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(71) Applicant *(for all designated States except US)*: **HYUNDAI PHARMACEUTICAL IND. CO., LTD** [KR/KR]; 1110-2, Hwagok-dong, Gangseu-gu, Seoul 157-019 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KIM, Hack-Joo** [KR/KR]; 141-33, Seongsan 2-dong, Mapo-gu, Seoul 121-252 (KR). **AN, Heung-man** [KR/KR]; #6-1205, Jinju Apt., 30-2, Gajwa 2-dong, Seo-gu, Incheon 404-252 (KR). **CHA, Min-Jong** [KR/KR]; 252-46, Neung-dong, Gwangjin-gu, Seoul 143-108 (KR).

(74) Agent: **PARK, Sa-Ryong**; Park Patent & Law, Rm. 301, Chongho Bldg., 823-5, Yeoksam 1-dong, Kangnam-gu, Seoul 135-081 (KR).

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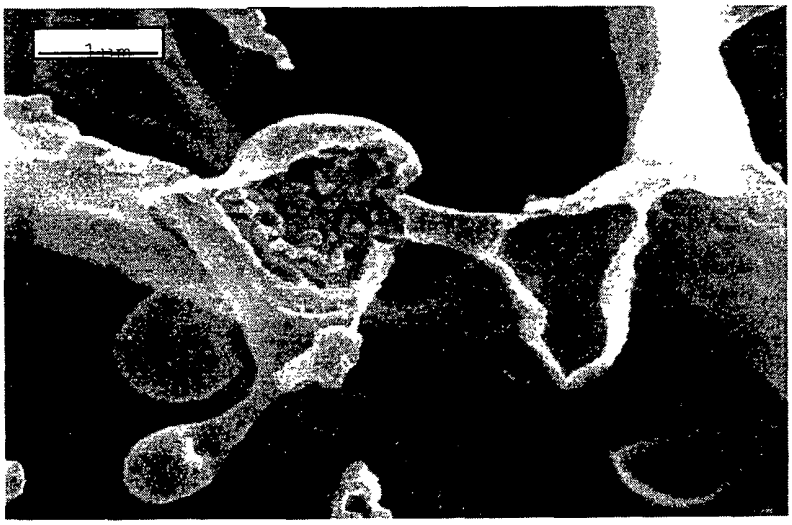
(54) Title: ORAL DELIVERY OF PEPTIDE



(57) Abstract: Proliposome of a peptidyl drug and enteric preparations containing said proliposome are disclosed by the present invention, wherein said proliposome is prepared by dissolving the peptidyl drug and phospolipid in an organic solvent and coating the resulting solution with water-soluble chitosan. The oral delivery system of peptide using the proliposome and the enteric preparation according to the present invention remarkably increases stability and bioavailability of a peptidyl drug.

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ORAL DELIVERY OF PEPTIDE

TECHNICAL FIELD

The present invention relates to a preparation for oral administration of peptide and protein drugs (hereinafter, referred to as "the peptide drug") and a method for producing the same.

BACKGROUND ART

According to a rapid progress of genetic recombination technique and solid-phase peptide synthetic technique, the kinds of peptide drugs which can be used in clinical field have been broadened on a sudden increasing tendency in the 1990's, which has been accelerated due to remarkable progress of biotechnology so that about 100 or more drugs have been developed and commercialized in the world at present.

However, most of peptide drugs as presently developed and commercialized are in the form of injections and therefore, have some disadvantages in that 1) injection to the patient is accompanied by a pain, 2) it has a difficulty in storage such as the necessity of storage in a refrigerator, and 3) a patient must attend a hospital when the drug is injected. Thus, although it is urgently required to develop the oral preparations of the peptide drug, such a development is under the first stage to provide only an insignificant outcome up to the present.

If the above-mentioned disadvantages involved in the injection of the peptide drug can be overcome by the oral preparation, it is expected that 1) the peptide drug can be formulated in the most suitable form for administration to the patient, 2) the stability of the preparation is increased so that the conditions of production, selling and consumption such as distribution, establishment of the effective period, etc. can be conveniently and advantageously varied as compared to the prior formulation, and 3) the market of the oral preparation preferred by the patient will rapidly substitute for the

market of prior drug formulations. Therefore, the technical study to develop the oral preparation has been actively made.

In general, the technical difficulty in developing the oral preparation of the peptide drug has been reported in well-known papers (Raymond M. Reilly, Rommel Domingo and Jasbir Sandhu. Drug Delivery Systems, 32, 4, 313-323, 1997; Jeoseph A. Fix, Pharmaceutical Research, vol. 13, No. 12, 1996; Amyn P. Sayani and Yie W. Chien, Critical Reviews in Therapeutic Drug Carrier Systems, 13, 1&2, 85-184, 1996; Jane P. F. Bai, Li-Ling Chang and Jian-Hwa Guo, Critical Reviews in Therapeutic Drug Carrier Systems, 12, 4, 339-371, 1995).

In developing the oral preparation of such peptide drugs, the major problems to be solved include 1) a low absorption in intestinal tract, 2) a loss of efficacy by proteinase present in small intestine and 3) a difficulty in maintaining the efficacy due to a short half-life within a living body after absorption and thus, the study places the focus such problems.

Among these problems, first the following methods have generally been used for solving a low absorption in intestinal tract: 1) Inclusion of drugs in a nano- or microsphere particle to increase their permeation through bio-membrane (Istvan Toth, International Journal of Pharmaceutical, 183, 1999, 51-55; E. Bjork, U. Isaksson, P. Edman, J. Drug Targeting 2, 501-507, 1995; Shinji Sakuma, Norio Suzuki, International Journal of Pharmaceutical, 149, 1997, 93-106); 2) Method for using the drug together with an absorption accelerant (Patrick J. Shinko, Yong-Hee Lee, Pharmaceutical Research, vol. 16, No. 4, 1999, 527-533; J. C. Scott-Moncrieff, Z. Shao, J. Pharm. Sci. 83, 1465-1469, 1994; E. A. Hosny, N. M. Khan, Drug Devel. Ind. Pharm. 21, 1583-1589, 1995; Isao Sasaki, Hideyuki Tozaki, Biol. Pharm. Bull. 22, 6, 611-615, 1999; Anthomy C. Chao, Joseph Vu Nguyen, International Journal of Pharmaceutical, 191, 1999, 15-24); 3) Methods for modifying peptide structures (Deven Shah, Wei Chiang, Journal of Pharmaceutical Sciences, vol. 85, No. 12, 1996, 1306-1309; Kazunori Iwanaga, Satoshi

Ono, Journal of Pharmaceutical Sciences, vol. 88, No. 2, 1999, 248-252; Istvan, John P. Malkinson, J. Med. Chem. 1999, 42, 4010-4013); 4) Method for modifying the structures of the drugs or using the drugs together with an enzyme inhibitor (P. Buhlnayer, A. Caseli, W. Fuhrer, J. Med. Chem. 31, 1839, 1998; A. Yamamato, T. Taniguchi, K. Rikyu, T. Tsuji, Pharm. Res. 11, 1496-1500, 1994); and 5) Inclusion of the drugs in a microsphere or modification of the drugs in order to increase the stability of drugs in a living body (David F. Ranney, Biochemical Pharmacology, vol. 59, 105-114, 2000; Ian M. Chapman, Ora H. Pescoviz, Gail Murphy, Journal of Clinical Endocrinology & Metabolism, 1997, vol. 82, No. 10, 3455-3463). Further, the study to develop the preparation of the peptide drug has been actively conducted through numerous ways (Z. Aydin, J. Akbuga, International Journal of Pharmaceutics 131, 101-103, 1996; K. Aledeh, E. Gianasi, I. Orienti and V. Zecchi, J. Microcapsulation, 1977, vol. 14, No. 5, 567-576; A. Pork, B. Amsden, K. de yao, Journal of Pharmaceutical Science vol. 83, No. 2, 1994, 178-185).

Further, as one method for developing the oral preparation of such peptide drugs a liposome has been utilized. This method has some advantages that 1) the drugs can be relatively readily included in a nano- or microsphere particle and 2) a liposome can be easily absorbed in intestinal mucous membrane, and therefore, has been widely studied in recent years. Prior arts relating to such method include a technique disclosed in a patent by Lipotec, S.A. (EP-0855179 A2). However, this method has several disadvantages in using the liposome as the medicinal product that in preparing the liposome 1) the use of water may cause the problem relating to a stability of the drugs, 2) the procedures for lyophilization or drying must be used in order to prepare the liposome in the form of a powder, 3) its stability in aqueous solution is low because of the properties of liposome itself including aggregation, sedimentation, fusion, oxidation, phospholipid hydrolysis, etc., and 4) it is difficult to provide the reproductivility and sterilization during the production procedures.

DETAILED DESCRIPTION OF INVENTION

In order to overcome the problems involved in the prior art as mentioned above the present inventors have studied and examined the preparation of peptide drugs with laying emphasis on the physical modification rather than chemical modification of peptide drugs for a long time, and thus completed the present invention.

Thus, the present invention provides a method for improving the problems involved in the prior art as mentioned above, in which 1) water-soluble chitosan is used to prepare proliposome as the precursor of liposome thereby increasing the absorption into intestinal mucous membrane, 2) an agent for controlling pH is used to increase a stability of peptide drugs in aqueous intestinal juice, 3) an additive such as absorption accelerator, etc. is added in order that peptide drugs can be smoothly absorbed into intestinal mucous membrane, and then 4) the product is formulated into a preparation suitable to oral administration and at the same time, 5) the preparation is covered with an enteric coating so that the drug can be readily migrated and absorbed into the intestine without destruction.

That is, the present invention can prepare a proliposome, as the precursor of liposome, in a high yield within a short time without conducting the procedure for lyophilization or evaporation in order to prepare liposome in the form of a powder, which may cause the problems occurring in the prior method for preparing the oral preparation using liposome as disclosed in EP-0855179, and therefore, has some advantages that the procedure of the process is simple, a stability of peptide drugs to moisture and temperature, which constitutes the disadvantage of peptide drugs, is increased, and chitosan is used as a carrier for preparing proliposome to increase a bioavailabilty.

Specifically, according to the present invention

1) using the water solubility of water-soluble chitosan, the organic solvent is used to prepare the peptide drug coated onto water-soluble chitosan in the form of a powder;

2) an enteric coating is applied to the drug in order to protect the peptide drug from gastric acid;

3) when the drug reaches the intestine, the powdery proliposome in the form of a powder forms a liposome within the intestine owing to the physical property of water-soluble chitosan to be dissolved in water, so that the drug can be smoothly absorbed into intestinal mucous membrane and at the same time, chitosan having a free cation provides a property of attaching to intestinal mucous membrane;

4) in order to prevent an easy decomposition of the drug in the intestine after oral administration due to an instability of most peptide drugs in an aqueous solution, an agent for controlling pH level is added to increase a stability of the drug in the intestine by taking an advantage that the most of peptide drugs are stable at pH level of 3 to 4; and

5) in order that peptide drugs can be readily absorbed into intestinal mucous membrane after a paratrans pattern between cells, an additive such as absorption accelerator is combined so that the peptide drugs can be easily absorbed into intestinal mucous membrane.

Hereinafter, the present invention will be more specifically explained.

Chitosan is a natural high molecular material and is a substance made from polysaccharide chitin which is widely distributed in shells of crustacean such as crab, shrimp, etc. and insect outer skins, mushrooms, cell walls of fungi and plays a role of supporting and preventing the living body containing them.

Specifically, chitin is a high molecular material in a very long chain structure without side chains, which is similar to celluloses, wherein carbon atom at 2-position is substituted with acetylamino radical (-$NHCOCH_3$) in place of hydroxyl radical (-OH). Chitosan is a material produced by deacetylation of acetyl radical attached to amino group present on carbon atom at 2-position in chitin and can have a lot of free cations so that it possesses various physiological activities.

For this reason, chitosan in a high quality has been developed and widely used in various industrial field such as food, cosmetics, pharmaceuticals and absorbents, activating agent for plant cells, aggregating agent for waste water disposal, etc. Particularly, in recent years it has been disclosed that an ability of chitosan to combine fat is far better than that of vegetable fibrin, and chitosan improves the immunity and resistance to diseases and further, has no toxicity in a living body and therefore, is harmless.

However, since chitosan which has been widely used in various field in these days cannot be dissolved in water or an alcohol but can be dissolved only in an aqueous solution of an organic acid such as formic acid, lactic acid, acetic acid, etc., or an inorganic acid such as dilute hydrochloric acid, it has the limited applicability in the field of pharmaceutical preparations.

Thus, the present inventors have studied to prepare the oral preparation of peptide drugs using water-soluble chitosan (JK FM-01: Ja Kwang Co., Ltd.), and then completed the present invention. Water-soluble chitosan used in the present invention is characterized in that 1) the degree of deacetylation is 85%-99%, 2) the solubility in water is 99.99% or more, 3) the molecular weight is 100,000 to 500,000 to be possibly used as the excipient in pharmaceutical products, and 4) it meets the standard requirements for a food additive.

That is, the major characteristic feature of chitosan used in the present invention is the water solubility of 99.99% or more. The reasons why the water solubility of chitosan used in the present invention is higher than that of the prior chitosan are that 1) the method for preparing chitosan by treating chitin with an aqueous alkaline solution, and conducting deacetylation procedure by hydrolyzing acetylamino group to amino group followed by a multi-step separation membrane procedure allows an increase in the degree of acetylation to 85 – 99 % while maintaining the water-solubility of chitosan, 2) chitosan can be prepared in a purity of 90% or more through a multi-step separation

membrane procedure, and 3) since amino group is activated during the deacetylation procedure for carbon atom at position 2 of chitosan, chitosan can be readily dissolved in water while maintaining the characteristic properties of chitosan when chitosan is contacted with water molecules.

Furthermore, the characteristic feature of the present invention is to prepare the oral preparation of peptide drugs by removing the problems of liposome which has been used in the field of pharmaceuticals and cosmetics and simultaneously taking advantages of water-soluble chitosan which can be dissolved in an aqueous solution while reserving the advantages of chitosan.

That is, since in 1965 liposome was reported as a mono-layer or multi-layer phospholipid double-membrane closing vesicle by Bamgham et al., (Diffusion of Univalent Ions across the Lamellae of Swollen Phospholipids, J. Mol. Biol., 13, 238-252, 1965), during the past scores of years liposome has been prepared according to numerous methods for preparation thereof by modifying 1) a surface charge, 2) a size and 3) a lipid content so as to meet with the use purpose and end use of liposome, and used in food, cosmetics and pharmaceutical products.

Particularly, in recent years liposome has been used in the pharmaceutical field to develop various pharmaceutical formulation for the purpose of 1) the sustained activity and targeting of the drug, 2) the alleviation of acute toxicity and the alleviation or enhancement of immunoreaction, 3) the stabilization of the drug and 4) the change of dosage formulation. However, liposomes which have been utilized for development of pharmaceutical formulations have disadvantages insufficient to be effectively used in the pharmaceutical products that their stability in the aqueous solution is low due to the properties of liposome itself including 1) aggregation, 2) sedimentation, 3) fusion, 4) oxidation and 5) phospholipid hydrolysis, and 6) the efficiency of sterilization and reproductibility during the process for mass production is low.

For the reasons above, the present inventors have extensively studied to remove

the problems involved in such prior liposome preparations for a long time, and as a result, surprisingly found that all the problems of the prior liposome preparations can be removed by preparing a proliposome in the form of a solid powder as the precursor of liposome using water-soluble chitosan as a carrier. Thus, we have completed the present invention.

Therefore, the purpose of the present invention is to provide a proliposome of the peptide drug prepared by using a water-soluble chitosan.

Another purpose of the present invention is to provide an oral preparation formulated from a proliposome of the peptide drug produced using a water-soluble chitosan according to a conventional pharmaceutical method.

Further purpose of the present invention is to provide a process for preparing an oral preparation formulated from a proliposome of the peptide drug produced using a water-soluble chitosan according to a conventional pharmaceutical method.

In general, the carrier for preparing proliposome must have the following properties: 1) it must be water-soluble since it is dissolved in water to be converted into liposome from proliposome, and 2) its solubility in the organic solvent used in the course of the process must be low. Water-soluble chitosan used in the present invention not only has the carrier properties of proliposome as mentioned above but also can increase the absorption of peptide drug in the living body in case of the oral administration of the drug due to an effect of increasing the adhesive property caused by an ability of free cations contained in chitosan itself to combine with lipid when it is dissolved by the action of water within the intestine.

Typical examples of peptide drugs which can be used in the present invention include aprotinin, buserelin, calcitonin, desmopressin, elcatonin, glucagon, gonadotropin, gonadorelin, goserelin, hirudin, leuprolein, lypressin, nafarelin, octreotide, oxytocin, protirelin, salcatonin, sermorelin, somatostatin, somatropin, terlipressin, tetracosacrin, thymopentin, triptorelin, vasopressin, albumin, insulin, interferon, immunoglobulin,

GM-CSF, G-CSF, glycoprotein, etc. In addition, of course, other peptide and protein drugs can also be included within the scope of the present invention.

Phospholipids used in preparing the proliposome containing peptide drug according to the present invention include a pharmaceutically acceptable phospholipid conventionally used for preparation of liposome, for example, phosphatidyl-DL-glycerol-dimyristoyl, L-α egg phosphatidyl choline, soybean phosphatidyl choline (lecithin), dipalmitoyl phosphatidyl choline (DPPC), dimyristoyl phosphatidyl choline (DMPC), cholesterol, stearylamine, diacetylphosphate, phosphatidyl serine, methoxypolyethylene glycol distearoyl phosphatidyl-ethanolamine, etc.

The peptide drugs composed of amino acids are generally contain asparagines or glutamine residue in their structure and these residues occur the reaction such as deamidation, beta-elimination, disulfide exchange, racemization, oxidation, etc., which may readily induce the structural degeneration to ultimately cause the lose of an activity as peptide drugs. As the method for preventing such problem, the present invention uses an agent for controlling pH level to pH 3-4 at which asparagines and glutamine residues do not occur chemical reaction when the oral preparation according to the present invention is readily dissolved in the intestine, to stably maintain the peptide drugs in the intestine.

As the agent for controlling pH level in the present invention, any agent which can be generally used for oral administration in the pharmaceutical products can be used and the typical example thereof includes citric acid, sodium citrate, adipic acid, sodium monohydrogen phosphate, etc. The agent for controlling pH level is preferably used in an amount of 1.0% to 50% on the basis of the total amount.

Another characteristic feature of the present invention is to stimulate the absorption of peptide drugs into the small intestine when the drug is administered via oral route, by using the absorption accelerator originated from natural products or food products which do not cause any damage to the epithelial cells of intestinal tract. The

absorption accelerators which can be used in the present invention include fatty acids or salts thereof such as capric acid, oleic acid, etc., cholate as the salt of bile acid, deoxycholic acid, salicylate-based absorption accelerator, or monoglyceride-based absorption accelerator. The absorption accelerator generally used in Example part of the present invention is preferably deoxycholate as the cholic acid-based salt which is preferably used in an amount of 0.1% to 10%.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a photograph showing a shape of the proliposome formed from salmon calcitonin and water-soluble chitosan and the inclusion of the drug;

Figure 2 is a photograph showing the process of dissolution in water as obtained from observation of the hydration process of the proliposome using water-soluble chitosan by mean of a microscope, wherein Figure 2a is a photograph showing proliposome powder and Figure 2b is a photograph showing the production of liposome from proliposome;

Figure 3 is a graph showing the particle size and distribution of liposome formed from hydration of the proliposome;

Figure 4 is a graph showing a change in the content of salmon calcitonin in the proliposome of salmon calcitonin, as one embodiment according to the present invention, in course of time; and

Figure 5 is a graph showing the drug permeability of various formulations containing the proliposome of salmon calcitonin, as one embodiment according to the present invention.

EMBODIMENT FOR CARRYING OUT THE INVENTION

The present invention will be more specifically illustrated by the following

examples and experiments. However, the scope of the present invention is not limited by these examples and experiments.

Example 1

Preparation of proliposome (Example of preparing salmon calcitonin)

4 g of water-soluble chitosan powder (106 μm - 300 μm) passed through a sieve was introduced into a 100 ml round-bottomed flask, mounted on a rotary evaporator and then dried under reduced pressure for 30 minutes at room temperature. 267 mg of egg lecithin as phospholipid was dissolved in 30 ml of chloroform and then mixed with a solution of 27 mg of salmon calcitonin, which was previously dissolved in 10 ml of methanol.

While the temperature in a 100 ml round-bottomed flask containing water-soluble chitosan is maintained at 20-30°C, a given amount (about 1 ml) of a mixed solution of liposome constituents dissolved in the organic solvent was added and then, the rotation of rotary evaporator was maintained at 120-150 rpm. When water-soluble chitosan is completely dried to be a free-flow state, the mixed solution of organic solvent was added again to repeatedly apply the coating. The final solution was added and dried and then, the product was re-dried in a freezing drier for 24 hours to remove the organic solvent (Yield: 95% or more).

Example 2

Preparation of proliposome (Example of preparing desmopressin)

3g of water-soluble chitosan powder (106 μm - 300 μm) passed through a sieve was introduced into a 100 ml round-bottomed flask, mounted on a rotary evaporator and then dried under reduced pressure for 30 minutes at room temperature. 160 mg of phosphatidyl-DL-glycerol-dimyristoyl as phospholipid was dissolved in 24 ml of chloroform and then mixed with a solution of 20 mg of desmopressin, which was previously dissolved in 12 ml of ethanol.

While the temperature in a 100 ml round-bottomed flask containing water-soluble chitosan is maintained at 20-30°C, a given amount (about 1 ml) of a mixed solution of liposome constituents dissolved in the organic solvent was added and then, the rotation of rotary evaporator was maintained at 120-150 rpm. When water-soluble chitosan is completely dried to be a free-flow state, the mixed solution of organic solvent was added again to repeatedly apply the coating.

The final solution was added and dried and then, the product was re-dried in a freezing drier for 24 hours to remove the organic solvent (Yield: 96% or more).

Example 3-33

The respective drug selected from the group consisting of aprotinin, buserelin, elcatonin, glucagon, gonadotropin, gonadorelin, goserelin, hirudin, leuprolein, lypressin, nafarelin, octreotide, oxytocin, protirelin, salcatonin, sermorelin, somatostatin, somatropin, terlipressin, tetracosacrin, thymopentin, triptorelin, vasopressin, albumin, insulin, interferon, immunoglobulin, GM-CSF, G-CSF and glycoprotein was used according to the similar method to Example 1 or 2 to prepare the corresponding proliposome containing respective peptide drug.

Experiment 1

Identification of inclusion of the drug within proliposome (Cryo-SEM identification)

In order to confirm the inclusion of the drug within proliposome containing salmon calcitonin, the proliposome not including the drug and the proliposome including the drug were comparatively observed by means of an ultra-low temperature electron microscope. For pretreatment of the sample, first, one drop (about 3 μl) of the liquid sample (liposome hydrated from proliposome) was dropped on a discus sample table having a diameter of 1 cm, and liquid nitrogen was filled in a nitrogen slushing chamber of Cryo-transfer system (CT 1500 Cryotrans, Oxford Instrument Ltd., UK). Then, the

sample was rapidly introduced in the chamber and fixed for one minute in vacuum.

Next, the sample was transferred to a cryo-chamber controlled to -170°C and the vacuum state was confirmed. Then, the cooled sample was voluntarily broken and cleaved, and the resulting broken sample was transferred to a sample table of a scanning electron microscope (JSM-5410LV, JEOL LTD., Japan) connected to the cryo-transfer system and the temperature was controlled to -70°C at which the sample was maintained for 5 minutes to sublimate the moisture form the sample surface.

After completion of sublimation, the sample was transferred again to the cryo-chamber and then subjected to gold coating. Under the accelerating voltage of 20 kV, the broken and cleaved section of the sample was observed to confirm the shape of liposome as formed and the inclusion of the drug of which the photograph is shown in Figure 1.

Experiment 2

Observation of dissolution of proliposome

In order to observe the hydration process of proliposome produced using water-soluble chitosan by means of an optical microscope, the proliposome granules were mounted on a slide of the microscope and the focus was adjusted. At 400X magnifications, one drop of water was dropped on the granule and after one minute the hydration process could be observed to confirm the dissolution process of the proliposome in water of which the photograph is shown in Figure 2.

Experiment 3

Analysis of the particle size of liposome as produced

5 ml of distilled water was added to 5 mg of water-soluble chitosan proliposome as prepared in Example 1 and shaken with vortex to dissolve the proliposome, which was then hydrated for 30 minutes. The particle size and distribution of liposome thus

prepared was measured by means of a particle size analyzer. The result thereof is shown as the graph in Figure 3.

Experiment 4

Quantitative analysis and stability of salmon calcitonin within the proliposome

In order to identify the stability and conduct the quantitative analysis, the prepared proliposome corresponding to about 5 μg of salmon calcitonin was accurately weighed, dissolved in 300 μl of methanol and then completely dissolved with vortex. After filtration through a 0.45 μm filter, an aliquot of 40 μl was applied to HPLC to quantify the content of salmon calcitonin in the proliposome. The result from determination of the stability at room temperature and under refrigerator is shown in Figure 4. The conditions for HPLC utilized for analyzing the content of the drug are as follows:

HPLC conditions: Column – C18 (Sucelpo Inc.)

Flow rate – 1 ml/min.

Solvent A – 0.1% trifluoro acetic acid/acetonitrile

Solvent B – 0.1% trifluoro acetic acid /water

Experiment 5

Preparation and enteric coating of oral formulation (tablet)

The tablet was prepared by a direct compression in the manner of dry process rather than wet process according to the general method for preparing a tablet as defined in the part of General Provisions For Preparation in the Korea Pharmacopoeia. The compositions of respective samples prepared in order to examine the effect of the present invention are shown in the following Table 1 and the respective samples prepared by a direct compression were coated with Cellulose Acetate phthalate(CAP) to

provide an enteric coating. The result of coating was confirmed by practicing the disintegrating test in artificial gastric juice(solution 1) and artificial intestinal juice(solution 2) using the general test method as defined in the Korea Pharmacopoeia and is described in the following Table 2.

Table 1

Classification	Formulation 1	Formulation 2	Formulation 3	Formulation 4	Formulation 5
Proliposome containing salcatonin	24%	24%	24%	24%	24%
Deoxycholic acid		5%		5%	16%
Citric acid			19%	48%	60%
Lactose	76%	71%	57%	23%	
Total	100%	100%	100%	100%	100%

Table 2

Classification	Formulation 1	Formulation 2	Formulation 3	Formulation 4	Formulation 5
Solution 1	Not dissolve	Not dissolve	Not dissolve	Not dissolve	Not dissolve
Solution 2	Dissolve	Dissolve	Dissolve	Dissolve	Dissolve

In order to verify the effect of the present invention, the samples were used to

practice both in vivo and in vitro tests.

Experiment 6

In vitro test using cells

(1) Preparation of a complete media

1 L x 2 of DMEM (Dulbecco's modified eagle medium), 7.4 g of sodium bicarbonate and 2.6 g of HEPES were added to 2 L of distilled water which was previously sterilized and cooled, and then stirred for about 1-1.5 hours in a plate stirrer.

After stirring, the pH level was adjusted with 1 N hydrochloric acid to 7.4 by means of a pH- meter. The solution was filtered through a filter (Corning Filter 430015) and then dispensed into 500 ml bottles 450 ml in each bottle by means of a 50 ml pipette.

To a 500 ml bottle containing 450 ml of the solution were added 50 ml of fetal bovine serum(FBS), 5 ml of streptomycin and 5 ml of mem nonessential amino acid solution(NEAA). In order to confirm whether the prepared complete medium was contaminated or not, about 6 ml of the complete medium was introduced into a 25 ml T-flask, and after observation by a microscope, was incubated for one day or more in an incubator to ascertain the contamination.

(2) Defrosting of $CaCO_2$ cells

The complete medium, which was prepared and then preserved in a refrigerator, was warmed in a water bath at 37°C for about 20 minutes and then mixed for 4 to 5 times using a 5 ml pipette. 5 ml of the medium was then introduced into a 15 ml centrifuge tube. Cell line (ATCC HTB-37, LOT 944495) was taken out of a nitro tank and thawed in a water bath at 37°C with slightly opening a cap of the bottle. After identifying that the cell line was completely thawed, the cap of the bottle reserving cell

line was opened and mixed 5 to 6 times with a 5 ml pipette. The whole content of the bottle was introduced into the above 15 ml centrifuge tube.

15ml centrifuge tube was centrifuged by means of a centrifuge at 1000 rpm for 7 minutes to separate the supernatant, which was taken off then removed. 1 ml of the medium was added thereto and then mixed 4 to 5 times with a pipette to lose the cells. The whole content of the solution contained in 15 ml conical tube was transferred to a 25 ml T-flask that was then preserved in an incubator.

(3) Change of feeding cell media

A 25 ml T-flask which was preserved in the incubator was taken out of the incubator and the media on the bottom of the flask was removed and replaced by warming the compete medium, which was previously prepared and preserved in a refrigerator, in a water bath at 37°C for about 20 minutes and then introducing 5 ml aliquot of the medium into 25 ml T-flask with taking care of not touching the pipette with the bottom of the flask.

(4) Subculture

Trypsin EDTA container and free media, complete medium, which were preserved in a refrigerator, were warmed in a water bath at 37°C for about 20 minutes, and then the medium contained in 25 ml T-flask preserved in the incubator was removed and 2 ml of trypsin EDTA was introduced into 25 ml T-flask.

Trypsin EDTA was allowed to uniformly distribute and the flask was then preserved in a water bath at 37°C for 4 minutes. 10 ml of the complete medium was added to 25 ml T-flask and the flask was shaken to separate the cells. The separated solution was transferred to 15 ml conical tube by means of a pipette, centrifuged at 1000 rpm for 5 minutes to remove the supernatant. 2 ml of the complete medium was added

to T-flask and the cells were allowed to be well loosen and then re-incubated in the incubator (In this experiment, the cells over 40 passages were used.).

(5) Coating of transwell membrane with collagen

10 ml of sterilized 0.1% acetic acid was added to 25 mg of rat tail collagen, which was then dissolved by stirring for 3 to 5 hours with addition of a magnetic bar. The resulting solution was diluted with 60% ethanol solution in the ratio of 1:1.5 to make the final concentration of 0.3 mg/ml. Then, 50 μl of collagen solution was uniformly distributed on the upper membrane of a transwell (Costar 3401) and dried by standing under UV light form 4 to 5 hours in a laminar flow with opening the cap.

(6) Seeding cells onto the transwell plate

0.5 ml and 1.5 ml of the complete medium were introduced into the upper compartment and the lower compartment, respectively, of the coated transwell and incubated for 15 minutes in the incubator. Then, the medium was removed and 1.5 ml of the complete medium was re-introduced into the lower compartment of the re-coated transwell. Cells over 40 passages under subculture were diluted to the concentration of 2.5-3 x 10^5 cells/ml and then introduced into the upper compartment of the coated transwell in an amount of 1.5 ml and incubated for 2 to 3 weeks so as to be used in the experiment.

(7) Identification of the properties of $CaCO_2$ cell monolayer

After 2 to 3 weeks from the inoculation of cells onto the transwell, transepithelial electrical resistance (TEE) was measured by means of a Millicell-ERS Resistance System to confirm the resistance of 250 Ωcm^2 or more which means that the monolayer of cells was well established. When the permeability experiment was

conducted using C^{14}-mannitol, the value of (<receiver dpm> / <donor dpm>) / hr / cm^2 was below 0.4%, which means that the cell monolayer was well established. The specific content of the experiment are as follows.

Drug concentration as used: 2-10 μM of C^{14}-mannitol having a specific activity of 50 mCi/mmol

Quantification of C^{14}-mannitol: 100 μl of the sample was taken and added to a LSC vial to which 2 ml of LSC cocktail was added and, after vortex for 10 seconds, the content of C^{14}-mannitol was measured in dpm by means of a liquid scintillation counter (LSC).

(8) Test for drug permeation (in case of salcatonin)

The examined medium present in the upper compartment of the transwell was removed using a pipette. The proliposome prepared in Example 1 above was dissolved in a transbuffer to the concentration of the drug (4.0 μg/ml) and then introduced into the upper compartment of the transwell, which was transferred to the new well containing a flashbuffer in intervals of 15 minutes after addition of the drug while slowly shaking at 85 rpm in a shaking water bath of 37°C.

The drug was sampled from the well in the donor part of transwell and then quantitatively analyzed with respect to salcatonin by means of ELISA and RIA kit (Pen. Lab.). The result is shown in Figure 5.

Experiment 7

In vivo test using rats

SD male rats (5 rats per group) weighing 250 g-300 g were received only drinking water from one day before the start of the experiment and adapted to the experimental environment. In order to insert cannula into femoral vein and artery 400

μl of the mixture of ketamine and lumpun in the ratio of 1:4 as an anesthetic agent was intramuscularly injected and then rats were fixed on the operating bed and femoral vein and artery were found in a leg using scissor and pincette and cannulated.

After cannulation, blood was sucked using a syringe from vein and artery to identify the blood collection. Then, the samples of formulation 1, 2, 3, 4 or 5 was orally administered to rats using an oral capsule sonde and blood was collected from femoral artery in an amount of 500 μl in each case in interval of a given time. The blood sample was collected while the corresponding amount of saline was injected into femoral vein.

The collected blood was centrifuged in a centrifuge at 10,000 rpm for 10 minutes to separate the supernatant from which the content of the drug was quantified using ELISA and RIA Kit (Pen. Lab.).

Separately, in order to compare an increase in bioavailability with each other, the same amount of the drug as orally administered was injected to femoral vein and the blood was pre-treated according to the similar manner to the method for collecting blood in case of each formulation. Then, the drug was quantified to calculate the bioavailability, which is described in the following Table 3.

Table 3

	Formulation 1	Formulation 2	Formulation 3	Formulation 4	Formulation 5
Bioavailability	0.1%	0.6%	2.5%	7.5%	5.8%

INDUSTRIAL APPLICABIITY

Thus, the present invention provides a method wherein 1) water-soluble chitosan is used to prepare proliposome as the precursor of liposome thereby increasing the absorption into intestinal mucous membrane, 2) an agent for controlling pH is used to increase a stability of peptide drugs in aqueous intestinal juice, 3) an additive such as absorption accelerator, etc. is added in order that peptide drugs can be smoothly absorbed into intestinal mucous membrane, and then 4) the product is formulated into a preparation suitable to oral administration and at the same time, 5) the preparation is covered with an enteric coating so that the drug can be readily migrated and absorbed into the intestine without destruction.

That is, the present invention can prepare a proliposome, as the precursor of liposome, in a high yield within a short time without conducting the procedure for lyophilization or evaporation in order to prepare liposome in the form of a powder, which may cause the problems occurring in the prior method for preparing the oral preparation using liposome as disclosed in EP-0855179, and therefore, has some advantages that the procedure of the process is simple, a stability of peptide drugs to moisture and temperature, which constitutes the disadvantage of peptide drugs, is increased, and chitosan is used as a carrier for preparing proliposome to increase a bioavailabilty.

CLAIMS

1. A proliposome prepared by dissolving a peptide drug and a phospholipid in an organic solvent and coating the resulting solution with a water-soluble chitosan.

2. The proliposome as defined in claim 1, characterized in that the peptide drug is selected from the group consisting of aprotinin, buserelin, calcitonin, desmopressin, elcatonin, glucagon, gonadotropin, gonadorelin, goserelin, hirudin, leuprolein, lypressin, nafarelin, octreotide, oxytocin, protirelin, salcatonin, sermorelin, somatostatin, somatropin, terlipressin, tetracosacrin, thymopentin, triptorelin, vasopressin, albumin, insulin, interferon, immunoglobulin, GM-CSF, G-CSF and glycoprotein.

3. The proliposome as defined in claim 1 or 2, characterized in that said water-soluble chitosan has the deacetylation degree of 85%-99% and the molecular weight of 100,000-500,000.

4. The proliposome as defined in claim 1 or 2, characterized in that the used phospholipid is selected from the group consisting of L-α egg phosphatidyl choline, soybean phosphatidyl choline, dipalmitoyl phosphatidyl choline, dimyristoyl phosphatidyl choline, cholesterol, stearylamine, diacetylphosphate, phosphatidyl serine and methoxypolyethylene glycol distearoyl phosphatidyl-ethanolamine.

5. An enteric-coated preparation of a proliposome of a peptide drug, which is prepared by dissolving a peptide drug and a phospholipid in an organic solvent, coating the resulting solution with a water-soluble chitosan to prepare a proliposome, formulating the proliposome into the pharmaceutically acceptable preparation according

to a conventional pharmaceutically acceptable method and then applying an enteric coating to the resulting preparation according to a conventional enteric-coating method.

6. The preparation as defined in claim 5, characterized in that the peptide drug is selected from the group consisting of aprotinin, buserelin, calcitonin, desmopressin, elcatonin, glucagon, gonadotropin, gonadorelin, goserelin, hirudin, leuprolein, lypressin, nafarelin, octreotide, oxytocin, protirelin, salcatonin, sermorelin, somatostatin, somatropin, terlipressin, tetracosacrin, thymopentin, triptorelin, vasopressin, albumin, insulin, interferon, immunoglobulin, GM-CSF, G-CSF and glycoprotein.

7. The preparation as defined in claim 5 or 6, characterized in that the proliposome is mixed with an agent for controlling pH to adjust the pH level in the range of 3-4.

8. The preparation as defined in claim 5, 6 or 7, characterized in that an absorption accelerator selected from the group consisting of fatty acids or salts thereof, bile acid , cholate, salicylic acid and salicylates is added as an absorption accelerator for the peptide drug.

FIG. 1

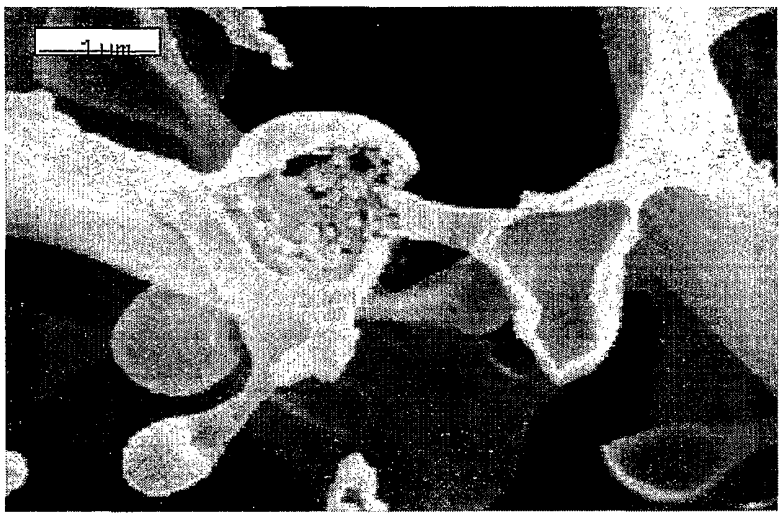


Fig. 2a

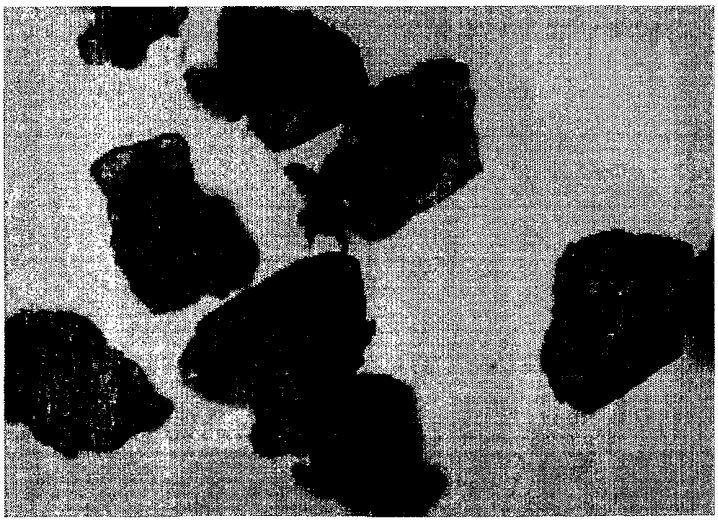


Fig. 2b

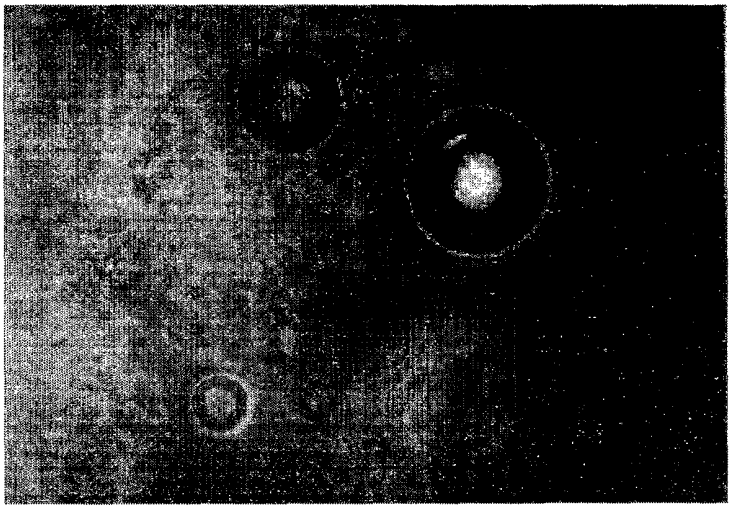


Fig. 3

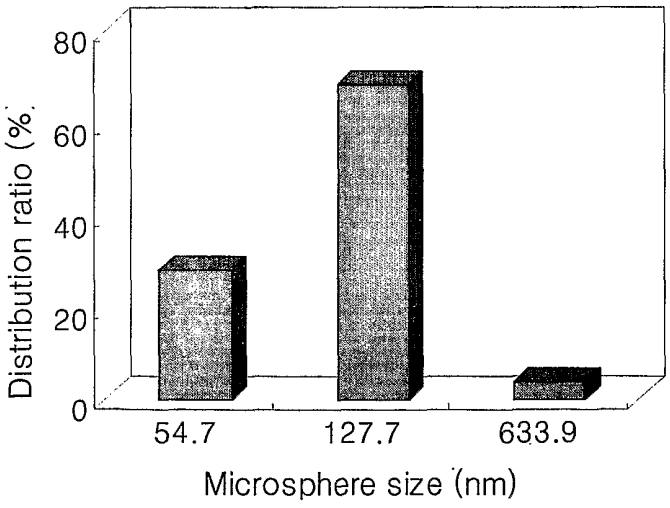

80
60
40
20
0
Distribution ratio (%)
54.7
127.7
633.9
Microsphere size (nm)

Fig. 4

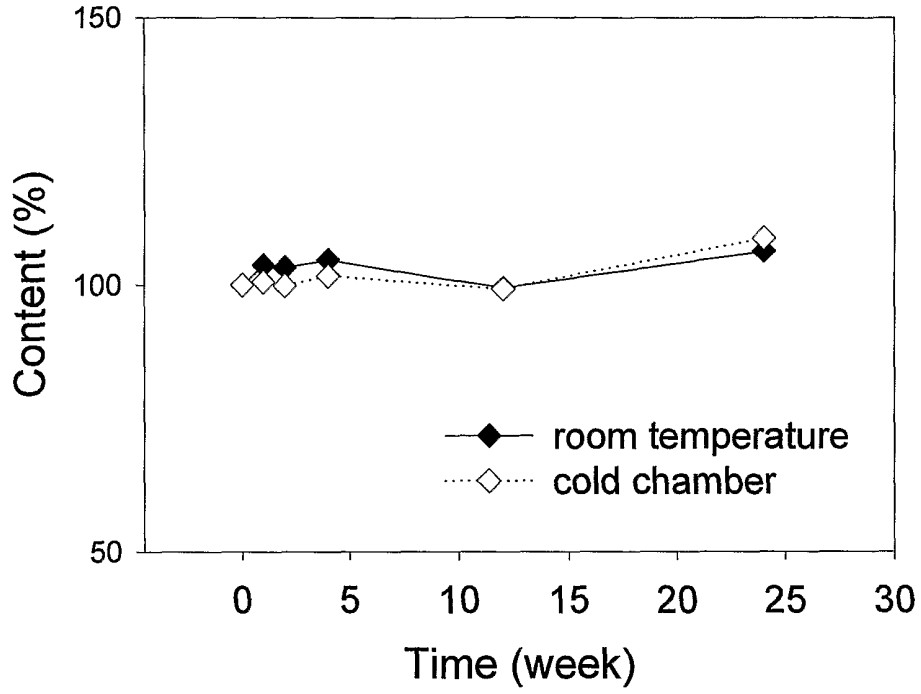
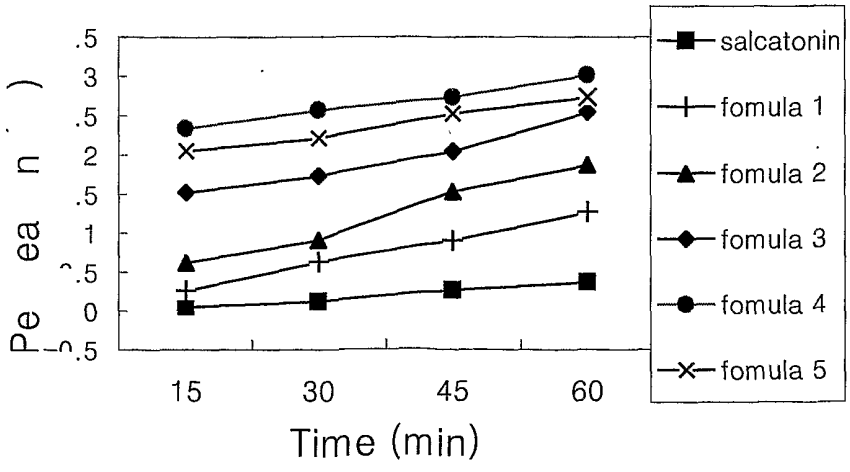

150
100
50
Content (%)
0
5
10
15
20
25
30
Time (week)
room temperature
cold chamber

Fig. 5


.5
3
.5
2
.5
1
.5
0
.5
15
30
45
60
Time (min)
salcatonin
fomula 1
fomula 2
fomula 3
fomula 4
fomula 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00892

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 A61K 9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)

CA Online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Takeuchi et al., 'Mucoadhesive liposomes coated with chitosan or Carbopol for oral administration of peptide drugs' In: Proc. Int. Symp. Controlled Release Bioact. Mater., 1999, 26th, 988-989, see the entire document.	1-8
Y	Takeuchi et al., 'Enteric absorption of insulin in rats from mucoadhesive chitosan-coated liposomes' In: Pharm. Res., 1996, vol. 13(6), 896-901, see the entire document.	1-8

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevence

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"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 APRIL 2001 (30.04.2001)	30 APRIL 2001 (30.04.2001)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea	Yoon, Kyung Ae
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5609

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