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Research paper

Hypoglycemic activity and oral bioavailability of insulin-loaded liposomes containing bile salts in rats: The effect of cholate type, particle size and administered dose

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ABSTRACT

Oral delivery of protein or polypeptide drugs remains a challenge due to gastric and enzymatic degradation as well as poor permeation across the intestinal epithelia. In this study, liposomes containing bile salts were developed as a new oral insulin delivery system. The primary goal was to investigate the effect of cholate type, particle size and dosage of the liposomes on the hypoglycemic activity and oral bioavailability. Liposomes containing sodium glycocholate (SGC), sodium taurocholate (STC) or sodium deoxycholate (SDC) were prepared by a reversed-phase evaporation method. After oral administration, all liposomes elicited a certain degree of hypoglycemic effect in parallel with an increase in blood insulin level. The highest oral bioavailability of approximately 8.5% and 11.0% could be observed with subcutaneous insulin as reference for SGC-liposomes in non-diabetic and diabetic rats, respectively. Insulin-loaded liposomes showed slower and sustained action over a period of over 20 h with peak time around 8–12 h. SGC-liposomes showed higher oral bioavailability than liposomes containing STC or SDC and conventional liposomes. The hypoglycemic effect was size-dependent with the highest at 150 nm or 400 nm and was proportionally correlated to the administered dose. The results supported the hypothesis of insulin absorption as intact liposomes.

1. Introduction

Recently, more and more bioactive peptides and proteins have been discovered and utilized as medicines for the treatment for diseases. However, the administration of these peptide and protein drugs so far has been almost exclusively limited to the parenteral route, which has various disadvantages, such as low patient compliance because of pain and injection inconvenience, allergic reactions, and sometimes in the case of insulin, hypoglycemia induced by frequent subcutaneous injections [1,2]. Non-parenteral administration of these drugs, especially via the oral route, represents one of the biggest challenges in modern pharmaceutical technology [3]. The oral bioavailability of naked protein and peptide drugs is negligible due to severe gastric and proteolytic inactivation in the gastrointestinal tract [4,5] as well as low permeability across the intestinal epithelia [6–8].

In order to improve their oral bioavailability, various approaches have been developed, including co-administration with absorption enhancers [9,10] and/or enzyme inhibitors [10,11], increasing lipophilicity and permeability by chemical modification [12] and incorporation into particulate nanocarriers [13–17]. Recently, a novel colloidal vehicle-liposomes containing bile salts, which is also called 'bilosomes', have drawn attention in the field of oral immunization. Oral administration of antigens, such as A/Panama [18], tetanus toxoid [19] and hepatitis B [20], by means of bilosomes has shown promising potential in stimulating both systemic and mucosal immune responses without obvious adverse effects. The addition of bile salts to the lipid bilayers of the liposomes was believed to make the vehicles resistant to the detrimental effects of physiological bile salts in the GI tract [21], and thus protect antigens from enzymatic degradation. Furthermore, bile salts embedded in the lipid bilayers of liposomes may perform membrane-destabilizing effect upon contact with the intestinal epithelia and facilitate internalization of the particles. Although the mechanisms of oral immunization, which commonly happens in the intestinal mucosa, differ significantly from the mechanisms of oral absorption, bilosomes seem to have potential to be used to enhance the oral bioavailability of protein drugs. A pro-liposome

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formulation of bilosomes has shown preliminary enhancing effect on oral bioavailability of a model peptide drug salmon calcitonin [22,23]. Until now, little is known about their potential to enhance the oral delivery of proteins.

Herein, we set forth to explore the potential and mechanisms of liposomes containing bile salts as novel oral delivery systems for proteins with recombinant human insulin (rhINS) as the model drug. In order to optimize the performance of this carrier, we used a special bile salt, sodium glycocholate (SGC), which possessed both permeation-enhancing and enzyme-inhibiting effect. Previous in vitro results on the protective effect of this carrier on encapsulated rhINS showed superiority of this carrier compared to conventional liposomes [24]. In this study, the hypoglycemic activities and oral bioavailability after oral administration of this vehicle were studied in both diabetic and non-diabetic rats. The primary goal was to confirm the enhancing effect on oral delivery of insulin and to investigate the underlying mechanisms by studying the effect of different kinds of cholates, particle size and administered dose.

2. Materials and methods

2.1. Materials

rhINS (27 IU/mg) was kindly gifted by Novo Nordisk (Copenhagen, Denmark). Sodium glycocholate (SGC) was purchased from Amresco (USA). Soybean phosphatidylcholine (SPC) and cholesterol (CH) were supplied by Lipoid (Germany). Sodium taurocholate (STC) and Sodium deoxycholate (SDC) were obtained from Sigma (Shanghai, China). Sephadex G-50 was purchased from Pharmacia (USA). Alloxan was obtained from Aladdin Chemistry Co., Ltd. (Shanghai, China). ELISA kit for human insulin was purchased from Westang Bio-Technology Co., Ltd. (Shanghai, China). Glucose GOD-PAD kit was obtained from Shanghai Rongsheng Biotech Co., Ltd. (Shanghai, China).

2.2. Animals

Male ICR mice (18–20 g) and male Wistar rats (200–250 g) were used throughout the study. The animals were raised in rooms controlled at 23 ± 1 °C and 55 ± 5% relative humidity with 12 h light/12 h dark time cycles. They received standard laboratory chow diet and tap water during acclimatization. We have obtained approval from the ethics review board of Novo Nordisk and have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations.

2.3. Preparation of liposomes containing insulin and bile salts

Liposomes containing bile salts were prepared by a reversed-phase evaporation method according to our previous report [24]. Briefly, soybean phosphatidyl choline (SPC) and bile salts (SGC/STC/SDC) were dissolved in absolute ether with a ratio of 4:1 (mol/mol), into which rhINS solution in Na_2HPO_4 buffer (4 mg/mL) was added. The mixture was ultrasonicated to form a w/o emulsion, which was subsequently evaporated under reduced pressure with a rotating speed of 50 rpm at 30 °C for 20 min to remove the organic solvent. After that, a citric acid-Na_2HPO_4 buffer (pH 5.6) was added and hydrated until a homogeneous dispersion was formed. Finally, this dispersion was extruded though a high-pressure homogenizer (AH 100 D, ATS Engineering Inc.) to obtain liposomes containing different kinds of bile slats with different sizes. Conventional liposomes (CH-liposomes) were prepared similarly as a control.

The particle size was evaluated by photon correlation spectroscopy using Malvern Zetasizer Nano instrument (Malvern, UK). rhINS entrapment efficiency was measured by using molecular exclusion chromatography with a 30 cm-long Sephadex G50 column as described in the previous study [24].

2.4. Conformational stability

The conformational stability of rhINS in the preparation was detected by circular dichroism spectroscopy (CD) [25]. Briefly, rhINS was first released by destroying the liposomal vesicles using 1% Triton X-100, and then separated from SPC and SGC/STC/SDC/CH by passing through an ENVI-18 solid extraction column (Supelco, USA). The column was first activated by methanol. After 1 mL released rhINS was mounted, 1 mL aqueous methanol (40%) was used to wash out SPC and the bile salts or CH, followed by another 1 mL aqueous methanol (60%) to elute rhINS. The collected rhINS was adjusted to a concentration of approximately 20 μg/mL for CD test. A rhINS solution with similar concentration, dissolved in 60% aqueous methanol, was used as a positive control. CD spectra were recorded at 25 °C on a Jasco-715 CD spectrophotometer (Tokyo, Japan), using a 0.1 cm path length cell at 200–250 nm with a step size of 1.0 nm and also a bandwidth of 1.0 nm. Five scans were performed and averaged for each sample.

2.5. Bioactivity study

The bioactivity of rhINS in various formulations was evaluated by measuring the hypoglycemic activity after subcutaneous injection of rhINS released from the liposome formulations. rhINS solution in 0.9% NaCl served as a positive control and destroyed blank SGC-liposomes as a negative control. The controls and released rhINS were subcutaneously injected to normal mice at a dose of 0.5 IU/kg. Blood samples were collected from the retro-orbital plexus before dosing and 1 h after dosing, and then centrifuged at 4000*g* for 5 min. Serum was collected, and blood glucose levels were determined using a Glucose GOD-PAD kit. Hypoglycemic percentage was expressed as the ratio of post-dosing to pre-dosing blood glucose level.

2.6. Induction of diabetes

Diabetes was induced in male Wistar rats by a single intra-peritoneal injection of alloxan at a dose of 120 mg/kg [26]. One week after the injection of alloxan, diabetes was confirmed by measuring glucose concentration in the blood samples from the tail vein. Only rats with fasting blood glucose levels more than 16 mmol/L were considered as diabetic and used in the present study.

2.7. The hypoglycemic activity and relative bioavailability studies

The rats were fasted overnight, but allowed free access to water before the experiment. The rats were randomly divided into several groups (six rats in each group) and given the various liposome formulations by oral gavage with 2 IU/kg subcutaneous rhINS as a reference, and one group was given physiological saline as base control [27]. If not specified otherwise, the oral dose of rhINS in all formulations was 20 IU/kg. Oral doses ranging from 2 to 20 IU/kg were also investigated to study the dose effect. Blood samples were collected from the tail vein prior to oral administration to establish baseline glucose levels and, at different times after dosing, blood samples were collected similarly. Plasma was separated by centrifugation to determine the glucose level using the glucose oxidase method (GOD kit, Shanghai Rongsheng Biotech Co., Ltd., Shanghai, China) and the insulin concentration using ELISA (ELISA kit, Shanghai Xitang Biotech Co., Ltd., China), respectively. Twelve hours after administration, the rats were allowed free access to food. The relative bioavailability based on either the hypoglycemic