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(54) Process for preparing liposome compositions

(57) A process for preparing liposome compositions capable of retaining larger amount of drugs with a small amount of phospholipid and to provide, therefore, safer medications of various drugs, which comprises dispersing freeze-dried multilamella vesicles or small unilamella vesicles in an aqeuous medium in the presence of both an active ingredient and a divalent cation to form large unilamella vesicles or large oligolamella vesicles entrapping said active ingredient.

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SPECIFICATION

Process for preparing liposome compositions

Vesicles, which are so-called "liposomes" consisting of phospholipid bilayers, can be prepared by dispersing a lipid in an aqueous solvent. The liposomes are useful as drug-carriers for administration to subjects, the drug being contained in aqueous compartments in the liposomes. The present invention relates to a process for the preparation of liposome compositions containing clinically active ingredients.

A process for preparing liposome compositions is disclosed in JPN Unexam. Pat. Pub. No. 53-142514 wherein liposome compositions which have been formulated with phospholipid, active ingredient and adjuvants are freeze-dried for stable storage.

Processes for preparing highly safe liposome compositions are also disclosed in JPN Unexam. Pat. Pub. Nos. 57-82310 and 57-82311 wherein freeze-dried vesicles are prepared with no organic solvent and are formulated into liposome compositions by the use of an aqueous medium containing or not containing active ingredient.

On the other hand, a process is disclosed in JPN Unexam. Pat. Pub. No. 58-152812, wherein SUV (small unilamella vesicles) or LUV (large unilamella vesicles) are prepared by dispersing a special phospholipid in an aqueous medium having a specific pH-value.

Conventional methods of liposome formulation, i.e., hydration methods, give only MLV (multilamella vesicles) which are made up of multiple phospholipid layers and aqueous compartments, unless the procedures are carried out under specific conditions, as shown in the JPN Unexam. Pat. Pub. No. 58-152812. When the lyophilizates of these MLV are dispered in the brine or buffer which is generally used as an aqueous medium, they are reformed into MLV. In the MLV, the rate of retention of aqueous medium per unit amount of phospholipid is smaller than in LUV. The captured volume of an MLV is, therefore, smaller and the up-take rate of an active ingredient thereinto is lower.

It has now been found that liposomes can be reformed into LUV or LOV when the lyophilizates or MLV or SUV are dispersed in an aqueous medium if a divalent cation is present in the aqueous medium within a specific range.

In JPN Pat. Application No. 59-171265, it was found that LUV or LOV could be regenerated if the ionic strength of monovalent cation was kept at 0.05 or below in the regeneration procedures. This meant that MLV, but never LUV or LOV could be regenerated if the ionic strength was over 0.05. However, according to the present invention, the presence of a specific amount of a divalent cation prevents the regeneration of MLV and gives desired LUV or LOV.

According to the present invention there is provided a process for preparing liposome compositions which comprises dispersing freeze-dried multilamella vesicles or small unilamella vesicles in an aqueous medium in the presence of one or more active ingredients and a divalent cation in order to regenerate large unilamella vesicles or large oligolamella vesicles entrapping said active ingredients.

Lecithins, i.e., saturated or unsaturated phosphatidyl choline, which are lipids which make up liposomes, may be used in the invention. These lecithins may be combined with, for example, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl inositol, diphosphatidyl glycolol, phosphatidate or sphingomyelin. They may also be combined with cholesterol or electrically charged substances (e.g., stearylamine, dicetylphosphate). Examples of such lipids are lechithins derived from egg yolk, soybean, or other animal or plant tissues, hydrogenates thereof, and synthetic lechithins, which may be employed solely or in a mixture thereof. For instance; dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine, 1-palmitoyl-2-strearoyl phosphatidylcholine and 1-stearoyl-2-palmitoyl phosphatidylcholine, can be employed solely or in a mixture.

The freeze-dried liposomes employed may be freeze-dried MLV or SLV prepared by known methods. They may be obtained by means of any method for lyophilization.

Examples of active ingredients used are anti-cancer agents such as 5-fluorouracil, neomycin and bleomycin; antibiotic agents such as chloramphenicol, tetracycline, cefalexin and latamoxef; enzymes or hologues such as urokinase; peptides such as interferon, interleukin, globulin and insulin; nucleic acids such as DNA and RNA; vitamins; or other agents such as sulfamethoxazole and phenobarbital.

The active ingredients to be entrapped may be (1) added to the liposome dispersion system just before freeze-drying, (2) dispersed along with the freeze-dried liposomes. The thus obtained mixtures may than be dispersed in an aqueous medium containing suitable divalent cation to regenerate a liposome composition. Alternatively, the active ingredient may be (3) dissolved or dispersed in the aqueous medium in which freeze-dried liposomes have been dispersed, for their regeneration.

Water, brine (e.g, isotonic saline), or buffer (e.g, phosphate buffer, trisaminomethane buffer), for example, may be employed as an aqueous medium in which the freeze-dried liposomes are dispersed: the choice depends on the purpose for which the resulting liposome composition is intended to be used.

Divalent cations used in the present invention include metallic ions, for example calcium, magnesium, zinc, manganese iron, cobalt and nickel; calcium, manganese and magnesium are particularly preferred. When freeze-dried SUV or MLV are dispersed in an aqueous medium containing a divalent cation, they are reformed into LUV or LOV: the optimum concentration of the divalent cation varies with species of ion used.

Thus there is an optimum concentration range for each ion; for instance, the optimum range for calcium is about 3×10^{-3} to 1×10^{-1}M, about 4×10^{-3} to 1×10^{-1}M for magnesium and about 8×10^{-4} to 1×10^{-1}M for manganese. The preferred species of metallic ions, and their concentrations, may be determined according to the use of the liposome compositions.

In accordance with regeneration of freeze-dried liposomes prepared from synthetic lecithin described in the aforementioned JPN Pat. Appln. No. 59-171265, it is preferred that the procedures are carried out at or above the gel-phase/liquid crystal-phase transition temperature.

The resulting liposome compositions may be orally or parenterally administered to subjects directly or as a purified dispersion by removing excess active agent outside the liposome by methods such as centrifugal separation, ultrafiltration or gelfiltration.

The invention further relates to a composition prepared by a process as hereinbefore described and adapted for pharmaceutical or medical use. The composition may be in unit dosage form.

The liposome compositions prepared according to the present invention have a high uptake-rate and therefore entrap the active ingredient therein in high efficiency. Since each regenerated liposome has a large captured volume, a large amount of the active ingredient is entrapped by a lesser amount of the phospholipid: this means that the liposome compositions keep such troubles away as toxicity accompanied by phospholipid when adminstered.

Additionally, both liposome and active ingredients can be stored in a stable state, because the active ingredient to be entrapped may be admixed at the tine when the freeze-dried liposomes are regenerated.

The present invention may be further explained and illustrated by the following examples, which do not limit the scope of the invention.

Example 1

Commercially available yolk lecithin (made by Merck & Co.) was further refined by silica gel chromatography. A chloroform solution of 354mg of the refined lecithin was placed in a 200ml round-bottom flask and evaporated to dryness by a rotary evaporator to give a thin layer of the phospholipid on the inner wall of the flask. To the adequately dried layer was added a mixture (30ml) of water with 1.5 parts of mannitol and the mixture was shaken by hand to prepare a dispersion of MLV. The dispersion was frozen with dry-ice/acetone and freeze-dried by a vacuum pump. 1% Aqueous human serum albumin (HSA) containing sodium chloride (NaCl 0.05M) and/or calcium chloride ($CaCl_2$ 0.03M) was added to the freeze-dried substance at a rate of 0.4 ml to 25 mg of the freeze-dried substance (10mg as lecithin) at room temperature. The mixture was allowed to stand for an hour while being occasionally shaken, combined with 5ml of an isotonic aqueous NaCl, and divided by means of ultra-centrifugal separation (85,000g × 60 minutes) into the external solution and liposomes. The isolated liposomes were dispersed again in another 5ml of said isotonic NaCl and centrifugally separated. By the quantitative analysis of HSA in the accumulative external solution, the up-take rate of HSA in the accumulative external solution, the up-take rate of HSA into liposomes on their regeneration were measured (Table 1).

Table 1 shows positive effect of calcium ion on and negative effect of sodium ion against the formation of liposomes having large captured volume.

HSA was quantitatively analyzed according to Lowry's method (Shin Jikken Kagaku-Koza 20-I, 130 published by Maruzen).

TABLE 1

	Ion	Uptake rate (%) of HSA	Captured Vol.* (μ 1/mg lipid)
1	None	37.3	12.4
2	0.03M of $CaCl_2$	45.1	15.5
3	0.05M of NaCl	11.6	2.1
4	0.03M $CaCl_2$, 0.05M NaCl	34.8	11.4

(*) The value of the captured volume is free from HSA (6.2% to uptake rate) absorbed on the liposome surfaces.

Example 2

In the same manner as in Example 1, 262mg of a hydrogenated yolk lecithin (iodine value 3, Asabi Chem. Ind.) was employed for the formation of the thin layer on the inner wall of a round-bottom flask. Purified water (20ml) was added thereto to give a dispersion of MLV.

The dispersion was freeze-dried to leave powder. To 10mg of the freeze-dried powder was added 0.4ml of aqueous solution of 5-flurouracil (5-FU, 10mg/ml) containing NaCl (0.05M) and/or $CaCl_2$ (0.03M), and the mixture was allowed to stand for about an hour then warmed up and kept at 60°C for 5 minutes. The mixture was allowed to stand at room temperature approximately for another hour and mixed with 5ml of isotonic aqueous NaCl. The obtained liposome dispersion was subjected to centrifugal separation (85,000 g × 60 minutes). The precipitated liposomes were collected and dispersed in another 5ml of isotonic NaCl solution, and isolated again by a centrifugal separator (85,000 g × 60 minutes). Finally, thus

isolated liposomes were broken by Triton X-100 whereby the released 5-FU was quantitatively analyzed by high performance liquid chromatography (column: Nucleosil $_{10}C_{18}$, solvent: 0.01M KH_2PO_4).

TABLE 2

	Ion	Uptake rate (%) of 5-FU	Captured Vol.* (μl/mg lipid)
1	0.03M $CaCl_2$	31.3	12.5
2	0.05M NaCl	22.6	9.0
3	0.05M NaCl, 0.03M $CaCl_2$	32.7	13.1

The results in Table 2 suggest that semi-synthetic phospholipids such as hydrogenated yolk lecithin, when the freeze-dried liposomes composed of them are regenerated, are reformed into liposomes having a large captured volume by the effect of calcium ion.

Example 3

In the same manner as in Example 1, MLV were prepared by use of dialmitoyl phosphatidylcholine (DPPC) and freeze-dried. The freeze-dried MLV (10mg each) were dispersed in an aqueous solution (0.4ml) of 19mM latamoxef containing 0.03M $CaCl_2$ and in not containing $CaCl_2$. The two mixtures were respectively warmed up and kept at 50°C for 5 minutes. The uptake rates of latamoxef and the captured volumes of the regenerated liposomes are shown in Table 3.

TABLE 3

	Ion	Uptake rate (%) of latamoxef	Captured Vol. (μl/mg lipid)
1	None	9.5	3.8
2	0.03M $CaCl_2$	36.5	14.6

Example 4

The freeze-dried MLV (10mg each) composed of DPPC prepared in Example 3 was added to 0.4ml each of 0.25% aqueous solution of 5-FU containing either one of the salts listed in Tables 4 to 7, and warmed up and kept at 50°C for 5 minutes. The uptake rates of 5-FU and the captured volumes of the regenerated liposomes are shown in Tables 4 to 7.

TABLE 4

	Monovalent cation	Divalent metallic ion $CaCl_2$	Uptake rate (%)	Captured Vol. (μl/mg lipid)
1	0.05M NaCl	None	4.7	1.9
2	0.05M NaCl	0.01M	40.2	16.1
3	0.05M NaCl	0.05M	38.2	15.3
4	0.05M CH_3COONa	None	3.2	1.3
5	0.05M CH_3COONa	0.05M	40.5	16.2
6	0.05M CH_3COONH_4	None	3.2	1.3
7	0.05M CH_3COONH_4	0.05M	27.2	10.9

TABLE 5

	Monovalent cation	Divalent metallic ion	Uptake rate (%)	Captured Vol. (μl/mg lipid)
1	0.05M NaCl	None	4.7	1.9
2	0.05M NaCl	0.01M $MgCl_2$	41.5	16.6
3	0.05M NaCl	0.01M $MnCl_2$	40.7	16.3
4	0.05M NaCl	0.05M $CoCl_2$	22.0	8.8
5	0.05M NaCl	0.05M $ZnSo_4$	27.7	11.1

TABLE 6

	Concentration of $CaCl_2$	Uptake rate (%)	Captured Volume (μl/mg lipid)
1	1.0M	0.7	0.03
2	3.0mM	45.9	18.4
3	0.5mM	3.0	1.2
4	0.1mM	43.9	17.6

TABLE 7

	Concentration of $MnCl_2$	Uptake rate (%)	Captured Volume (μl/mg lipid)
1	0.2M	11.0	4.4
2	5.0mM	43.2	17.3
3	0.5mM	4.8	1.9
4	0.2mM	43.0	17.2

CLAIMS

1. A process for preparing liposome compositions which comprises dispersing freeze-dried multilamella vesicles or small unilamella vesicles in an aqueous medium in the presence of one or more active ingredients and a divalent cation in order to regenerate large unilamella vesicles or large oligolamella vesicles entrapping said active ingredients.

2. A process as claimed in claim 1, wherein said active ingredients are admixed before the freeze-drying of said multilamella vesicles or small unilamella vesicles.

3. A process as claimed in claim 1, wherein said active ingredients are admixed with said freeze dried multilamella vesicles or small unilamella vesicles and then dispersed in the aqueous medium.

4. A process as claimed in claim 1, wherein the active ingredient is added in advance to the aqueous medium.

5. A process as claimed in any one of the preceding claimed wherein the vesicles are composed of lecithins.

6. A process as claimed in any one of the preceding claims wherein the dispersal of the vesicles in the aqueous medium is carried out at or above the gel-phase/liquid crystal-phase transition temperature.

7. A process as claimed in any one of the preceding claims wherein the divalent cation used is selected from calcium, manganese and magnesium.

8. A process as claimed in claim 7, wherein when the divalent cation is calcium, the cation concentration range is about 3×10^{-3} to 1×10^{-1}M.

9. A process as claimed in claim 7, wherein when the divalent cation is magnesium, the cation concentration range is about 4×10^{-3} to 1×10^{-1}M.

10. A process as claimed in claim 7, wherein when the divalent cation is manganese, the cation concentration range is about 8×10^{-4} to 1×10^{-1}M.

11. A process as claimed in claim 1 substantially as hereinbefore described.

12. A process as claimed in claim 1 substantially as hereinbefore described with reference to any one of the Examples.

13. A composition prepared by a process as claimed in any one of the preceding claims and adapted for pharmaceutical or veterinary use.

14. A composition as claimed in claim 13 and in unit dosage form.

15. A composition as claimed in claim 13 or claim 14 for use in the treatment of diseases and ailments.

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