µmol, which reduces the enzymatic reducing activity of the macrophages toward tetrazolium salts by 50%.

b. IC_{50}: Concentration, expressed in µmol, which reduces the intracellular infection of the macrophages J-774-G8 infected by Candida tropicalis by 50%.

c. TI Therapeutic index; ratio between TOX_{50} and IC_{50}.

EXAMPLE 7: Preparation of microcrystals of amphotericin B containing cholesterol

The combination of amphotericin B with sterols has been tried out (WITZKE et al., [4]), in particular with cholesterol and ergosterol. The incorporation of sterol into liposomes containing amphotericin B reduces their toxicity (SZOKA et al., [3]).

The addition of cholesterol has the same effect on the microcrystals of amphotericin B, while increasing their activity.

Microcrystals of amphotericin B containing phosphatidylcholine (PC) and cholesterol (CH) were prepared in accordance with the following protocol:

a) Preparation: 15.7 ml of phosphatidylcholine (PC) at a concentration of 10 mg/ml in $CHCl_3$ (0.2 mM), 370 ml of amphotericin B at a concentration of 0.5 mg/ml in chloroform/methanol (1:1 by volume),

equivalent to 0.2 mM, and 58 mg of cholesterol (CH) in solution in 10 ml of methanol (0.15 mM) are introduced into a 500 ml round-bottomed flask.

The solvent is evaporated under vacuum at 30°C using a rotary evaporator.

The microcrystals are formed by addition of 10 ml of water and sonication for 15 min. in an ultrasound bath JULABO model USR 6.

b) Purification: The preparation is purified as in Example 6. The fraction of the first peak, which has the highest concentration of amphotericin B, contains 2.55 mg of ampho/ml, 9.11 mg of PC/ml and 1.93 of Ch/ml, corresponding to a molar ratio: PC/Ch/amphotericin B of 4/2/1.

c) Acute toxicity in mice: At a dose of 25.5 mg/kg of amphotericin B, administered intravenously to 2 female OF1 mice, the preparation of microcrystals of amphotericin B containing cholesterol induces a maximum weight loss of 8% on day 6 in 1 mouse and a weight gain of 9% on day 6 in the 2nd mouse (on average: no loss in weight). The average weight of the 2 mice on day 19 is 100% of the initial weight.

d) Direct antifungal activity: The antifungal activity of the amphotericin B control (DMSO and RPMI) and the preparation of microcrystals of amphotericin B containing cholesterol are of the same order. The MIC is between 0.05 and 0.1 µM.

e) In vitro activity toward infected macrophages: The MIC of the preparation of microcrystals of amphotericin B containing cholesterol is 0.1 µM whereas that for the amphotericin B dissolved in DMSO is 0.4 µM. Amphotericin B in the form of microcrystals containing cholesterol is therefore at least 4 times less toxic and 4 times more active on infected macrophages than amphotericin B.

BIBLIOGRAPHIC REFERENCES CITED IN THE DESCRIPTION

[1]: WRIGHT et al. The Journal of antibiotics 1982 Vol. 35 No. 7 pp. 911-914.

[2]: LOPEZ-BERESTEIN et al. The Journal of infections [sic] diseases Vol. 145, No. 5, May 1983 pp. 939-945.

[3]: SZOKA et al. Antimicrobial Agents and Chemotherapy, March 1987, pp. 421-429 Vol. 31, No. 3.

[4]: WITZKE et al. Biochemistry 1984, 23, 1668-1674.

CLAIMS

Claims except for SPAIN and GREECE

1. Process for the preparation of lipid microparticles of crystalline appearance comprising a substance which is insoluble in water, having an affinity for phospholipids, and at least one phospholipid, characterized in that:

a) the organic solvent(s) is(are) evaporated from a solution of phospholipids and the said substance, and

b) the film obtained after evaporation of the solvent(s) is resuspended in an aqueous solution with vigorous stirring, the molar ratio between the phospholipid(s) and the substance being between 0.1 and 2.

2. Process according to Claim 1, characterized in that the molar ratio between the phospholipid(s) on the one hand and the substance on the other hand is between 0.8 and 1.2.

3. Process according to one of the preceding claims, characterized in that the phospholipid is phosphatidylcholine.

4. Process according to one of the preceding claims, characterized in that the various solvents of the solutions employed are chosen from among the usual organic solvents, especially methanol, chloroform and mixtures thereof.

5. Process according to one of the preceding claims, characterized in that step a) is subdivided as follows:

a_1) the solvent(s) is(are) evaporated from the solution of phospholipid(s),

a_2) the lipid film obtained after evaporation is redissolved by adding a solution of the said substance,

a_3) the solvent(s) is(are) evaporated again from the solution of phospholipid(s) and the said substance.

6. Process according to one of the preceding claims, characterized in that the vigorous stirring in step b) is brought about by treatment with ultrasound.

7. Process according to one of the preceding claims, characterized in that the solution of phospholipid(s) is a solution in chloroform or in chloroform/methanol.

8. Process according to one of the preceding claims, characterized in that the solution of the said substance is a solution in chloroform/methanol.

9. Process according to one of Claims 1 to 8, characterized in that the said substance is chosen from substances which are antagonists of the acether of the "platelet activating factor".

10. Process according to Claim 9, characterized in that the substance in question is chosen from lignans and neolignans.

11. Process according to Claim 9 or 10, characterized in that the substance is chosen from kadsurenone or derivatives thereof and the synthetic dinorlignan L-652-731 and derivatives thereof.

12. Process according to one of Claims 1 to 8, characterized in that the substance in question is chosen from ginkgolides and derivatives thereof.

13. Process according to Claim 10, characterized in that the substance is chosen from PAF-acether antagonist substances which have a structure analogous to that of the said PAF-acether.

14. Process according to one of Claims 1 to 8, characterized in that the said substance is chosen from amphotericin B and derivatives thereof which have an antifungal activity.

15. Process according to one of the preceding claims, characterized in that sterol(s), especially cholesterol and/or ergosterol, is(are) added to the solution of the said substance.

16. Process according to Claim 15, characterized in that the molar ratio between the phospholipid(s) on the one hand and the sterol(s) on the other hand is between 1 and 2.

17. Process according to one of Claims 9 to 13 characterized in that in step b) of the process the film obtained after evaporation of the solvent is resuspended

in a solution buffered at a pH of between 5 and 8, preferably at pH 5.9.

18. Process according to Claim 17, characterized in that in step b) of the process the film obtained after evaporation of the solvent is resuspended in a solution of acetate or phosphate buffer.

19. Process according to one of Claims 14 to 16 characterized in that in step b) of the process the aqueous solution is a 0.1 M saccharide or NaCl solution.

20. Process according to one of the preceding claims, characterized in that after step b) of the process the lipid microparticles of crystalline appearance are purified by centrifugation and washing with distilled water or by an aqueous solution.

21. Lipid microparticles of crystalline appearance obtained in particular by employing the process according to one of the preceding claims.

22. Lipid microparticles of crystalline appearance which are stable in aqueous medium and comprise a substance which is insoluble in water and has an affinity for phospholipids, and at least one phospholipid, characterized in that the molar ratio (phospholipid(s)/active substance) is less than or equal to 2.

23. Lipid microparticles of crystalline appearance comprising an active substance chosen from amphotericin B and antifungal derivatives thereof, and at least one phospholipid, in a molar ratio (phospholipid(s)/active substance) of less than or equal to 2.

24. Microparticles according to one of Claims 21 to 23, characterized in that the molar ratio (phospholipid(s)/substance) is between 0.8 and 1.4.

25. Microparticles according to one of Claims 21 to 24, characterized in that the phospholipid is phosphatidylcholine or derivatives thereof.

26. Microparticles according to one of Claims 21, 22, 24 and 25, characterized in that the insoluble substance which has an affinity for phospholipids is an anti-PAF-acether agent.

27. Microparticles according to Claim 26,

characterized in that the substance is chosen from the ginkgolides and derivatives thereof.

28. Microparticles according to Claim 26, characterized in that the substance is chosen from lignans and neolignans such as kadsurenone and derivatives thereof and the synthetic dinorlignan L-652-731 and derivatives thereof.

29. Lipid microparticles of crystalline appearance according to one of Claims 21 to 25, characterized in that the substance is chosen from amphotericin B and antifungal derivatives thereof.

30. Lipid microparticles of crystalline appearance according to one of Claims 21 to 29, characterized in that sterol(s) is(are) added to the substance.

31. Lipid microparticles microcrystals [sic] of crystalline appearance according to Claim 30, characterized in that the molar ratio (phospholipid(s)/ substance + sterol(s)) is between 0.8 and 1.4.

32. Lipid microparticles microcrystals [sic] of crystalline appearance according to one of Claims 28 to 31, characterized in that they comprise amphotericin B, cholesterol and phosphatidylcholine in a molar ratio of 1:2:4 respectively.

33. Microparticles according to one of Claims 21 to 32, characterized in that they are in lyophilized form.

34. Pharmaceutical composition, characterized in that it contains as active principle microparticles according to one of Claims 21 to 33.

35. Pharmaceutical composition according to Claim 34, characterized in that it is presented in injectable or pulverizable form.

36. Pharmaceutical composition according to Claim 34, characterized in that it is presented in lyophilized form.