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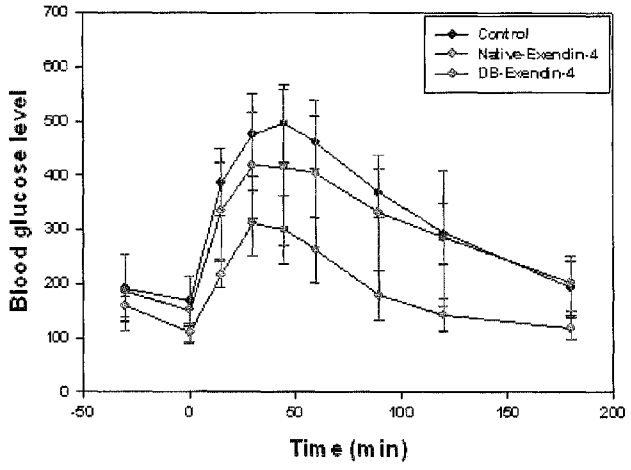
(54) **Title**: EXENDIN DERIVATIVE LINKED BIOTIN, METHOD FOR THE PREPARATION THEREOF AND PHARMACEUTICAL COMPOSITION COMPRISING THE SAME

[Fig. 11]




(57) **Abstract**: Disclosed are exendin-3 or exendin-4 derivatives modified with biotin, a preparation method thereof and a pharmaceutical composition containing the same. More specifically, disclosed are exendin-3 or exendin-4 derivatives in which the lysine residue of exedin is modified with biotin. The disclosed exendin-3 or exendin-4 derivatives modified with biotin show biological activity similar to that of native exendin and at the same time, have increased in vivo stability and are easily absorbed through the mucosa. Thus, biotin-modified exendin-3 or exendin-4 derivatives are useful for treating diseases, which can be caused by the excessive secretion of insulin, the lowering of plasma glucose, the inhibition of gastric or intestinal motility, the inhibition of gastric or intestinal emptying or the inhibition of food intake. Particularly, the biotin-modified exendin-3 or exendin-4 derivatives are useful for the treatment of diabetes, obesity and irritable bowel syndromes.

WO 2009/107900 A1

# Description

# EXENDIN DERIVATIVE LINKED BIOTIN, METHOD FOR THE PREPARATION THEREOF AND PHARMACEUTICAL COMPOSITION COMPRISING THE SAME

## Technical Field

[1] The present invention relates to exendin-3 or exendin-4 derivatives modified with biotin or its conjugate, a preparation method thereof and a pharmaceutical composition containing the same.

## Background Art

[2] Glucagon-like peptide-1 (hereinafter to be referred to as "GLP-1") induces numerous biological effects such as stimulating insulin secretion, inhibiting glucagon secretion, inhibiting gastric emptying, inhibiting gastric motility or intestinal motility, enhancing glucose utilization, and inducing weight loss. It is known that GLP-I may further act to prevent the pancreatic β-cell deterioration that occurs as type II diabetes, non-insulin dependent diabetes mellitus (NIDDM), progresses, and to recover insulin secretion by stimulating the production of new β-cells. Particularly, a significant characteristic of GLP-1 is its ability to stimulate insulin secretion without the associated risk of hypoglycemia that is seen when using insulin therapy or some types of oral therapies that act by increasing insulin expression. In addition, GLP-I is very effective in the treatment of type II diabetes because it does not involve side effects, such as the apoptosis and necrosis of pancreatic β-cells, which result from the long-term administration of the blood glucose-lowering drug sulfonylurea and the like.

[3] However, the usefulness of therapy involving GLP-1 peptides has been limited by the fact that GLP-1 is poorly active, and the two naturally occurring truncated peptides, GLP-1(7-37)OH and GLP-1(7-36)$NH_2$, are rapidly cleared *in* vivo and have extremely short *in vivo* half lives. Particularly, it is known that endogenously produced dipeptidyl-peptidase IV (hereinafter to be referred to as "DPP-IV") inactivates circulating GLP-1 peptides by removing the N-terminal histidine (7) and alanine (8) residues and is a major reason for the short *in vivo* half-life [see O' Harte et al., 2000].

[4] For this reason, various approaches have been attempted either to use DPP-IV inhibitors (P93/01, NVP-LAF237, NVP-DPP728, 815541A, 823093, MK-0431, etc.) to inhibit the degradation of GLP-1, or to use GLP-1 receptor agonists or GLP-1 derivatives (exendin, liraglutide, GLP-1/CJC-1131, etc.) to extend the half life of GLP-

1 peptides while maintaining the biological activity or reduce the rate of the removal of GLP-1 peptides from the body.

[5] Also, exendins, another group of peptides that lower blood glucose levels, were suggested for the first time by John Eng (see US Patent No. 5424286, exendin-3 [SEQ ID NO: 1] HSDGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS and exendin-4). Exendin-4 has the following sequence and shows partial sequence similarity (53%) to GLP-1(7-36)$NH_2$ [see Goke et al., 1993].

[6] **His** $^{1}$-Gly-Glu-Gly-The-Phe-The-Ser-Asp-Leu-Ser- **Lys** $^{12}$ - Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-**Lys** $^{27}$ - Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-$NH_2$ (SEQ ID NO: 2: HGEGTFTSDL SKQMEEEAVR LFIEWLKNGG PSSGAPPPS).

[7] Meanwhile, the exendins are found in the venom of *Helodermatidae* or beaded lizards. Exendin-3 is present in the venom of *Heloderma horridum*, the Mexican beaded lizard, and exendin-4 is present in the venom of *Heloderma suspectum*, the Gila monster. Also, exendin-4 differs from exendin-3 at only positions two and three. It has resistance to degradation by DPP-IV in mammals and has a longer half-life than GLP-1 having a half-life of less than 2 minutes for DPP-IV [see Kieffer TJ et al., 1995 ]. The results of *in vivo* experiments revealed that exendin-4 shows a half-life of 2-4 hours and can reach a sufficient blood level when it is intraperitoneally administered 2-3 times a day [see Fineman MS et al., 2003]. Also, exendin-4 is known to regulate gastric motility, reduce food intake and inhibit plasma glucagon (US Patent Nos. 6858576, 6956026 and 6872700). With respect to the blood glucose-regulating action of exendin-4, it was reported that, when exendin-4 was administered alone or in combination with an antidiabetic agent (such as sulfonylurea or metformin) for 28 days, it lowered the level of glycosylated hemoglobin (HbA1C) (which means the amount of hemoglobin bound to glucose in blood) to less than 1% [see Egan JM et al., 2003]. Recently, synthetic exendin-4 (commercially available under the trade name of Byetta™) was approved for use by the US FDA.

[8] Meanwhile, vitamins, which are essential nutrients necessary for a variety of biological processes, are involved directly in human metabolism and growth, particularly the production of digestive enzymes, antibodies and fatty acids, and when they are deficient, various diseases occur. However, for humans and mammals, it is necessary to obtain such vitamins from external sources because they have no ability to synthesize vitamins. For this reason, transport systems capable of absorbing such vitamins are very well developed in the human small intestine, and such vitamins are

absorbed into various intestinal sites of the human body through a active transport system, a concentration-dependent passive transport system, an intracellular transport system, a receptor-mediated endocytotic pathway or the like depending on various environmental conditions such as concentration or pH. For example, thiamin and niacin are mostly absorbed in the duodenum, and cyanocobalamin is absorbed throughout the small intestines. They have specific transport systems, the most well-known system of which is a Na-dependent multivitamin transport system, which is a biotin transport system, a kind of active transport system. This system is known to be distributed equally in various human organs, such as liver, kidneys or heart, in addition to small intestines. The affinity constant of this system for biotin in small intestines is known to be about 2.6 nM.

[9] Accordingly, the present inventors have conducted studies to develop exendin derivatives specifically modified with biotin at a specific position of exendin, which are highly pure, increase the *in vivo* residence time of exendin, are easily absorbed through the mucosa and have the pharmacokinetic profiles and pharmacological properties similar to the therapeutic effects of native exendin by injection, as well as a preparation method thereof and a pharmaceutical composition containing the same.

## Disclosure of Invention

## Technical Problem

[10] It is an object of the present invention to provide exendin derivatives modified with biotin.

[11] Another object of the present invention is to provide exendin-3 or exendin-4 derivatives modified with biotin.

[12] Still another object of the present invention is to provide a method for preparing said exendin-3 or exendin-4 derivatives modified with biotin.

[13] Yet another object of the present invention is to provide a pharmaceutical compositi on containing said exendin-3 or exendin-4 derivatives modified with biotin.

## Technical Solution

[14] To achieve the above objects, the present invention provides: 1) exendin derivatives modified with biotin; and 2) a composition for preventing or treating diseases, which are caused by the excessive secretion of insulin, the lowering of plasma glucose, the inhibition of gastric or intestinal motility, the inhibition of gastric or intestinal emptying or the inhibition of food intake, the composition containing said biotin-modified exendin derivatives as active ingredients.

[15] Hereinafter, the present invention will be described in detail.

[16] In one aspect, the present invention provides biotin-modified exendin derivatives or pharmaceutically acceptable salts thereof.

[17] The exendin that is modified according to the present invention may be native exendin or recombinant exendin, and is preferably exendin-3 or exendin-4.

[18] Also, the exendin derivative modified with exendin according to the present invention may be a form in which exendin-4 is modified with biotin at lysine 12 (Lys$^{12}$-mono-biotin-exendin-4; hereinafter to be referred to as "MB1-exendin-4", a form in which exendin-4 is modified with biotin at lysine 27 (Lys$^{27}$-mono-biotin-exendin-4; hereinafter to be referred to as "MB2-exendin-4"), or a form in which exendin-4 are modified with biotin at lysines 12 and 27 (Lys$^{12,27}$-di-biotin-exendin-4; hereinafter to be referred to as "DB-exendin-4". The most preferred is the form in which exendin-4 is modified with biotin at lysines 12 and 27.

[19] In another aspect, the present invention provides a method for preparing said biotin-modified exendin derivatives, the method comprising the steps of: (1) adding biotin, exendin and a reducing agent to a buffer solution or an organic solvent and allowing the mixture to react; (2) storing the reaction mixture of step (1) at a given temperature for a given time in a light-shielded condition; (3) removing unreacted reactants from the reaction mixture of step (2); and (4) separating and purifying biotin-modified exendin from the product of step (3), from which the unreacted reactants have been removed.

[20] In step (1) of the method according to the present invention, the reaction molar ratio of biotin to exendin is preferably selected in the range of 1-4. The selection of the reaction molar ratio can be performed in consideration of the molecular structure and molecular weight of biotin, the pH of the reaction solution, reaction temperature, reaction time, etc.

[21] Said buffer solution or organic solvent is not specifically limited, and a buffer solution, which is conventionally used in the art, can be suitably selected depending on biotin which is used for the modification of exendin.

[22] The storage temperature and time in step (2) of the method according to the present invention are preferably suitably adjusted depending on biotin used in the modification of exendin, as described above with respect to the reaction molar ratio. For example, the reaction mixture may be stored at 4℃ for 6 hours or at room temperature for a shorter time. The storage temperature and time are connected with the reactivity of biotin used for the modification of exendin. During the storage step, the modification

reaction is carried out, and after the passage of a suitable amount of time, the modification reaction can be stopped using a glycine solution or a trifluoroacetic acid solution.

[23] The removal of unreacted reactants in step (3) of the inventive method can be performed through a method which is conventionally used in the art. For example, the unreacted reactants may be removed by dialysis using a suitable buffer solution, such as PBS (phosphate buffered saline).

[24] The separation and purification in step (4) of the inventive method can be performed, but not limited to, using size-exclusion chromatography, reverse-phase high-performance liquid chromatography, etc.

[25] In still another aspect, the present invention provides a composition for preventing or treating diseases which are caused by the excessive secretion of insulin, the lowering of plasma glucose, the inhibition of gastric or intestinal motility, the inhibition of gastric or intestinal emptying or the inhibition of food intake, the composition comprising said biotin-modified exendin derivatives as active ingredients.

[26] The inventive composition containing the biotin-modified exendin derivatives as active ingredients may be formulated into a variety of oral or parenteral dosage forms for clinical administration, but the scope of the present invention is not limited thereto.

[27] The inventive composition may be formulated using conventional diluents or excipients, such as fillers, extenders, binders, wetting agents, disintegrants, surfactants and the like. Solid preparations for oral administration include tablets, pills, powders, granules, capsules, etc. These solid preparations are formulated by mixing the inventive composition with at least one excipient, for example, starch, calcium carbonate, sucrose, lactose or gelatin.

[28] In addition to simple excipients, lubricants such as magnesium stearate or talc are also used. Liquid preparations for oral administration include suspensions, solutions, emulsions, syrups, etc., and may include commonly used, simple diluents such as water and liquid paraffin, and if desired, may further include various excipients, for example, wetting agents, sweeteners, aromatics and preservatives. Preparations for parental administration include sterile aqueous solutions, non-aqueous solutions, suspensions, emulsions, freeze-dried preparations, suppositories, etc. For non-aqueous solutions and suspensions, propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable esters such as ethyloleate may be used. In addition, calcium or vitamin $D_3$ may be added to the inventive composition to enhance the effect of treating proliferative diseases or autoimmune diseases.

[29] The dosage of the inventive composition may vary depending on the patient's weight, age, sex, general health conditions, diet, administration time, administration route, excretion rate and disease severity, but an effective dosage of the composition may be administered several times for 1-2 weeks. In addition, the composition may be administered once or several times within a daily effective dosage range.

## Advantageous Effects

[30] The exendin modified with biotin according to the present invention can have an increased *in vivo* half-life and show a biological activity similar to that of native exendin. Also, by selectively limiting the position of conjugation and the number of conjugations, side effects resulting from such factors can be minimized. In addition, the exendin-3 or exendin-4 derivatives modified with biotin according to the present invention are useful for preventing and treating diseases such as diabetes or obesity, which are caused by the excessive secretion of insulin, or diseases such as irritable bowel syndromes, which are caused by the lowering of plasma glucose, the inhibition of gastric or intestinal mobility, the inhibition of gastric or intestinal emptying or the inhibition of food intake.

## Brief Description of the Drawings

[31] FIG. 1 shows an HPLC chromatogram of a mixture of exendin-4, $Lys^{12}$-Mono-Biotin-Exendin-4 (hereinafter referred to as "MB1-Exendin-4), $Lys^{27}$-Mono-Biotin-Exendin-4 (hereinafter referred to as MB2-Exendin-4) and $Lys^{12,27}$-Di-Biotin-Exendin-4 (hereinafter referred to as DB-Exendin-4) according to the present invention.

[32] FIG. 2 shows HPLC chromatograms of exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4, separated from the mixture of FIG. 1.

[33] FIG. 3 shows the MALDI-TOF mass spectra of exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4 according to the present invention.

[34] FIG. 4 shows the MALDI-TOF mass spectra of exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4 after enzymatic digestion with lysine-C.

[35] FIG. 5 shows the results of analysis of time-residual amount of exendin-4 and biotin-modified exendin-4 in trypsin enzyme.

[36] FIG. 6 shows the results of analysis of time-residual amount of exendin-4 and biotin-modified exendin-4 in intestinal juice.

[37] FIG. 7 shows the results of insulin secretion-stimulating tests of exendin-4 and biotin-modified exendin-4 in rat pancreatic islets.

[38] FIG. 8 shows the results of analysis for the receptor binding of exendin-4 and DB-exendin-4, analyzed using an insulin-secreting cell line (INS-1).

[39] FIG. 9 is a graphic diagram showing the change in the blood glucose level of test animals, after exendin-4 and DB-exendin-4 were administered intraperitoneally to the animals.

[40] FIG. 10 shows the results of blood glucose lowering of exendin-4 and DB-exendin-4, obtained on the basis of the results of FIG. 9.

[41] FIG. 11 is a graphic diagram showing the change in the blood glucose level of test animals, exendin-4 and DB-exendin-4 were administered orally to the animals.

[42] FIG. 12 shows the results of blood glucose lowering of exendin-4 and DB-exendin-4, obtained on the basis of the results of FIG. 11.

[43] FIG. 13 is a graphic diagram showing the change in the blood glucose level of test animals after exendin-4 and DB-exendin-4 were administered orally to the animals in a concentration-dependent manner.

[44] FIG. 14 shows the results of blood glucose lowering of exendin-4 and DB-exendin-4, obtained on the basis of the results of FIG. 13.

[45] FIG. 15 shows the changes in blood concentration of exendin-4 and DB-exendin-4 after exendin-4 and DB-exendin-4 were intravenously injected and orally administered to rats.

[46] FIG. 16 is a graphic diagram showing the area under the blood concentration curve between 0 min and 180 min, obtained on the basis of the results of FIG. 15.

## Mode for the Invention

[47] Hereinafter, the present invention will be described in further detail with reference to examples. It is to be understood, however, that these examples are illustrative only, and the scope of the present invention is not limited thereto.

[48] **Examples**

[49] **Example 1: Preparation of exendin-3 or exendin-4 derivatives modified with biotin**

[50] 100 ㎕ of triethylamine (TEA) (Sigma, 9% TEA-containing DMSO solution) was added to 100 ㎕ of exendin-3 or exendin-4 (Bachem, 10 mg/ml in 0.3% TEA-containing DMSO solution), and then 100 ㎕ of biotin-NHS (Sigma, 0.8 mg/ml in 0.3% TEA-containing DMSO solution) was added thereto and well stirred. The molar ratio of exendin-3 or exendin-4 to Biotin-NHS was 1:2, and the mixture was allowed to react at room temperature for 60 minutes. The reaction was stopped with 300 ㎕ of 1% trifluoroacetic acid (TFA)-containing distilled water.

[51] **Example 2: Separation, purification and analysis of exendin-3 or exendin-4 derivatives modified with biotin**

[52] The exendin-4 derivatives, prepared in Example 1, were separated using reverse-phase high-performance liquid chromatography (hereinafter referred to as "RP-HPLC"). As columns, Jupiter RP-18 (250 x 10 mm, 5 μm, Phenomenex, USA) and Capcell-pak RP-18 (250 x 4 mm, 5 μm, Shiseido, Japan) were used, and as mobile phase solvents, 36-41% solvent B (0.1% TFA-containing acetonitrile) and 64-59% solvent A (0.1% TFA-containing distilled water) were used. The mobile phase was linearly changed while maintaining a flow rate of 1 ml/min. Each peak was quantified using a UV spectrophotometer at 215 nm. The biotin-modified exendin-3 or exendin-4 derivatives, prepared through the above-described method, were analyzed with a MALDI-TOF mass spectrometer to determine the number of biotin conjugates. Also, the derivatives were digested with the protease lysine-C, and then analyzed with a MALDI-TOF mass spectrometer. Each of the purified conjugates was dissolved in 50 μℓ of triethylamine-HCl buffer (10 mmol/L; pH 7.4) at a concentration of 1 mg/ml, and then 50 μℓ of an enzyme (1 mg/ml) was added thereto and allowed to react at 37 ℃ for 1 hour. 5 μl of 10% (w) TFA was added to the reaction mixture in order to stop the lysine-C digestion, and then the reaction mixture was analyzed with a MALDI-TOF mass spectrometer.

[53] FIGS. 1 and 2 show an HPLC chromatogram of a mixture after completion of the reaction of biotin with exendin-4 and an HPLC chromatogram of modified exendin-4 finally separated from the mixture. Four different materials shown in FIG. 2 were separated from the reaction mixture. As shown in FIG. 3, it was found through MALDI-TOF mass spectrometry that the molecular weights of the separated materials were consistent with those of unreacted exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4, respectively. Also, the separated modified exendin-4 derivatives had a purity of more than 98%.

[54] The materials separated from the reaction mixture of biotin with exendin-4 were enzymatically digested with lysine-C, and then each of the reaction solutions was analyzed by MALDI-TOF mass spectrometry. The analysis results are shown in FIG. 4. As shown in FIG. 4, it was found that the materials were unreacted exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4, respectively.

[55] **Example 3: Analysis of biological stability of exendin-4 derivatives** modified with biotin

[56] The biological stability of the modified exendin-4 derivatives, prepared and

separated in Examples 1 and 2, was performed through the analysis of time-residual amount using trypsin, which is an enzyme mainly degrading exendin-4, and an intestinal homogenate.

[57] **<3-1> Analysis of stability of biotin-modified exendin-4 derivatives in** trypsin enzyme

[58] 20 ㎕ of each of exendin-4 and biotin-modified exendin-4 derivatives was added to 20μl of 2 mM trypsin (25 mM phosphate buffer, pH 6.5) and then allowed to react in aqueous solution at 37 ℃. The reaction was stopped with 100 ㎕ of 1% TFA-containing distilled water, and each reaction solution was analyzed using HPLC at varying points of time. The HPLC analysis was performed in the same manner as described in Example 2, and was carried out using a 36 ~ 42% solvent B as a mobile phase at a flow rate of 1 ml/min for 10 minutes.

[59] FIG. 5 shows the results of analysis for the time-residual amount of exendin-4 and biotin-modified exendin-4 derivatives in trypsin enzyme. As shown in FIG. 5, DB-exendin-4 had significantly strong resistance to degradation in trypsin as compared to exendin-4. The results of analysis for the stability of biotin-modified exendin-4 derivatives in trypsin enzyme are shown in Table 1 below. As shown in Table 1, the half-lives of exendin-4, MB-exendin-4 and DB-exendin-4 were 2.3 min, 2.7 min and 28.1 min, respectively, that is, the half-lives of MB-exendin-4 and DB-exendin-4 were 1.2 times and 12.2 times as high as exendin-4, respectively.

[60] Table 1

[Table 1]

[Table ]

Stability in trypsin enzyme

| **Kind of GLP-1** | **Half-life** | **Folds increase** |
|---|---|---|
| Exendin-4 | 2.3 | - |
| MB-exendin-4 | 2.7 | 1.2 |
| DB-exendin-4 | 28.1 | 12.2 |

[61] **<3-2> Analysis of stability of biotin-modified exendin-4 derivatives in** intestinal homogenate

[62] Each of 20 ㎕ of exendin-4 and biotin-modified exendin-4 derivatives was added to 20 ㎕ of an intestinal homogenate, and then allowed in aqueous solution at 37 ℃. The reaction was stopped with 100 ㎕ of 1% TFA-containing distilled water, and each

reaction solution was analyzed using HPLC at varying points of time. The HPLC analysis was performed in the same manner as described in Example 2, and was carried out using a 36-45% solvent B as a mobile phase at a flow rate of 1 ml/min for 15 minutes.

[63] FIG. 6 shows the results of analysis for the time-residual amount of exendin-4 and biotin-modified exendin-4 derivatives in intestinal homogenate. As shown in FIG. 6, it can be seen that DB-exendin-4 had significantly strong resistance to degradation in intestinal homogenate as compared to native exendin-4. The results of analysis for the stability of biotin-modified exendin-4 derivatives in intestinal homogenate are shown in Table 2 below. As can be seen in Table 2, the half-lives of exendin-4, MB-exendin-4 and DB-exendin-4 were 2.4 min, 2.7 min and 15.9 min, respectively, suggesting that the half-lives of MB-exendin-4 and DB-exendin-4 were 1.1-fold and 6.6-fold, respectively, higher than that of exendin-4.

[64] Table 2

[Table 2]

[Table ]

Stability in intestinal homogenate

| **Kind of GLP-1** | **Half-life** | **Folds increase** |
|---|---|---|
| Exendin-4 | 2.4 | - |
| MB-exendin-4 | 2.7 | 1.1 |
| DB-exendin-4 | 15.9 | 6.6 |

[65] **Example 4: Measurement of biological activities of biotin-modified** exendin-4 derivatives

[66] The biological activities of position isomers of the biotin-modified exendin-4 derivatives, prepared and separated in Examples 1 and 2, were measured through an insulin secretion stimulating test using rat pancreatic islets and through receptor binding analysis using an insulin secreting cell line (INS-1 cell line).

[67] **<4-1> Insulin secretion stimulating test**

[68] For an insulin secretion stimulating test, pancreatic islets were separated from laboratory rats (Sprague Dawley rats) by collagenase digestion and Ficoll density gradient separation. The separated pancreatic islets were cultured in a cell incubator for 2-3 days, and then placed in a 24-well plate, containing 1 ml of KRH buffer (containing 16.7 mM glucose), at a density of 20 islets/well. Then, each of exendin-4,

MB-exendin-4 and DB-exendin-4 was added to the islets at varying concentrations of 0.1, 1, 10 and 100 nM, and the islets were cultured in a cell incubator for 2 hours. After completion of the culture, 200 $\mu\ell$ of a culture sample was collected from the culture medium, and the concentration of insulin in the sample was measured using an insulin enzyme immunoassay kit.

[69] FIG. 7 shows the results of insulin secretion stimulating tests of exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4. As shown in FIG. 7, it was observed that exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4 stimulated insulin secretion in a concentration-dependent manner. Such effects were stably increased in a concentration-dependent manner between exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4.

[70] **<4-2> Receptor binding test**

[71] The interaction of exendin-4 and biotin-modified exendin-4 derivatives with a GLP-1 receptor, the receptor of insulin-secreting cells, was analyzed through a receptor binding test. For the test, insulin-secreting INS-1 cells were placed in a 12-well plate at a concentration of 2.5 x $10^5$ cells/well, and then cultured for 2 days, such that the cells stably adhered to the culture plate. After the cell adhesion, the culture medium was replaced with binding buffer, and $^{125}$I-exendin-4 (9-39) was added thereto to a final concentration of 30 pM. Following this, each of exendin-4 and biotin-modified exendin-4 derivatives was added thereto to a final concentration of 0.001-100 nM, and then subjected to a competitive receptor binding assay at room temperature for 2 hours. After completion of the test, the cells were washed three times with cold phosphate buffer to remove unbound $^{125}$I-exendin-4. Finally, the cells were lysed with lysis buffer, the lysed cells were collected, and the amount of $^{125}$I-exendin-4 bound to the cells was measured using a gamma-ray spectrometer.

[72] FIG. 8 shows the binding behavior of the insulin secreting cell surface with the GLP-1 receptor. As shown in FIG. 8, the competitive binding of $^{125}$I-exendin-4 was decreased with the increase in the concentration of the sample. Also, the position isomers showed a difference in binding strength according to the modification position. The EC50 (concentration upon the binding of 50% $^{125}$I-exendin-4) values of exendin-4, MB1-exendin-4, MB2-exendin-4 and DB-exendin-4, obtained through the test, were 0.9, 1.2, 1.8 and 1.5 nM, respectively.

[73] Through such effects under cell culture conditions and the receptor binding tests, it can be seen that the receptor-binding abilities of the biotin-modified exendin-4 isomers were higher in the order of DB-exendin-4 < MB2-exendin-4 < MB1-exendin-4 <

exendin-4, but such difference was not attributable to the difference in the insulin secretion-stimulating ability as shown in FIG. 7. Accordingly, it could be seen that the biotin-modified exendin-4 derivatives had biological activity equal to that of native exendin-4.

[74] **Example 5: Measurement of biological activity of biotin-modified** exendin-4 derivatives in animal model

[75] **<5-1> Oral glucose tolerance test in animal model**

[76] In order to measure the oral glucose tolerance of exendin-4 and biotin-modified exendin-4 derivatives in an animal model, 6-week-old male db/db mice (C57/BLKS/J-db/db, Korea Research Institute of Bioscience and Biotechnology) were intraperitoneally injected with 100 $\mu\ell$ of each of exendin-4 and biotin-modified exendin-4 derivatives (1 nmole/kg) at -30 min. Then, 200 $\mu\ell$ of glucose (200 mg/ml) was orally administered to the mice, and at -30, 0, 15, 30, 60, 120 and 180 min, the change in the glucose level of the blood collected from the tail vein was observed.

[77] As shown in FIG. 9, it could be seen that the groups administered with the drugs, and a control group (placebo, injected with saline), showed a significant difference in oral glucose tolerance. The control group showed a rapid increase in blood glucose level according to the administration of glucose and a slow lowering in blood glucose level. On the other hand, the groups administered with the drugs showed a relatively low increase in blood glucose level and a relatively rapid lowering in blood glucose level. A graphic diagram of the area under the glucose concentration curve between 0 min and 180 min, obtained on the basis of the results of FIG. 10, is shown.

[78] As shown in FIG. 10, the test group that was administered DB-exendin-4 or exendin-4 showed a very rapid blood-glucose removal ability compared to that of the control group (injected with saline). This is believed to be attributable to the increased insulin secreting ability of DB-exendin-4 or exendin-4 in pancreatic islets. Accordingly, it could be seen that DB-exendin-4 chemically modified with biotin showed biological activity equal to that of native exendin.

[79] **<5-2> Measurement of intraperitoneal glucose tolerance test in animal** model

[80] The absorption behavior and efficacy of biotin-modified exendin-4 derivatives after oral administration in an animal model were observed by examining the change in the intraperitoneal glucose tolerance in the animals.

[81] In order to measure the intraperitoneal glucose tolerance of exendin-4 and biotin-modified exendin-4 derivatives in an animal models, 6-week-old male db/db mice

(C57/BLKS/J-db/db, Korea Research Institute of Bioscience and Biotechnology) were orally administered with 100 ㎕ of each of exendin-4 and DB-exendin-4 (1 mg/mouse, based on exendin-4) at -30. Then, the animals were intraperitoneally injected with 200 ㎕ of glucose (200 mg/ml), and -30, 0, 15, 30, 60, 120 and 180 min, the change in the glucose level of the blood collected from the tail vein was observed.

[82] As shown in FIG. 11, it could be seen that the groups administered with the drugs, and a control group (placebo, injected with saline), showed a significant difference in intraperitoneal glucose tolerance. The control group showed a rapid increase in blood glucose level according to the administration of glucose and a slow lowering in blood glucose level. However, the group administered with DB-exendin-4 showed a relatively slow increase in blood glucose level and a relatively rapid lowering in blood glucose level. Also, the group administered with native exendin-4 showed a slight increase in intraperitoneal glucose tolerance compared to the control group, but showed a decrease in intraperitoneal glucose tolerance compared to the group administered with DB-exendin-4. This is believed to be because the stabilization behavior and absorption of native exendin-4 and DB-exendin-4 in intestines are different between native exendin-4 and DB-exendin-4.

[83] A graphic diagram of the area under the glucose concentration curve between 0 min and 180 min, obtained based on the results of FIG. 11, is shown in FIG. 12. As shown i n FIG. 12, it could be seen that the intraperitoneal glucose tolerance was lower in the order of DB-exendin-4, exendin-4 and the control group. Also, this difference in efficacy is believed to be attributable to the difference in stability and absorption.

[84] Through Example <5-2>, it could be seen that DB-exendin-4 when administered orally showed a more excellent anti-diabetic effect, that is, an increase in intraperitoneal glucose tolerance, compared to that of native exendin-4. To more closely examine this fact, the change in oral glucose tolerance according to the oral dosage of DB-exendin-4 was examined. In addition, in order to examine the difference between this changed glucose tolerance and the glucose tolerance in normal animals, the glucose tolerance in normal animals as a control group was examined.

[85] **<5-3> Examination of efficacy according to oral dosage**

[86] This example was conducted in the same manner as described in Example <5-2>. Specifically, DB-exendin-4 was orally administered at dosages of 5, 1 and 0.2 mg/mouse at -30 min, 200 ㎕ of glucose (200 mg/ml) was intraperitoneally injected, and at -30, 0, 15, 30, 60, 120 and 180 min, the change in the glucose level of the blood collected from the tail vein was observed. In the case of normal animals, the same

dosage (relative to weight) of glucose as described above was administered, and then the change in blood glucose level was observed at the same points of time as described above.

[87] FIG. 13 shows the change in the intraperitoneal glucose tolerance of the animals, administered with varying dosages of DB-exendin-4, and in the intraperitoneal glucose tolerance of normal animals. It can be seen that the normal animals showed the most excellent glucose tolerance behavior and were restored to a normal blood glucose level at 120 min after the administration of glucose. However, the groups administered with DB-exendin-4 showed a slight increase in glucose tolerance with the increase in the dosage of DB-exendin-4. Also, the groups administered with DB-exendin-4 at dosages of 5 and 1 mg/mouse were restored to a normal blood glucose level at 2 hours, whereas the group administered with DB-exendin-4 at a dosage of 0.2 mg/mouse showed slightly reduced glucose tolerance and was maintained at a high blood glucose level, even at 2 hours after the administration of DB-exendin-4.

[88] A graphic diagram of the area under the glucose concentration curve between 0 min and 180 min, obtained based on the results of FIG. 13, is shown in FIG. 14. As shown in FIG. 14, it could be seen that the groups administered with DB-exendin-4 showed a decrease in intraperitoneal glucose tolerance with the decrease in the dosage of DB-exendin-4.

[89] **Example 6: Examination of pharmacokinetic behavior of exendin-4** derivatives modified with biotin

[90] From the results of Examples 3-5 above, it was found that DB-exendin-4 of the present invention was excellent in biological stability, biological activity and blood glucose lowering effects in diabetic animals, and thus shows the characteristics of sustained drugs.

[91] In order to examine the cause of such sustained characteristics, the pharmacokinetic behaviors of exendin-4 and DB-exendin-4 were observed. Laboratory rats (SD rats) weighing about 200 g were intravenously injected and orally administered with the drug at a dosage of 1 nmole/rat (10 $\mu$g/rat), and then the change in plasma blood drug concentration with time was measured using an ELISA kit. The plasma sample was collected from an inserted jugular vein catheter.

[92] The change in plasma drug concentration after the intravenous injection and oral administration of exendin-4 and DB-exendin-4 to the SD rats is shown in FIG. 15. As shown in FIG. 15, the DB-exendin-4 of the present invention reached the maximum plasma concentration at 15-30 min after oral administration, and then showed a slow

decrease in the plasma concentration. However, exendin-4 showed a rapid decrease in the plasma concentration for a short time after intravenous injection and reached the basal concentration (<2 ng/㎖) after 3 hours after intravenous injection. Also, it could be observed that there was no great change in the plasma concentration of exandin-4 after oral administration.

[93] A graphic diagram of the area under the blood concentration curve between 0 min and 180 min, obtained based on the results of FIG. 15, is shown in FIG. 16. As shown in FIG. 16, it could be seen that the plasma concentration of DB-exendin-4 after oral administration reached 3.96% of the plasma concentration after intravenous injection of exendin-4. Accordingly, it could be found that DB-exendin-4 according to the present invention was effectively absorbed through oral administration, such that it could show effects.

[94] Thus, from the results of Examples 3-6 above, it could be seen that exendin-4 chemically modified with biotin could have significantly increased stability through the bioconjugation process without reducing the activity of exendin and show improved anti-diabetic effects, resulting from excellent stability and absorption in intestines.

[95] In addition, it could be seen that DB-exendin-4 showed a change in glucose tolerance through the regulation of dosage, that is, the organic relationship between dosage and efficacy, and could realize glucose tolerance in diabetic animals, similar to that in normal animals, through oral administration.

## Industrial Applicability

[96] As can be seen from the foregoing, the present invention provides exendin derivatives modified with biotin, a method for preparing the same, and a pharmaceutical composition containing the biotin-modified exendin derivatives. The exendin-4 derivatives modified with biotin according to the present invention are useful for preventing or treating diseases such as diabetes or obesity, which are caused by the excessive secretion of insulin, or diseases such as irritable bowel syndrome, which are caused by the lowering of plasma glucose, the inhibition of gastric or intestinal mobility, the inhibition of gastric or intestinal emptying or the inhibition of food intake. Also, the exendin-4 derivatives modified with biotin can be applied as oral exendin preparations because most of exendin-related materials are currently developed as injection dosage forms, and agents for treating type II diabetes form a very large market.

# Claims

[1] Exendin derivatives containing biotin in exendin-3 of SEQ ID NO: 1.

[2] Exendin derivatives containing biotin in exendin-4 of SEQ ID NO: 2.

[3] The exendin derivatives of Claim 1 or 2, wherein the biotin is bound to at least one of lysine residue 12 and lysine residue 27 of exendin-4.

[4] The exendin derivatives of Claim 3, wherein the biotin is bound to lysine residue 12 and lysine residue 27 of exendin-4.

[5] A method for preparing biotin-conjugated exendin derivatives, the method comprising the steps of:

i) adding exendin-3 of SEQ ID NO: 1 or exendin-4 of SEQ ID NO: 2, biotin and a reducing agent to a buffer or an organic solution, and allowing the mixture to react;

ii) storing the reaction mixture of step i) at a given temperature for a given time in a light-shielded condition;

iii) removing unreacted reactants from the reaction mixture of step ii); and

iv) separating and purifying biotin-modified exendin from the product of step iii), from which the unreacted reactants have been removed.

[6] The method of Claim 5, wherein the exendin derivatives are the exendin derivatives of Claim 1 or 2.

[7] The method of Claim 5, wherein, in the step i), the reaction molar ratio of biotin to the exendin-3 of SEQ ID NO: 1 or the reaction molar ratio of biotin to the exendin-4 of SEQ ID NO: 2 is in the range of 1-4.

[8] A pharmaceutical composition for preventing or treating diabetes, which contains the exendin derivatives of Claim 1 or 2 as active ingredients.

[9] The pharmaceutical composition of Claim 8, which is used to prevent or treat diabetes caused by excessive secretion of insulin.

[10] The pharmaceutical composition of Claim 8, wherein the diabetes are type II diabetes.

[11] A pharmaceutical composition for preventing or treating obesity, which contains the exendin derivatives of Claim 1 or 2 as active ingredients.

[12] The pharmaceutical composition of Claim 11, which is used to prevent or treat obesity induced by excessive secretion of insulin.

[13] A pharmaceutical composition for preventing or treating irritable bowel syndromes, which contains the exendin derivatives of Claim 1 or 2 as active in-

gredients.

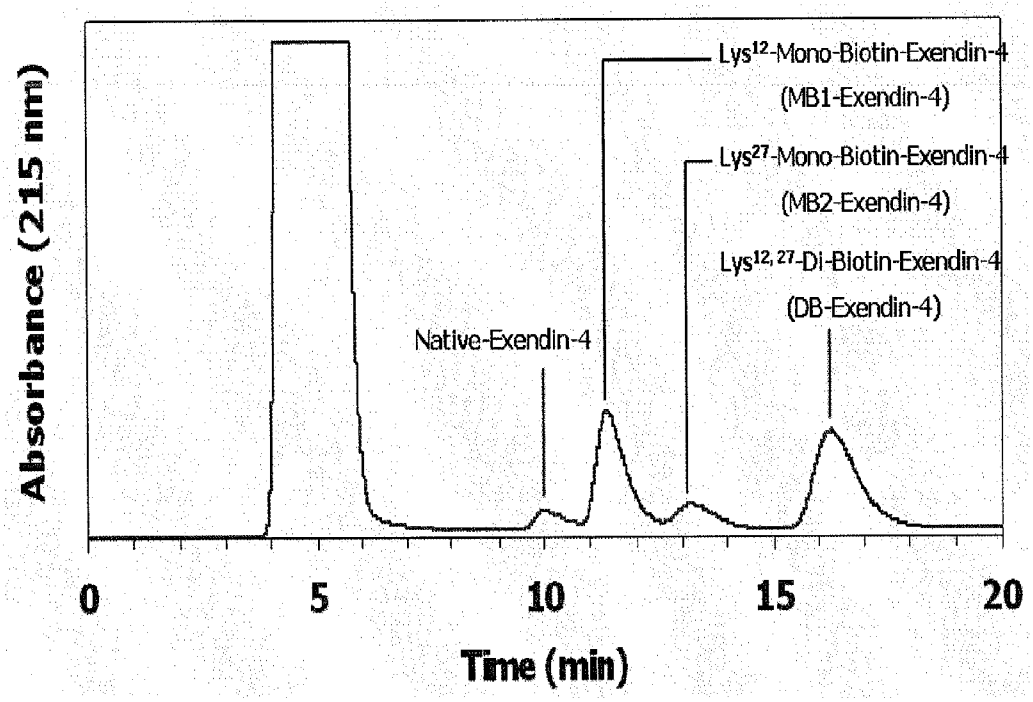
[14] The pharmaceutical composition of Claim 13, which is used to prevent or treat irritable bowel syndromes, which are caused by lowering of plasma glucose, inhibition of gastric or intestinal motility, inhibition of gastric or intestinal emptying, or inhibition of food intake.

[15] The pharmaceutical composition of Claim 8, wherein the composition is formulated in the form of oral preparations, including tablets, pills, powders, granules, capsules, suspensions, solutions, emulsions or syrups, or parenteral preparations, including external preparations, suppositories or sterile injection solutions.

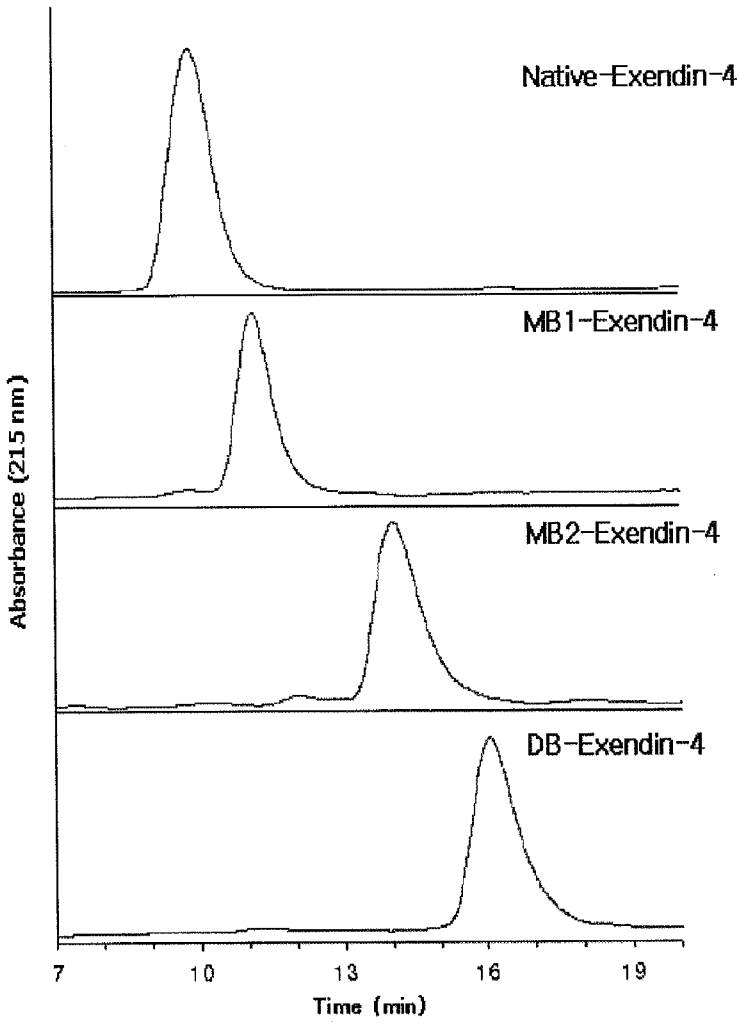
[16] The pharmaceutical composition of Claim 11, wherein the composition is formulated in the form of oral preparations, including tablets, pills, powders, granules, capsules, suspensions, solutions, emulsions or syrups, or parenteral preparations, including external preparations, suppositories or sterile injection solutions.

[17] The pharmaceutical composition of Claim 13, wherein the composition is formulated in the form of oral preparations, including tablets, pills, powders, granules, capsules, suspensions, solutions, emulsions or syrups, or parenteral preparations, including external preparations, suppositories or sterile injection solutions.

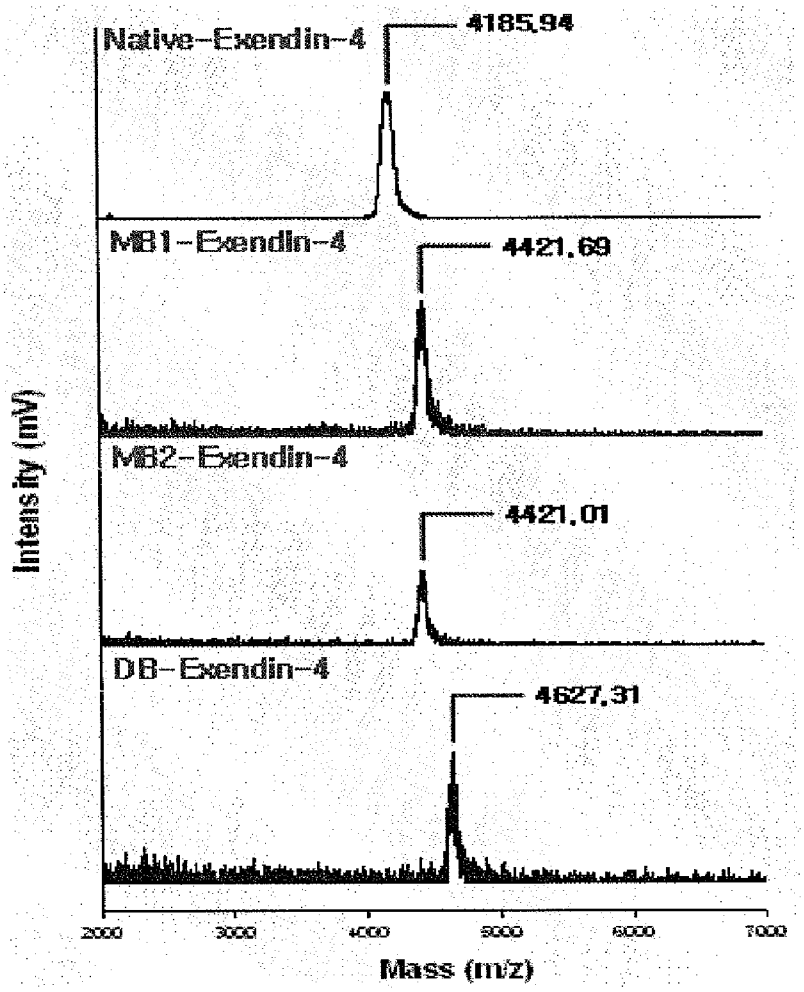
[Fig. 1]


Absorbance (215 nm)
Lys12-Mono-Biotin-Exendin-4
(MB1-Exendin-4)
Lys27-Mono-Biotin-Exendin-4
(MB2-Exendin-4)
Lys12,27-Di-Biotin-Exendin-4
(DB-Exendin-4)
Native-Exendin-4
0
5
10
15
20
Time (min)

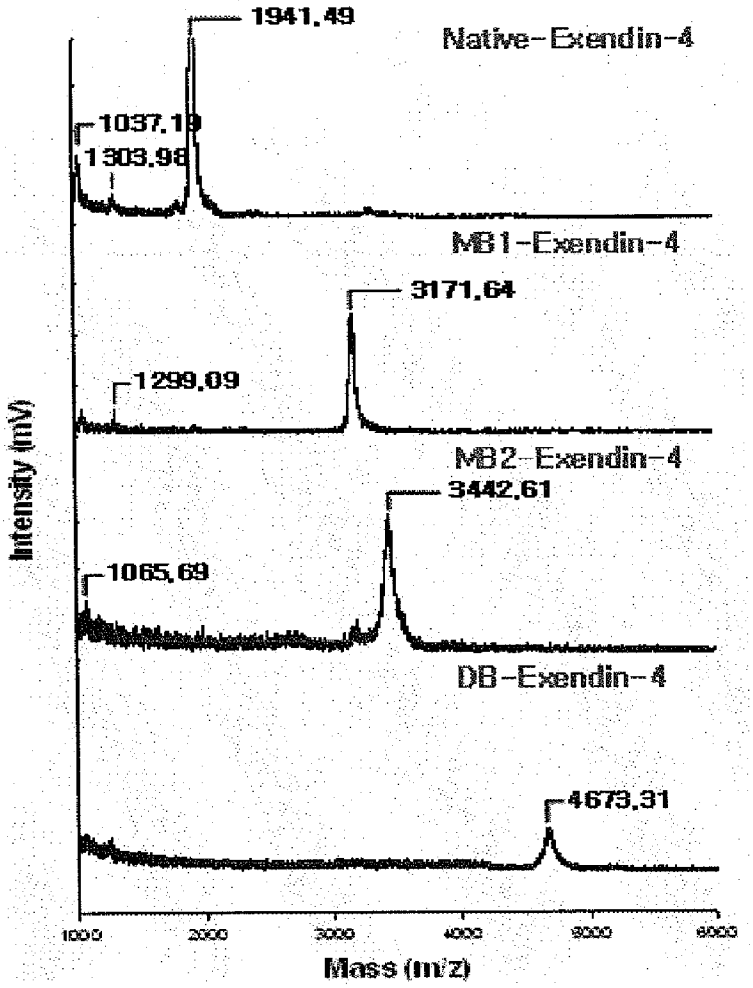
[Fig. 2]


Native-Exendin-4
MB1-Exendin-4
MB2-Exendin-4
DB-Exendin-4
Absorbance (215 nm)
7
10
13
16
19
Time (min)

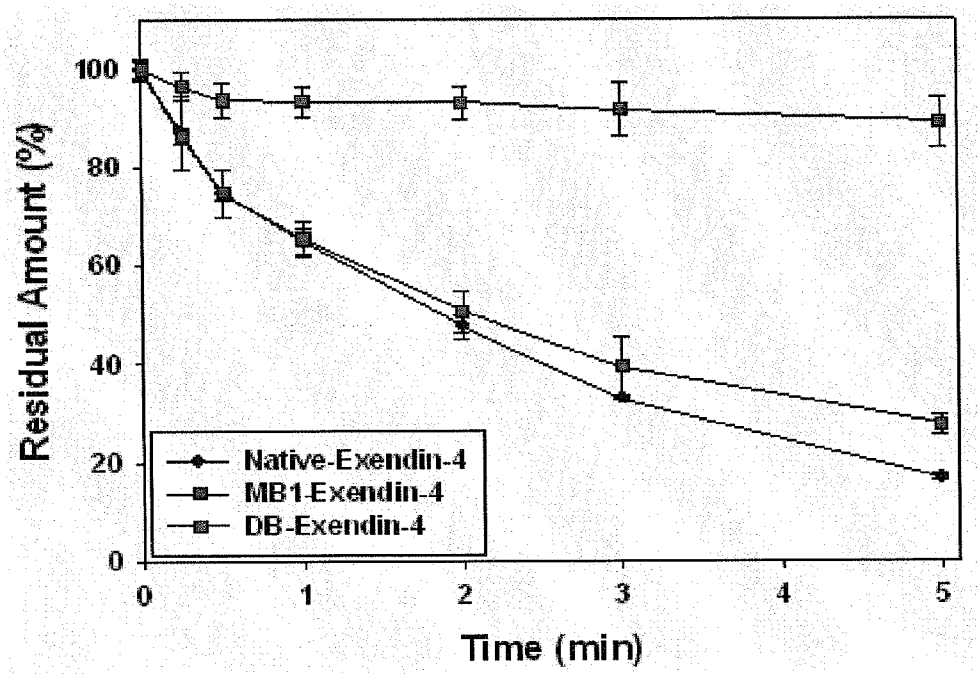
[Fig. 3]


Native-Exendin-4
4185.94
MB1-Exendin-4
4421.69
MB2-Exendin-4
4421.01
DB-Exendin-4
4627.31
Intensity (mV)
2000
3000
4000
5000
6000
7000
Mass (m/z)

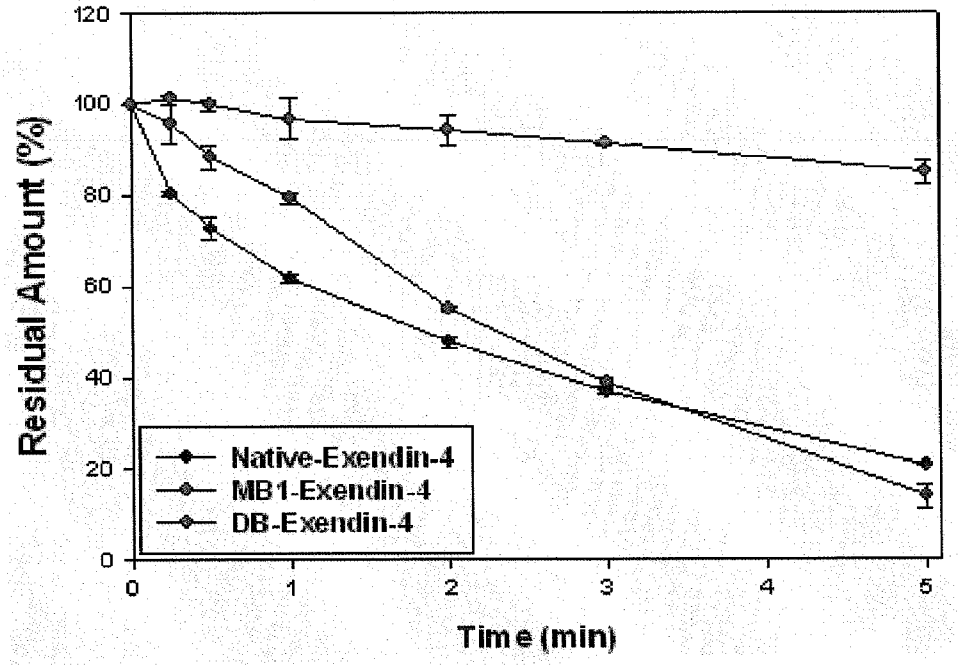
[Fig. 4]


1941.49
Native-Exendin-4
1037.19
1303.98
MB1-Exendin-4
3171.64
1299.09
MB2-Exendin-4
3442.61
1065.69
DB-Exendin-4
4673.31
Intensity (mV)
1000
2000
3000
4000
5000
6000
Mass (m/z)

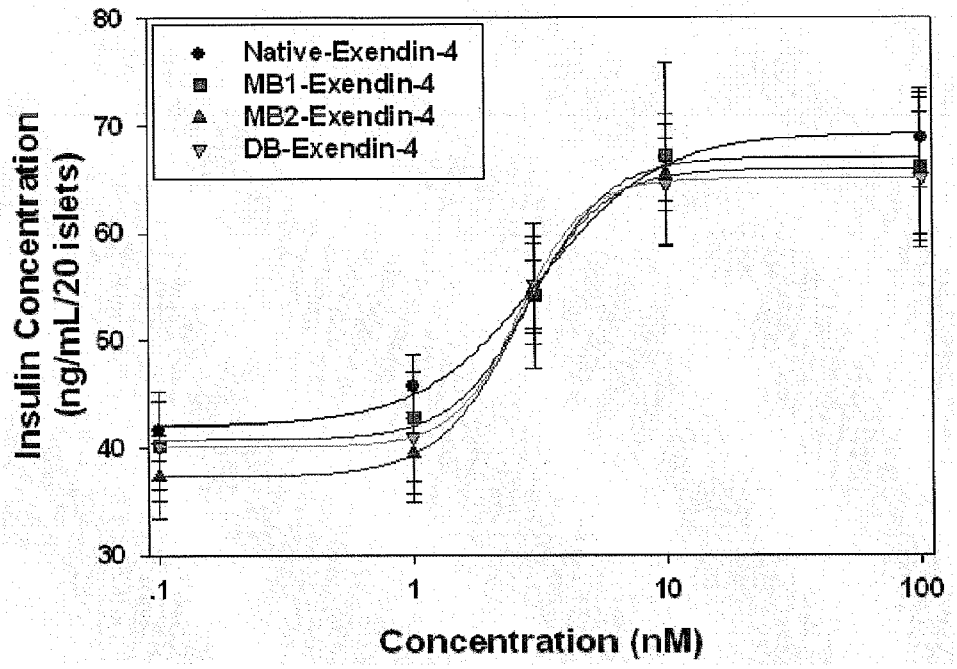
[Fig. 5]


Residual Amount (%)
100
80
60
40
20
0
0
1
2
3
4
5
Time (min)
Native-Exendin-4
MB1-Exendin-4
DB-Exendin-4

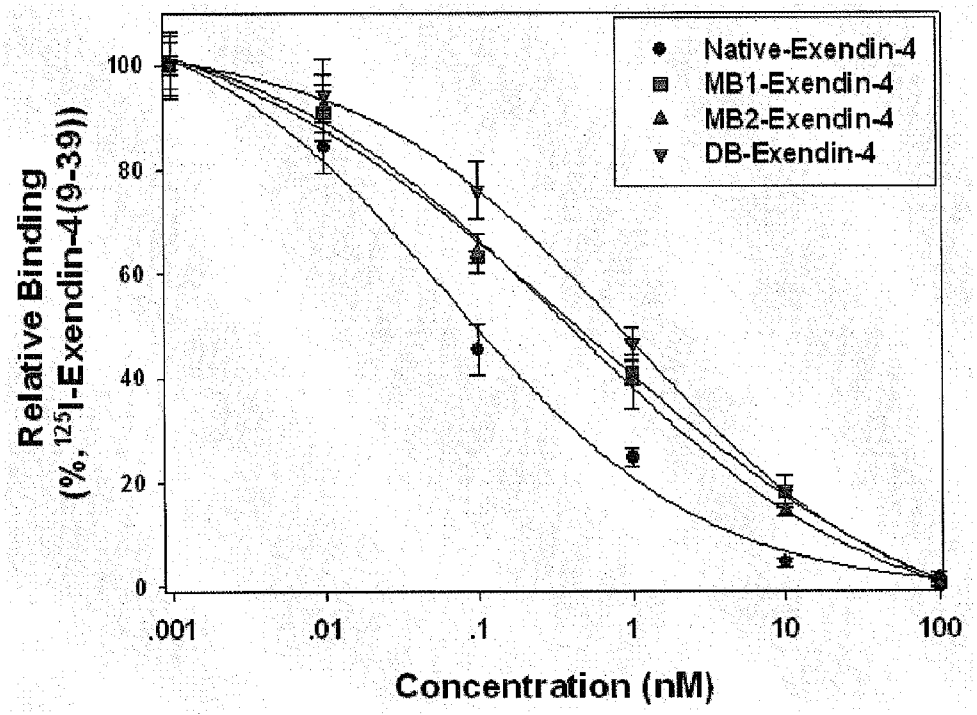
[Fig. 6]


Residual Amount (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
Time (min)
Native-Exendin-4
MB1-Exendin-4
DB-Exendin-4

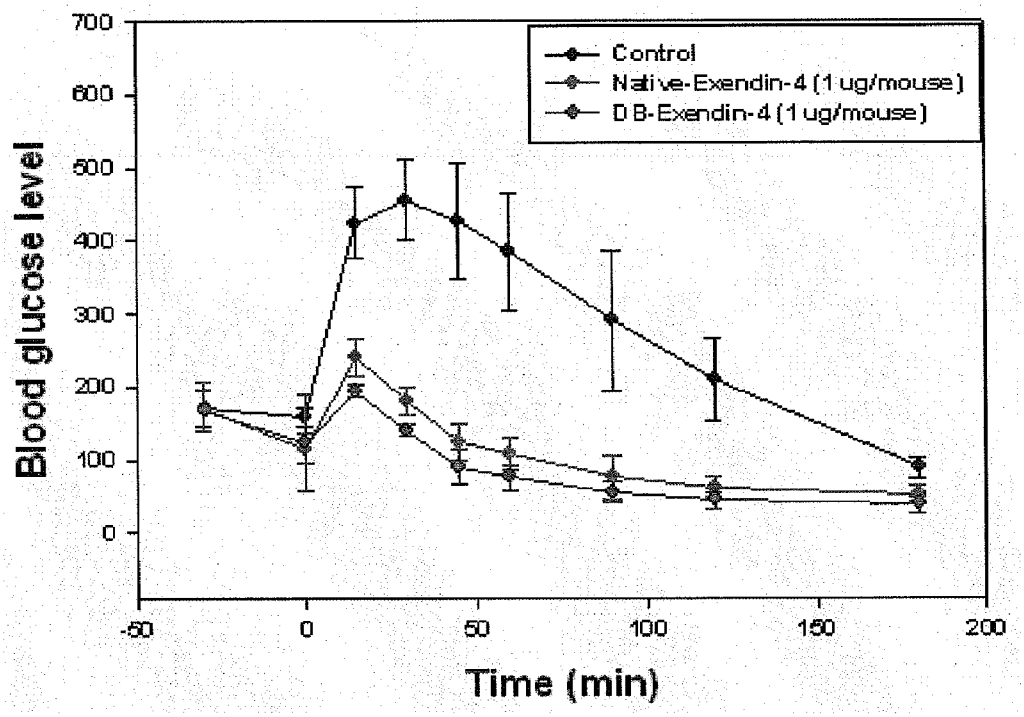
[Fig. 7]


Insulin Concentration (ng/mL/20 islets)
80
70
60
50
40
30
.1
1
10
100
Concentration (nM)
Native-Exendin-4
MB1-Exendin-4
MB2-Exendin-4
DB-Exendin-4

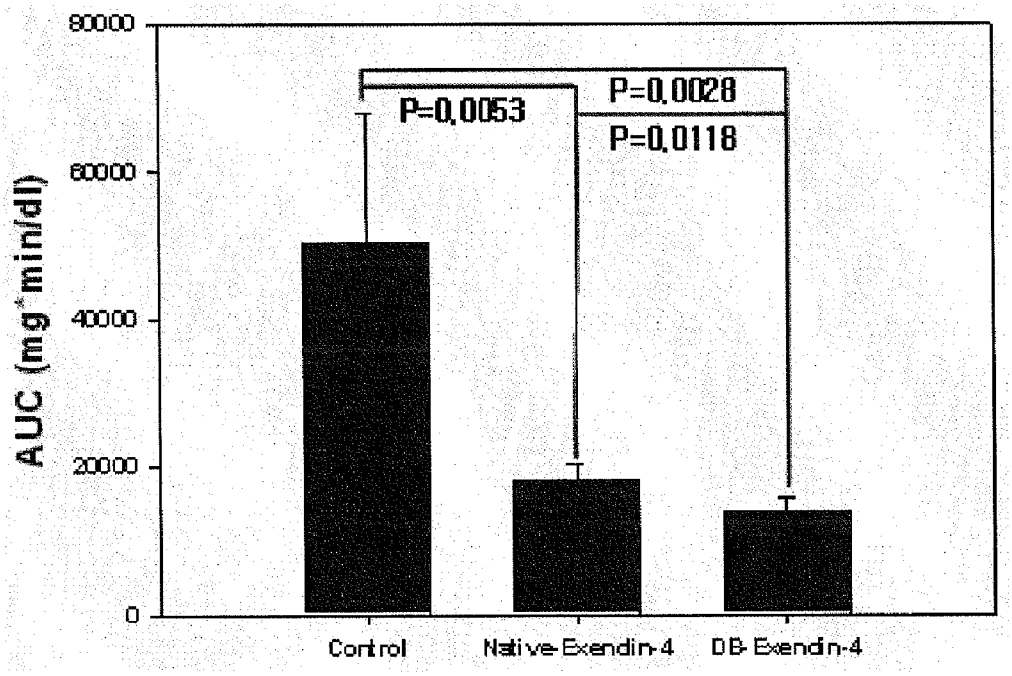
[Fig. 8]


Native-Exendin-4
MB1-Exendin-4
MB2-Exendin-4
DB-Exendin-4
Relative Binding (%, [125]I-Exendin-4(9-39))
100
80
60
40
20
0
.001
.01
.1
1
10
100
Concentration (nM)

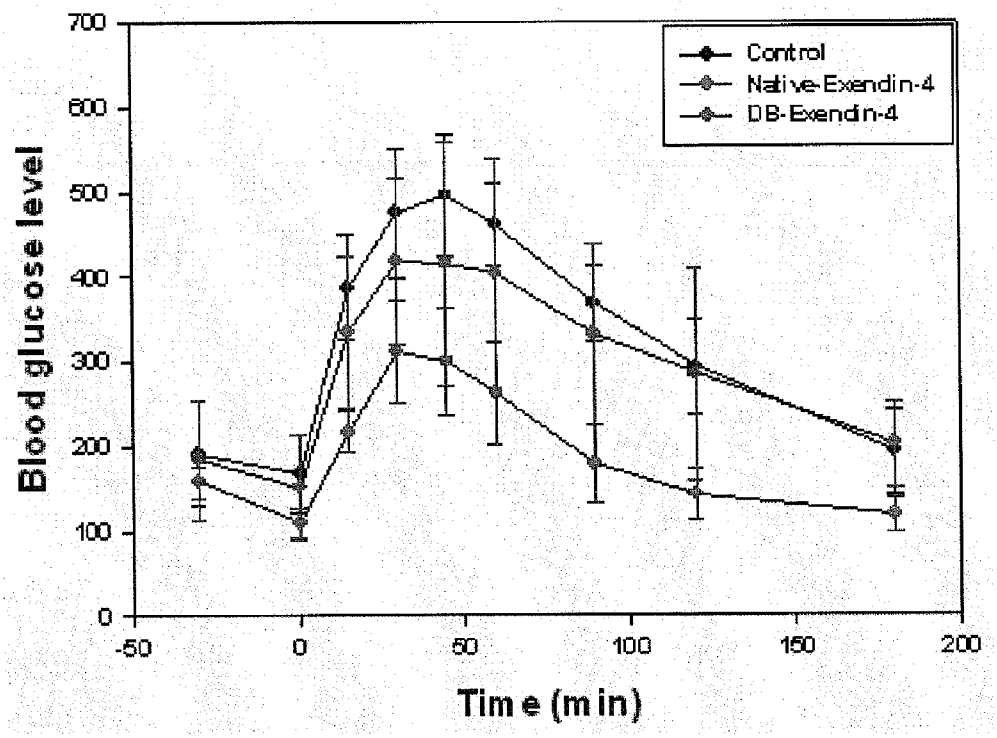
[Fig. 9]


Control
Native-Exendin-4 (1ug/mouse)
DB-Exendin-4 (1ug/mouse)
Blood glucose level
700
600
500
400
300
200
100
0
-50
0
50
100
150
200
Time (min)

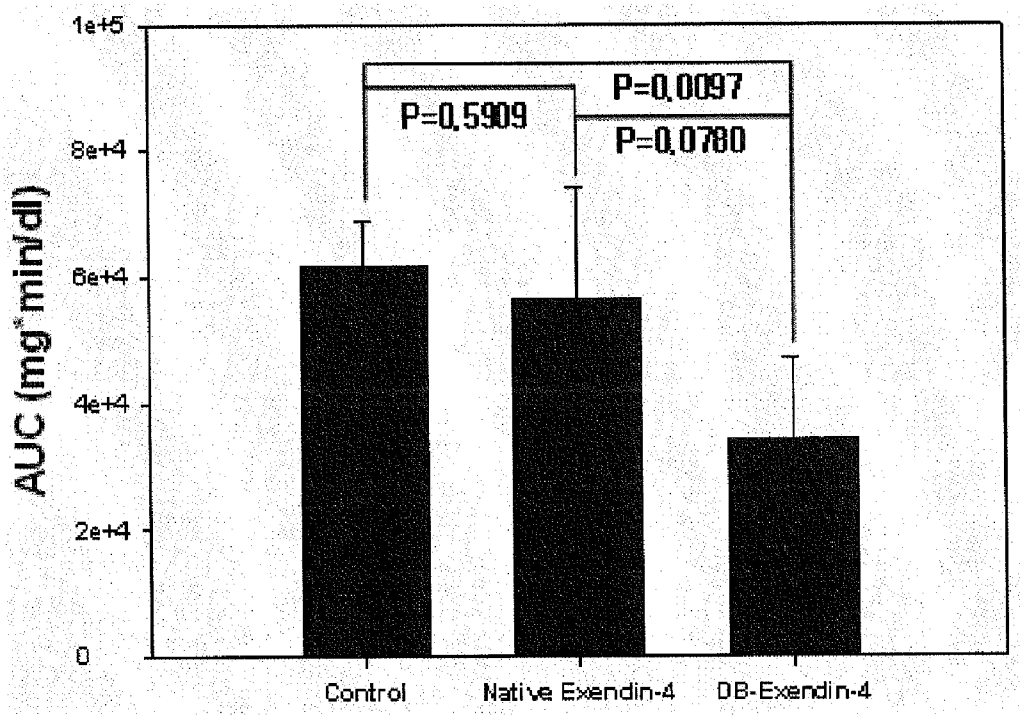
[Fig. 10]


80000
60000
40000
20000
0
AUC (mg*min/dl)
P=0.0053
P=0.0028
P=0.0118
Control
Native-Exendin-4
DB-Exendin-4

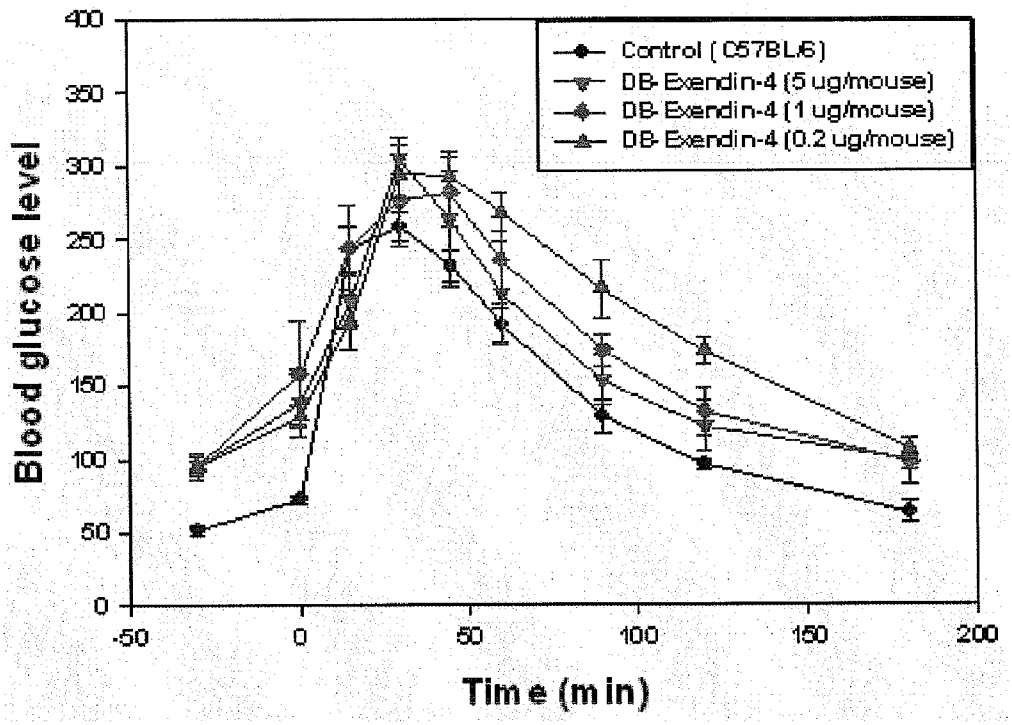
[Fig. 11]


700
600
500
400
300
200
100
0
-50
0
50
100
150
200
Blood glucose level
Time (min)
Control
Native-Exendin-4
DB-Exendin-4

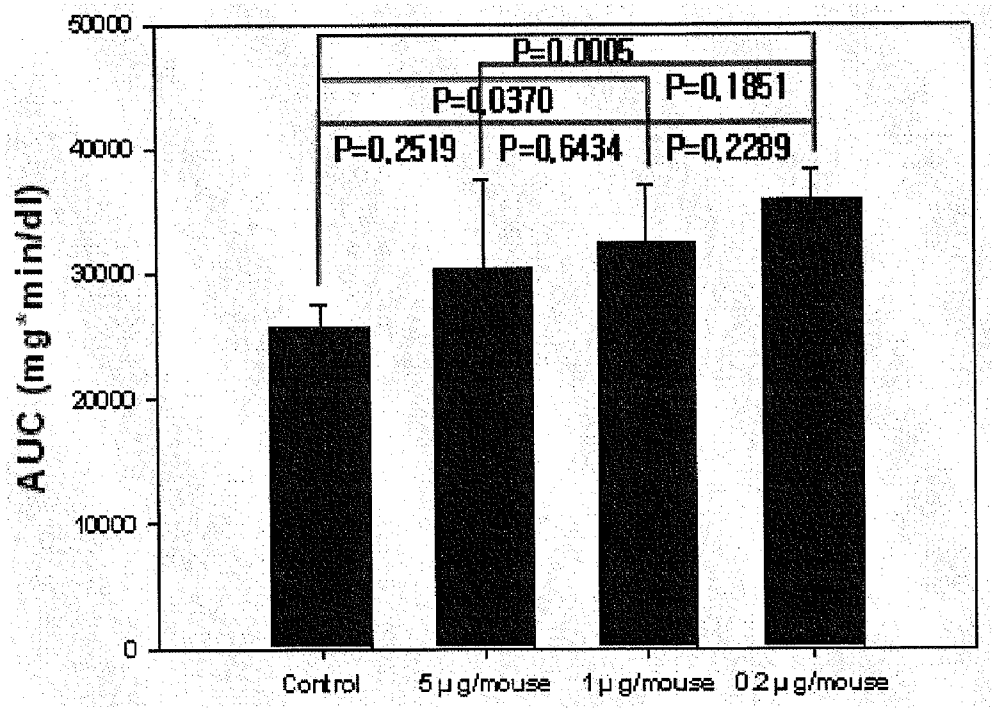
[Fig. 12]


1e+5
8e+4
6e+4
4e+4
2e+4
0
AUC (mg*min/dl)
P=0.5909
P=0.0097
P=0.0780
Control
Native Exendin-4
DB-Exendin-4

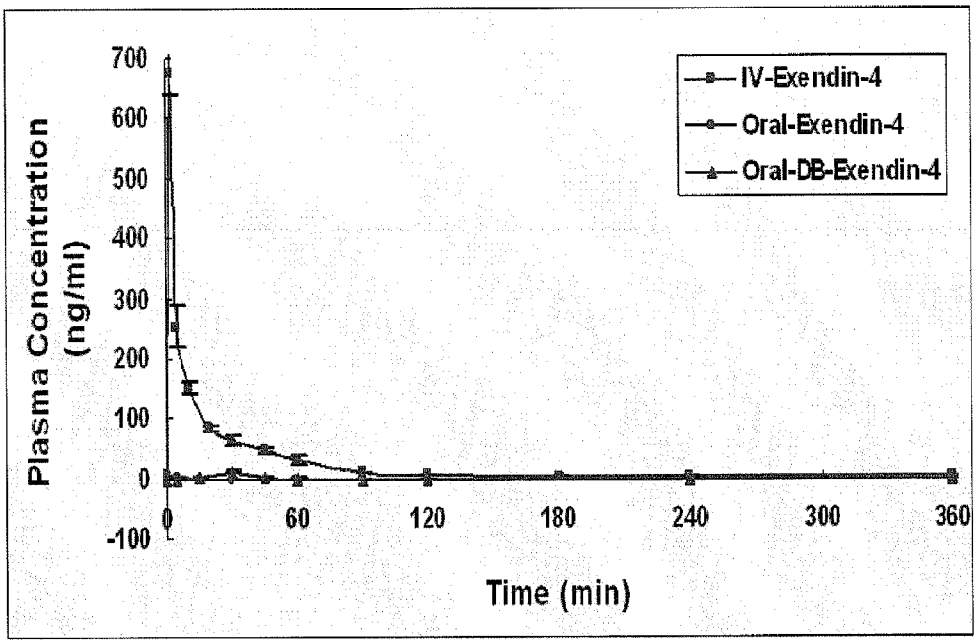
[Fig. 13]


400
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
Blood glucose level
Time (min)
Control (C57BL/6)
DB-Exendin-4 (5 ug/mouse)
DB-Exendin-4 (1 ug/mouse)
DB-Exendin-4 (0.2 ug/mouse)

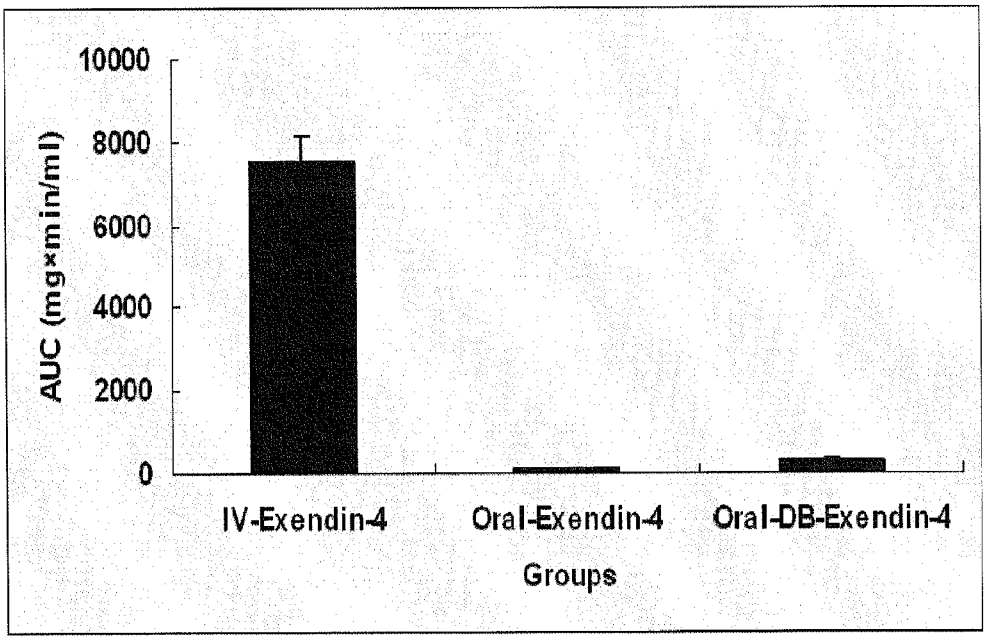
[Fig. 14]


P=0.0005
P=0.0370
P=0.1851
P=0.2519
P=0.6434
P=0.2289
AUC (mg*min/dl)
50000
40000
30000
20000
10000
0
Control
5μg/mouse
1μg/mouse
0.2μg/mouse

[Fig. 15]


IV-Exendin-4
Oral-Exendin-4
Oral-DB-Exendin-4
Plasma Concentration (ng/ml)
700
600
500
400
300
200
100
0
-100
0
60
120
180
240
300
360
Time (min)

[Fig. 16]


10000
8000
6000
4000
2000
0
AUC (mg×min/ml)
IV-Exendin-4
Oral-Exendin-4
Oral-DB-Exendin-4
Groups

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/KR2008/002694**

**A. CLASSIFICATION OF SUBJECT MATTER**

***C07K 14/575(2006.01)i***

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 C07K 14/572

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

eKIPASS, NCBI PubMed database, Delphion Research Intellectural Property Network database, google scholar, BLAST database
"exendin", "biotin", "conjugation", "lysine"

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | KR 10-0746658 (SUNGKYUNKWAN UNIVERSITY FOUNDATION FOR CORPORATE COLLABORATION) 06 August 2007<br>See the abstract and claims. | 1-17 |
| A | SHECHTER Y. et al.'[2-Sulfo-9-fluorenylmethoxycarbonyl]3-exendin-4: a long-acting glucose-lowering prodrug' Biochemical and biophysical research communications, 2003, Vol. 305, No. 2, pages 386-391.<br>See the abstract and Results. | 1-17 |
| A | PHILIP J. et al.'Systemic Administration of the Long-Acting GLP-1 Derivative NN2211 Induces Lasting and Reversible Weight Loss in Both Normal and Obese Rats 'Diabetes, 2001, Vol. 50, pages 2530-2539.<br>See the abstract and Results. | 11-12 |
| A | US 5,424,286 A(ENG; JOHN) 13 June 1995<br>See the abstract and claims. | 1-2 |

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

☒ See patent family annex.

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**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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