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Preparation and characterization of salmon calcitonin–sodium triphosphate ionic complex for oral delivery

Hea Eun Lee [a], Min Jung Lee [a], Cho Rong Park [a], A. Young Kim [a], Kyung Hwa Chun [a], Hee Jin Hwang [a], Dong Ho Oh [a], Sang Ok Jeon [a], Jae Seon Kang [b], Tae Sung Jung [c], Guang Jin Choi [a], Sangkil Lee [a,*]

[a] Department of Smart Foods and Drugs, Inje University, Gimhae, Gyeongnam 621-749, Republic of Korea
[b] College of Pharmacy, Kyung Sung University, Daeyun 3-dong, Nam-gu, Pusan 314-79, Republic of Korea
[c] Cell Medicine Co., Ltd 607 Obang-dong Gimhae, Gyeungnam 621-749, Republic of Korea

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ABSTRACT

Even though salmon calcitonin (sCT) has been known as a potent hypocalcemic agent, only injection or nasal spray products are available on the market. In order to develop oral delivery system of the agent, a novel sCT–sodium tripolyphosphate (STPP) ionic complex was fabricated and also characterized. For the optimization of the ionic complexation, the effect of incubation time and molar ratio between sCT and STPP was evaluated. Particle size of the ionic complex in aqueous media, SEM images, DSC, FT-IR, *in vitro* release test, stability within the simulated intestinal fluid, and hypocalcemic effect were evaluated. The optimal molar complexation ratio of sCT to STPP was ranged from 1:5 to 1:10 and the complexation efficiency was about 95%. The SEM image has shown that the freeze dried ionic complex has rough morphology in their surface and the particle size in PBS (pH 7.4) was about 220 nm. The DSC and FT-IR results provided evidences for ionic interaction between $-NH_2$ groups and –P O groups of sCT and STPP, respectively. The sCT ionic complex has shown sustained sCT releasing characteristics for 3 weeks. The sCT–STPP ionic complex was protective to enzymatic attack and *in vivo* animal data revealed that the present ionic complex would show continuous hypocalcemic effect. Conclusively, the present sCT–STPP ionic complex formulation thought to be a novel oral delivery candidate for the treatment of osteoporosis.

1. Introduction

Calcitonin is a cyclic polypeptide hormone comprised of 32 amino acids. This peptide has amino acid sequences of Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH_2, which is secreted from the parafollicular cells of the thyroid gland in mammals. Calcitonin regulates calcium homeostasis of body and prevents osteoclastic bone resorption [1–3]. Calcitonin is found in pig and human and even in the ultimobranchial gland of birds and fish. However, salmon calcitonin has been used preferentially as a therapeutic agent because of its higher potency than any other sources [1,4,5].

Salmon calcitonin (sCT) has been commercialized as injection and nasal spray formulations so far. The main limitations of injection formulation are nausea and especially low patient compliance such as needle phobia and pain [6]. The nasal formulations are thought to be useful for its simple usage. However, this formulation is inconvenient because it induces irritations to nasal mucosa and causes side effects such as rhinitis, rhinorrhea, and allergic rhinitis. These side effects are unavoidable since the nasal formulations inevitably contain absorption enhancers to increase trans-mucosal calcitonin delivery [7].

Oral drug delivery system can be considered as an alternative formulation strategy, which is expected to improve the patient compliances [6,7]. Liposomes or polymer nanoparticulate drug delivery systems have been studied widely for the purpose [8–10]. However, these methods have some issues during formulation process. Firstly, the polymeric encapsulation process utilizes harsh conditions such as high speed mixing procedure, hot air, and organic solvents for the solubilization of encapsulating materials including polymers and lipids. These severe conditions eventually give rise to denaturation or aggregation of macromolecular drugs such as proteins and peptides. Secondly, the traditional encapsulation methods were unsatisfactory to achieve high encapsulation efficiency of drugs. Finally, the formulation methods are sometimes complicated and require expensive equipments to fabricate the delivery systems [11–13].

Oral bioavailability of sCT has been known as less than 0.1% due to extensive proteolytic degradation in gastrointestinal (GI) tract and poor permeation across the intestinal epithelial cells [3,7,8,11,14]. In order to develop oral peptide drug delivery systems, particle size, stability within the GI tracts, and appropriate partition coefficient enough to be solubilized or permeate to the GI membranes should be considered [15].

* Corresponding author. Tel.: +82 55 320 3783; fax: +82 55 327 4955.
E-mail address: skdavid@inje.ac.kr (S. Lee).

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Employing ionic complexation reaction, designed to occur between sCT and sodium triphosphate (STPP), a novel and simple sCT oral formulation was developed in the present study. STPP is a polyanion, which has been regarded as GRAS (generally recognized as safe). It is used as an excipient for a food additive so the final products can be utilized for oral drug delivery formulation without significant issues [16]. In this study, it was hypothesized that the ionic complexation reaction between sCT and STPP would not only be simple and fast to fabricate but also enable the sCT stable within GI tract. Moreover, this method would achieve high complexation efficiency, control release profile of sCT, and show hypocalcemic effect. To characterize sCT–STPP ionic complex, particle size using particle size analyzer and SEM image analysis were performed. Additionally, physico-chemical interaction was evaluated using DSC (Differential scanning calorimetry) and FT-IR (Fourier transform infra red) spectroscopy. Stability within the GI tract was also investigated. Finally, *in vitro* release study and *in vivo* animal study using S.D. rats were carried out to confirm the feasibility of the developed delivery systems together with significant efficacy.

2. Materials and methods

2.1. Materials

Salmon calcitonin (3431.9 Da) was obtained from Bachem AG (Bubendorf, Switzerland). Sodium tripolyphosphate (STPP) and pancreatin were purchased from Sigma Aldrich (St. Louis, MO, USA). Salmon calcitonin ELISA kit was purchased from Phoenix Pharmaceuticals, Inc. (CA, USA). HPMC capsules (2.40×7.30 mm) and oral administration device (Model DJE-22) was purchased from Natsume Co., Ltd (Japan). All other reagents were of reagent grade or higher.

2.2. Preparation of sCT–STPP ionic complex

Aqueous sCT solution (1.0 w/v%) and various concentrations of STPP in water were prepared at 4 °C condition. Various concentrations of STPP and the sCT solutions were mixed together and the molar ratios between sCT and STPP were 1:0.1, 1:0.5, 1:1, 1:5, 1:10 and 1:15, respectively. sCT solution was injected slowly into 10 ml of the STPP solution using a peristaltic syringe pump (KDS 100, Kd Scientific, USA) with a 23 gauge needle at the rate of 1.0 ml/min and stirred under mild condition for 1 h. After stirring, the mixture was incubated more for 1 h at 4 °C with mild stirring. The final solutions were centrifuged at 3000 rpm for 30 min and freeze dried at the condition of −73 °C and 0.4 mbar for 24 h.

2.3. Determination of the ionic complexation efficiency

The ionic complexation efficiency was calculated using the following equation. For the determination of complexation efficiency, the reaction solution was centrifuged and the concentration of sCT from the supernatant was measured with ELISA kit.

$$\textit{Complexation efficiency} = \left[1-\left(\frac{\textit{sCT within supernatant}}{\textit{Total amount of sCT}}\right)\right]\times 100$$

2.4. Characterization of the sCT–STPP ionic complex

2.4.1. Particle size analysis

The mean particle size and polydispersity index (PI) of the sCT–STPP ionic complex were measured using particle size analyzer (model 90 PLUS, BIC Co., Ltd, USA). To measure the particle size and PI, the sCT–STPP ionic complex was carefully suspended into pH 7.4 PBS and measured promptly before its sedimentation. All measurements were performed in triplicate.

2.4.2. Scanning electron microscopy (SEM)

The morphology of sCT–STPP ionic complex was evaluated using scanning electron microscopy (SEM). Freeze dried sCT–STPP ionic complex was coated with gold ions by gold sputter module in a high-vacuum evaporator. The sample was imaged with a SEM (S4300-SE, Hitachi, Japan) using a 5 kV accelerating voltage, a 18 mm working distance, a 70 μm objective aperture, and a probe current of 6×10^{-11} A.

2.4.3. Differential scanning calorimetry (DSC)

Thermograms of sCT, STPP, and sCT–STPP ionic complex were obtained with a DSC (Model 2920, TA Instruments, USA). Briefly, accurately weighed 1 mg of each sample was placed onto standard aluminum pans and sealed. An empty pan was used as a reference. DSC scans were performed at a heating rate of 10 °C/min in a nitrogen atmosphere. To calibrate temperature and energy scale of the DSC instrument, aluminum oxide was used as a standard reference material.

2.4.4. FT-IR (Fourier transform infrared) spectroscopy study

The FT-IR spectrum of sCT, STPP and sCT–STPP ionic complex were obtained to observe the interaction between sCT and STPP. All samples were mixed with KBr powder and the KBr disks were prepared by pressing force of 7.54 kgf/cm^2. The IR absorbency scans were analyzed at the range from 400 to 4000 cm^{-1} and the changes in the intensity of the sample peaks were measured with FT-IR spectrophotometer (FT-IR-8201 PC, Shimadzu, Japan).

2.5. In vitro release study

In vitro sCT release from sCT–STPP ionic complex was studied in simulated intestinal fluid (PBS, pH 7.4). Microtubes containing 1.5 mg of sCT–STPP ionic complex and 700 μl of PBS were incubated at 37 °C. At predetermined time intervals, the samples were centrifuged at 10,000 rpm for 5 min and an aliquot of 400 μl was taken from the supernatant. After removal of the supernatant, same volume of fresh buffer solution was replaced. The amount of sCT within the collected supernatant was measured by ELISA ($n=3$).

2.6. Stability of the sCT–STPP ionic complex within simulated gastrointestinal enzyme solution

The stability of sCT–STPP ionic complex was examined using modified intestinal solution [17,18]. Briefly, pH 6.8, 1% w/w pancreatin solution was prepared following USP XXIV. To which sCT and the sCT–STPP ionic complex were added and incubated at 37 °C. Samples were collected predetermined time intervals (1 h, 2 h, 3 h, and 4 h) and the amount of free sCT and sCT within sCT–STPP ionic complex was analyzed using HPLC. The mobile phase was acetonitrile: de-ionized water containing 0.1% trifluoroacetic acid = 35: 65 (v/v), flow rate was 0.8 ml/min, injection volume was 20 μl, and detection wavelength was 210 nm.

For the analysis of sCT from sCT–STPP ionic complex, the simulated intestinal solution was centrifuged for 3 min at 7500 rpm. The supernatant was removed and the sediment was collected carefully. The sediment was then washed with pH 7.4 PBS three times and pH 3.0 PBS was added to the sCT–STPP ionic complex to dissociate sCT from sCT–STPP ionic complex. Because the present drug delivery system was based on the ionic complexation between sCT and STPP, it was assumed that the ionic complex could be dissociated by controlling environmental pH.

2.7. In vivo hypocalcemic effect of sCT–STPP ionic complex

To evaluate the hypocalcemic effect of sCT–STPP ionic complex, animal experiments were performed using male Sprague–Dawley rats weighing 200–240 g (7–8 weeks bred). The SD rats were divided into three groups and 500 IU/kg of sCT was administered using the method