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Salmon calcitonin-loaded Eudragit® and Eudragit®-PLGA nanoparticles: *in vitro* and *in vivo* evaluation

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Abstract

The main objective of this study was to prepare salmon calcitonin (sCT)-loaded Eudragit®RSPO, Eudragit®L100 and Eudragit®-poly(lactic-co-glycolic acid) blend nanoparticles for *in vitro* and *in vivo* evaluation as an oral drug delivery system. The prepared nanoparticles ranged in size from 179.7 to 308.9 nm with a polydispersity index between 0.051 and 2.75, and had surface charges ~ -11 to $+6$ mV. Efficient sCT encapsulation and release was observed with all the nanoparticle formulations. The polymer type was an important factor that influenced the release characteristics and the *in vivo* hypocalcemic effect. Nanoparticle formulations were also prepared with sodium taurodeoxycholate (NaTDC) and characterized. No statistically significant difference was noted between the hypocalcemic effect of any of the nanoparticle formulations with and without NaTDC ($p > 0.05$). The use of Eudragit®RSPO nanoparticles appears to be a potential approach for the oral delivery of sCT.

Keywords: sCT, Eudragit®L100, Eudragit®RSPO, PLGA, oral nanoparticles, NaTDC

Introduction

Therapeutic proteins currently represent a significant segment of the new pharmaceuticals coming onto the market every year. The progress in biotechnology has accelerated the economical and large-scale production of therapeutically active peptides and proteins. Currently, these biomaterials hold a strong position in the nanotechnology area for medical applications (Sarmento, 2008).

At present, the most commonly used route for administration of protein and peptide drugs is the parenteral route. Unfortunately, parenteral application is invasive in nature and causes pain, resulting in poor patient compliance. The alternate routes (oral, buccal, intranasal, pulmonary, transdermal, ocular and rectal) have been tried for the delivery of these drugs, but have been met with varying degrees of success. The oral route is the most convenient in terms of delivery and patient compliance, especially compared to parenteral route, because it avoids the pain and discomfort associated with injections (Shaji and Patole, 2008). However, despite its desirability, oral application presents serious difficulties due to the nature of the gastrointestinal tract (GIT, e.g. the highly acidic pH and the presence of pepsin in the stomach and pancreatic enzymes in the intestine and brush-border enzymes). These conditions can cause protein degradation and loss of drug activity. At the same time, macromolecular drugs need to be able to cross the intestinal membrane barrier to reach the systemic circulation. However, these drugs are too large and too hydrophilic to cross this barrier. For these reasons, many protein and peptide drugs have low oral bioavailability (Bowman and Leong, 2006).

Many studies have endeavoured to improve the oral delivery of peptides and protein drugs. Polymeric nanoparticle systems are one of several approaches that have been explored to enhance the oral bioavailability of these drugs. Polymeric nanoparticles are attractive vehicles in pharmaceutical technology field due to their inherent stability in

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the GIT compared to other colloidal carriers, especially liposomes (des Rieux et al., 2006). These nanoparticles can protect encapsulated drugs against the harsh environment of the GIT and also enhance their transmucosal transport (des Rieux et al., 2006).

Salmon calcitonin (sCT), a peptide drug composed of 32 amino acids, is currently marketed as injectable and nasal spray forms for the treatment of bone diseases (osteoporosis, Paget's disease) (Guggi et al., 2003). It has not yet reached its full market potential due to the low patient compliance associated with the injectable dosage forms and the nasal delivery system. Oral administration of sCT is not favoured due to the enzymatic degradation of sCT by luminally secreted serine proteases (Guggi and Bernkop-Schnürch, 2003).

A number of surfactants, including bile salts, have been employed in attempts to improve intestinal absorption of sCT. For example, Song et al. (2005) prepared proliposomes containing sCT and NaTDC and showed that duodenal administration to rats resulted in a 7.1-fold increase in the bioavailability of sCT. Bile acids are produced in the liver and recirculated 10–20 times per day as part of the enterohepatic circulation. Bile salts are absorbed in two ways: passively by the membrane of intestinal epithelial cells and actively by the ileum part of the intestine. Thus, coupling of drugs to the selective absorption of bile salts is a useful way to improve the bioavailability of drugs. Bile salts also increase the paracellular and transcellular transport by disturbing tight junctions in the epithelial lining (Samstein et al., 2008; Jung et al., 2009).

In this study, we investigated the effectiveness of an oral delivery system for sCT composed of cationic and anionic nanoparticles prepared using polymethacrylate polymers (Eudragit®L100 and Eudragit®RSPO) and blends of poly (lactide-co-glycolide) (PLGA) and polymethacrylates. In addition, we examined the effect of bile salt on intestinal absorption of sCT by preparing similar Eudragit® and Eudragit®-PLGA nanoparticles (NPs) which also contained NaTDC.

Materials and methods

Materials

sCT was obtained from PolyPeptide Laboratories (San Diego, CA). Eudragit®RSPO (ammonio methacrylate copolymer; MW ~150 000) and Eudragit® L100 (methacrylic acid-methyl methacrylate copolymer (1:1); MW ~135 000) was obtained from Rohm GmbH&Co. KG (Darmstadt, Germany). Acetone, NaTDC and polyvinyl alcohol (PVA, MW 30 000–70 000) were purchased from Sigma-Aldrich Co. LLC (USA), PLGA (50:50; Resomer® RG 502H) from Boehringer Ingelheim Pharma GmbH & Co., KG (Ingelheim, Germany). QuantiChrom™ Calcium Assay Kit was purchased from BioAssay Systems (Hayward, CA). All other reagents and solvents were of analytical grade.

Methods

Preparation of sCT-loaded Eudragit and Eudragit-PLGA nanoparticles

Eudragit® and Eudragit®-PLGA nanoparticles containing sCT were prepared by a nanoprecipitation method (Song et al., 2008; Jawahar et al., 2009). Briefly, Eudragit® RSPO or a Eudragit®RSPO-PLGA mixture (1:2 w/w) were dissolved in 3 mL of acetone, while Eudragit®L100 dissolved in 3 mL of ethanol and Eudragit®L100-PLGA mixture (1:2 w/w) was dissolved in 3 mL of ethanol:acetone mixture (1:2 v/v). The organic phase was added to 6 mL of PVA *aqueous solution* (3% w/v) containing sCT (5 mg) and then stirred using an ultrasonic probe (at 40% power) (Sonoplus, HD 2070; Bandelin, Electronics, Berlin, Germany) for 1.30 min. After evaporation of the organic phase under reduced pressure in a rotary evaporator at 40°C, nanoparticles were recovered by centrifugation at 17 000 rpm for 30 min at 4°C. Nanoparticles were then washed twice with distilled water. After the final washing, the nanoparticles were re-suspended in distilled water and frozen at −20°C in refrigerator overnight, then lyophilized overnight using a Freeze-drier (Alpha 1-2 LD plus LT, Martin Christ, Germany) at condenser *temperature* of −55°C and 0.021 mbar. All batches of nanoparticles were produced at least in triplicate. The same procedure was followed for the preparation of nanoparticles with NaTDC also added to the 6 mL PVA (3% w/v).

Characterization of nanoparticles

Morphological properties of nanoparticles

The surface morphology of the nanoparticle formulations was examined by means of atomic force microscopy (AFM; NanoMagnetics Instruments Ltd., UK). Freshly prepared nanoparticles were washed three times with deionized water and then re-suspended in deionized water. A drop of this nanoparticle suspension was placed on a glass slide, air-dried, and AFM images were taken (Cetin et al., 2007).

Particle size and zeta potential of nanoparticles

The particle size (*Z*-average mean) and zeta potential of the nanoparticles were determined using a Zetasizer 3000HS (Malvern Instruments Ltd., UK). The particle size and zeta potential measurements of nanoparticles were performed in triplicate, following dilution of the nanoparticle suspension in distilled water at 25°C.

Determination of sCT encapsulation efficiency of nanoparticles

The encapsulation efficiency (EE) and drug loading capacity (LC) were determined indirectly by centrifugation method (Saha et al., 2010). This method was used to separate the NPs formed from the aqueous medium containing unentrapped sCT (free sCT; sCT_f) by centrifugation at 17 000 rpm, 4°C for 30 min. The amount of sCT_f in the

Table 1. Intra- and inter-day precision and accuracy of the HPLC method (mean ± SD, $n = 6$).

Added (μg/mL)	Intra-day			Inter-day		
	Measured (μg/mL)	Precision (%RSD)	Accuracy	Measured (μg/mL)	Precision (%RSD)	Accuracy
25	23.50 ± 0.95	4.02	−6	22.72 ± 1.92	8.45	−9.12
300	300.16 ± 9.08	3.02	0.053	288.87 ± 5.79	2.00	−3.71
750	753.04 ± 51.68	6.86	0.40	772.93 ± 33.33	4.31	3.05

Note: RSD: relative standard deviation; Accuracy: [(measured − added)/added] × 100.

supernatant was measured by the HPLC method. All samples were measured in triplicate. The EE of sCT-NPs was calculated as the ratio of sCT loaded into the NPs compared to the total amount of sCT (sCT_t) used for preparation of the original mixture, as follows (Saremi et al., 2011):

$$EE\% = \frac{(sCT_t - sCT_f)}{sCT_t} \times 100.$$

Drug LC was calculated as follows (Saremi et al., 2011):

$$LC\% = \frac{(sCT_t - sCT_f)}{\text{weight of nanoparticles}} \times 100.$$

In vitro *release study*

An incubation method was used to investigate sCT release from nanoparticles (Li et al., 1998; Li et al., 2001; Mukherjee et al., 2008). The release studies were conducted in two media, simulated gastric fluid without enzymes (0.2 M HCl solution, pH 1.2), to evaluate the gastroresistance of a dosage form for about 2 h (the time of gastric emptying) and simulated intestinal fluid without enzymes (0.2 M phosphate buffer, PB, pH 6.8) (Cruz et al., 2010). Nanoparticles (15 mg) were suspended in 1.5 mL of release medium and then the samples were immersed in a constant temperature (37 ± 0.5°C) water bath with agitation at 50 rpm in a horizontal laboratory shaker. At predetermined time intervals, samples (0.25 mL) were withdrawn from the release medium and replaced with fresh buffer. Prior to analysis, all samples were centrifuged at 17 000 rpm for 30 min at 4°C (Li et al., 2001). sCT content in the supernatant was measured by means of a validated HPLC method.

HPLC method

The HPLC system consisted of a Thermoquest Spectra System P 1500 isocratic pump coupled with a Spectra System UV 6000 LP photodiode array detection system, a Spectra System AS 3000 autosampler, a SCM 1000 vacuum membrane degasser, a SN 4000 system controller. A C 18 column (Supelcosil^{TM} LC-18-DB, 25 cm × 4.6 mm × 5 μm) was used as the stationary phase. The mobile phase consisted of 50% of mobile phase A (tetramethyl-ammonium hydroxide (3.26 g) in water adjusted to pH 2.5 with phosphoric acid:acetonitrile (900:100 v/v)) and 50% of mobile phase B (tetramethyl-ammonium hydroxide (1.45 g) in water adjusted to pH 2.5 with phosphoric acid:acetonitrile (400:600 v/v)). The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 10 μL. The detector wavelength was set at 230 nm for quantification of sCT (European Pharmacopoeia, 2001). The calibration curve constructed over the concentration range of 5–1000 μg/mL. The method was validated as to specificity, recovery, linearity, the limit of quantification (LOQ), the limit of detection (LOD), precision and accuracy as reported in the International Conference on Harmonization (ICH, 1997) guidelines. The linearity of the method was determined by performing injections at six different concentration levels in the linear range. Sensitivity of the method was evaluated by determining the LOD and LOQ. Three different concentrations of standard sCT (25, 300 and 750 μg/mL; $n = 6$) were analysed on three consecutive days (inter-day) and three times within the same day (intra-day) for evaluation of precision and accuracy of the method. The regression equation of the calibration curve is $y = 6381.4x + 4183.1$ (where y is the peak area and x is the concentration of sCT in μg/mL, $r = 0.9998$, $n = 6$). LOD and LOQ were estimated from signal-to-noise ratio. The values of LOQ and LOD were 5 and 1 μg/mL, respectively. Precision and accuracy of the assay method were determined for both intra- and inter-day variations using the quality control samples of 25, 300 and 750 μg/mL. The intra- and inter-day precision and accuracy values of the method were given in Table 1.

In vivo *hypocalcemic potency of sCT-loaded nanoparticles*

The animal experiments were conducted according to the ethical norms approved by the Ethics Committee of Ataturk University (No: B.30.2.ATA.0.03.00.00/255). Female Sprague-Dawley rats (200–250 g) were provided from the Experimental Animal Teaching and Research Center of the Experimental Animal Laboratory at Ataturk University.

The efficacy of NPs after oral administration was examined by randomly dividing rats into 10 groups ($n = 6$ for each studied group). The rats were fasted overnight prior to the experiment and remained fasted throughout its duration, but were allowed free access to water. The following formulations were administered to individual rats by oral gavage with a 16G × 3″ (76.2 mm) feeding needle (Harvard Apparatus, Holliston, MA): acetate buffer solution, oral sCT solution (500 μg/kg) or sCT-loaded NPs suspension (equivalent to 500 μg/kg sCT). The solutions and suspensions were prepared in acetate buffer (10 mM, pH 3.5) in which sCT has high stability (Cholewinski et al., 1996; Mansoor et al., 2005) and were orally administered at a volume of 0.4 mL/rat. Whole blood was collected using

Lithium-Heparin coated-Microvette® CB 300 capillary tubes (Braintree Scientific, Braintree, MA) from the s*aphenous vein* and was immediately separated by centrifugation (10 000 rpm, 10 min). The plasma was frozen until assayed for calcium to determine hypocalcemic effects of the drug. Plasma calcium level was determined using a QuantiChrom™ Calcium Assay Kit.

The maximum percentage reduction in plasma calcium levels relative to the basal value (maxd%) was calculated. The total decrease in plasma calcium levels (D%) was calculated using a modified method modified by Hirai et al. (1981), as follows:

$$D\% = \frac{(AUC_{\text{control}} - AUC_{\text{sCTorformulations}})}{AUC_{\text{control}}} \times 100,$$

$AUC_{\text{sCTorformulations}}$ and AUC_{control} were the areas under the plasma calcium concentration versus time curves, up to 48 h after oral administration of samples and placebo, respectively.

Statistical analysis

The different sample groups were compared using a non-parametric Mann–Whitney U-test, and p-values less than 0.05 were considered statistically significant.

Results

Nanoparticles obtained by the nanoprecipitation technique were characterized in terms of size, zeta potential, *in vitro* release, and *in vivo* efficiency. The sCT-loaded nanoparticles had an almost spherical morphology (Figure 1). The particle sizes of sCT-loaded nanoparticles ranged from ~157 nm to ~309 nm (Table 2). The particle sizes of all nanoparticle formulations decreased with the addition of NaTDC in the formulation due to its surfactant nature ($p < 0.05$). Similar results were reported by Jung et al. (2009). The zeta potentials of nanoparticles are given in Table 2. Eudragit®RSPO and Eudragit®L100

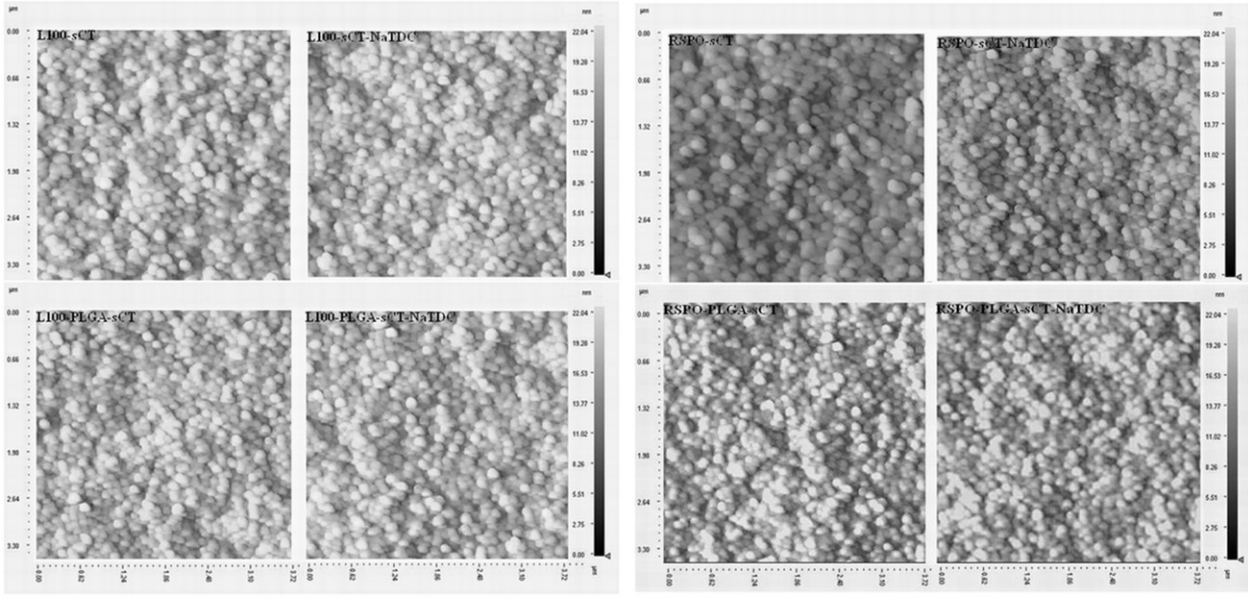

Figure 1. AFM images of nanoparticle formulations.
Notes: L100-sCT: sCT-loaded Eudragit®L100 NPs; L100-sCT-NaTDC: sCT and NaTDC-loaded Eudragit®L100 NPs; RSPO-sCT: sCT-loaded Eudragit®RSPO NPs; RSPO-sCT-NaTDC: sCT and NaTDC-loaded Eudragit®RSPO NPs; L100-PLGA-sCT: sCT-loaded Eudragit®L100-PLGA NPs; L100-PLGA-sCT-NaTDC: sCT and NaTDC-loaded Eudragit®L100-PLGA NPs; RSPO-PLGA-sCT: sCT-loaded Eudragit®RSPO-PLGA NPs and RSPO-PLGA-sCT-NaTDC: sCT and NaTDC-loaded Eudragit®RSPO-PLGA NPs.

Table 2. The characteristics of Eudragit® and Eudragit®-PLGA nanoparticles (mean ± SD; $n = 3$).

Formulation	Particles size (nm)	Zeta potential (mV)	Encapsulation efficiency (%)	Drug loading (%)
L100-sCT NPs	179.65 ± 1.62	−6.40±0.23	69.13 ± 2.82	9.90 ± 3.22
L100-sCT-NaTDC NPs	161.20 ± 0.36	−8.55 ± 0.32	61.02 ± 3.10	8.04 ± 2.92
RSPO-sCT NPs	247.25 ± 2.75	5.97 ± 0.04	64.20 ± 2.31	9.49 ± 1.28
RSPO-sCT-NaTDC NPs	180.20 ± 0.42	5.17 ± 0.58	59.40 ± 2.59	7.99 ± 2.94
RSPO-PLGA-sCT NPs	308.85 ± 2.08	4.00 ± 0.09	62.40 ± 2.87	7.36 ± 2.21
RSPO-PLGA-sCT-NaTDC NPs	157.40 ± 1.00	−0.68 ± 0.26	55.90 ± 2.34	6.17 ± 1.83
L100-PLGA-sCT NPs	234.10 ± 0.051	−10.97 ± 0.41	67.60 ± 1.36	7.98 ± 1.29
L100-PLGA-sCT-NaTDC NPs	224.23 ± 2.20	−15.93 ± 0.81	58.50 ± 2.31	6.45 ± 2.12

containing nanoparticle formulations showed positive and negative zeta potential values, respectively. These data reflect the charges of polymers: the polycationic Eudragit®RSPO bears positive charges and the polyanionic Eudragit®L100 has negative charges. The Eudragit®L100-sCT and Eudragit®L100-PLGA-sCT nanoparticles showed increasingly more negative charges. Similarly, the positive charge of Eudragit®RSPO-sCT and Eudragit®RSPO-PLGA-sCT nanoparticles decreased with the addition of NaTDC to the formulations, due to its anionic nature (Table 2, $p < 0.05$).

The EE of the nanoparticles ranged from 55.90% to 69.13%, as given in Table 2. Very slight differences were observed between the EE of nanoparticle formulations without NaTDC. As indicated in Table 2, the encapsulation efficiencies of nanoparticle formulations had a negative relationship with the addition of NaTDC ($p < 0.05$). Jung et al. (2009) used bile salts of various contents (0.75–7.5 mg) for the preparation of sCT-loaded PLGA nanoparticles and showed that the EE of nanoparticles significantly decreased with increases in the amount of bile acid in formulations. They also reported that bile acids were very active as emulsifiers during the emulsification process, but not as active as an interfacial barrier in preventing sCT molecules from diffusing into the water phase. The *in vitro* release of sCT-loaded nanoparticles was investigated in PB (pH 6.8) and also HCl (pH 1.2) at 37 ± 0.5°C. The cumulative percentage release is shown in Figure 2.

The pharmacological effects of sCT-loaded nanoparticles were evaluated in rats. Changes in plasma calcium levels after oral administration of sCT (100 μg of sCT/rat weighing 200 g) and sCT-loaded nanoparticles (equivalent to 100 μg of sCT/rat weighing 200 g) are shown in Figure 3 as the maximum decrease in plasma calcium levels (maxd%) relative to the basal levels and the total decrease in calcium level (*D*%) following oral administration (Table 3, Figure 4). Significant differences in plasma calcium reduction between each sCT-loaded nanoparticle formulation and control group were observed ($p < 0.05$).

When sCT-loaded Eudragit®RSPO nanoparticles were orally administered to the rats, the plasma calcium concentration was significantly reduced compared to the plasma of rats fed sCT solution or the other sCT-loaded nanoparticle formulations (Table 3, Figures 3 and 4). These low plasma calcium levels were maintained for at least 24 h, indicating the effectiveness of Eudragit®RSPO nanoparticles in enhancing the absorption of sCT. A significant difference was noted between *D*% values of sCT solution and each nanoparticle formulation ($p < 0.05$; Figure 4). The order of this hypocalcemic effect of nanoparticle formulations was Eudragit®RSPO-sCT* > Eudragit®RSPO-PLGA-sCT* > Eudragit®L100-sCT** = Eudragit®L100-PLGA-sCT** (*$p < 0.05$; **$p > 0.05$, *D*% compared). No statistically significant difference was found between *D*% of each nanoparticle formulation in the presence or absence of NaTDC ($p > 0.05$, Figure 4).

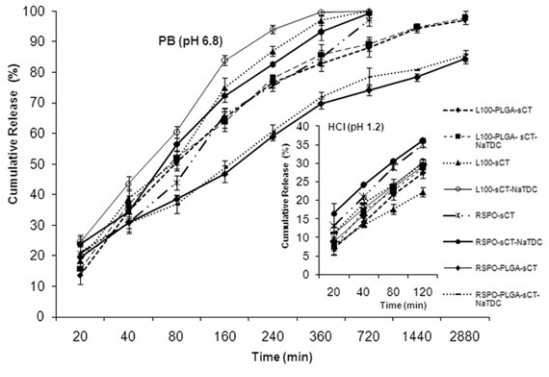

Figure 2. The *in vitro* release profiles of sCT from nanoparticles (mean ± SD; $n = 3$).

Discussion

As a drug for the treatment of osteoporosis in women, sCT works by preventing bone breakdown and increasing bone density. It is used nasally as a spray once a day, alternating with an injection used once a day or once every other day (Mark and Ettinger, 2003). Because sCT has very low permeability across biological membranes and also is metabolized by intestinal serine proteases (Cetin et al., 2008), replacement of this conventional delivery by an oral delivery system has proven difficult. Shah and Khan (2004) investigated the maximum permeability region of sCT in GIT of rats, and also determined the transport mechanisms of sCT through Caco-2 cell monolayer. They found that the permeability of sCT through Caco-2 cells took place via passive diffusion and the permeation of sCT through the mucosa of GIT in rats showed regional differences. The maximum permeation was obtained in ileum followed by colon, jejunum, duodenum and stomach. The absorption of sCT has been reported to be low (less than 0.1% of BA) after direct administration into the duodenum, ileum and colon in rats and dogs (Sinko et al., 1995; Hee et al., 2000).

The main obstacles for the oral delivery of sCT are low bioavailability and its instability problems (Garcia-Fuentes et al., 2005). The enhancement of the transport of peptides/proteins via nanoparticles is provided by different mechanisms (mucoadhesion, particle endocytosis and permeation-enhancing effect) depending on the nanoparticles composition (Garcia-Fuentes et al., 2005; des Rieux et al., 2006). In addition, nanoparticles protect the labile drugs against the GIT harsh environment (des Rieux et al., 2006). Nanoparticles can enhance drugs' absorption by optimizing their interaction with the absorption site in the GI-tract walls or by directly transporting these drugs through the intestinal mucosa to systemic circulation. In GI tract, there are several possibilities for the uptake of nanoparticles such as transcellular uptake, paracellular uptake and an uptake via the M cells, mainly located in Peyer's patches. Many factors (GI tract's physiology, the particle size/type/charge, hydrophilic/hydrophobic balance, polymer type used, the presence of a ligand, etc.) play an important role for uptake of nanoparticles via these routes. Several polymers (chitosan, polyacrylates, etc.) interacting were used to enhance paracellular uptake of peptides through interactions

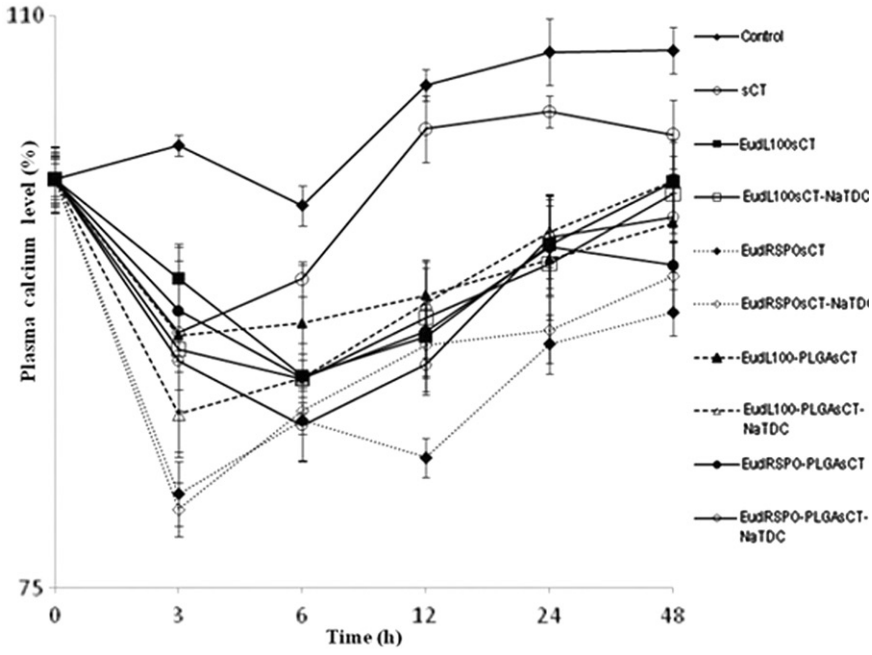

Figure 3. Plasma calcium levels, after oral administration to rats, of sCT in solution and nanoparticles formulations at doses of 100 μg/rat (weighing 200 g) (mean ± SD; $n = 6$ rats/group).
Notes: A statistically significant difference was found between the control and each sCT-loaded nanoparticle formulation ($p < 0.05$); a significant difference between sCT solution and each sCT-loaded nanoparticle formulation was found from 3 to 48 h ($p < 0.05$; except 48 h of L100-sCT NPs ($p > 0.05$); 3 and 48 h of L100-sCT-NaTDC NPs ($p > 0.05$); 3 and 6 h of L100-PLGA-sCT NPs ($p > 0.05$); 48 h of L100-PLGA-sCT-NaTDC NPs ($p > 0.05$); 3 h of RSPO-PLGA-sCT NPs ($p > 0.05$); 3 and 48 h of RSPO-PLGA-sCT-NaTDC NPs ($p > 0.05$)).

Table 3. Plasma calcium lowering effects of sCT solution and sCT-loaded nanoparticle formulations following oral administration at 100 μg doses to rats weighing 200 g (mean ± SD; $n = 6$ rats/group).

Formulation	Time to maximum decrease in plasma calcium (h)	maxd% in plasma calcium
sCT	3	9.35 ± 1.8
RSPO-sCT NPs	3	19.24 ± 2.0
RSPO-sCT-NaTDC NPs	3	20.18 ± 1.4
RSPO-PLGA-sCT NPs	6	12.14 ± 2.3
RSPO-PLGA-sCT-NaTDC NPs	6	15.05 ± 3.8
L100-sCT NPs	6	12.01 ± 1.5
L100-sCT-NaTDC NPs	6	12.20 ± 2.4
L100-PLGA-sCT NPs	3	10.00 ± 2.0
L100-PLGA-sCT-NaTDC NPs	3	14.38 ± 1.6

Note: maxd%: the maximum percentage reduction in plasma calcium levels relative to the basal value.

between the negatively charged cell membrane and the positive charges of the polymer, or by complexing Ca^{+2} involved in the structure of tight junctions (Jung et al., 2000; des Rieux et al., 2006; Mohanraj and Chen, 2006; Pinto et al., 2006).

Bioadhesive nanoparticles adhere to the mucosal surface led to the modification of mucosal surface properties via bioadhesive polymers and improve the residence time and contact of the drug with the epithelium. Thus, the compound concentration increases in the absorption site (Garcia-Fuentes et al., 2005). In this way, active substance dilution and degradation by luminal contents can be minimized, resulting in an enhanced absorption into systemic circulation (Makhlof et al., 2011).

In this study, the use of the nanoprecipitation technique allowed simple, rapid and uncomplicated production of nanoparticles in the size range ~150–300 (Table 2). All preparation procedures could be performed in one step and nanoparticles are quickly generated. This method utilizes two miscible solvents. Addition of polymer solution into the non-solvent results in nanoparticle formation due to the rapid desolvation of the polymer. The rapid nanoparticle formation is based on the Marangoni effect, which is a phenomenon of interfacial turbulence triggered by surface tension. Small nanoparticles (100–300 nm) with a narrow size distribution are usually prepared by nanoprecipitation methods using different polymers (e.g. PLGA, Eudragit, cellulose derivatives, poly ε-caprolactones, etc) (Bilati et al., 2005; Cetin et al., 2010).

The zeta potential values of the prepared nanoparticles showed the effect of Eudragit®RSPO and Eudragit®L100 on the surface charge of the nanoparticles. The formulations containing Eudragit®RSPO had positive zeta potential values because of the quaternary ammonium groups of Eudragit®RSPO, while nanoparticles containing Eudragit®L100 had negative zeta potential values because of the anionic groups of Eudragit®L100. Glowka et al. (2010) prepared nanoparticles with positive zeta potential using PLGA and positively charged Eudragit®RS blends. They reported that PLGA backbone chains were surrounded with Eudragit® RS and, as a result, the amine groups in the Eudragit®RS backbone became positioned on the outer side and a positive surface charge was

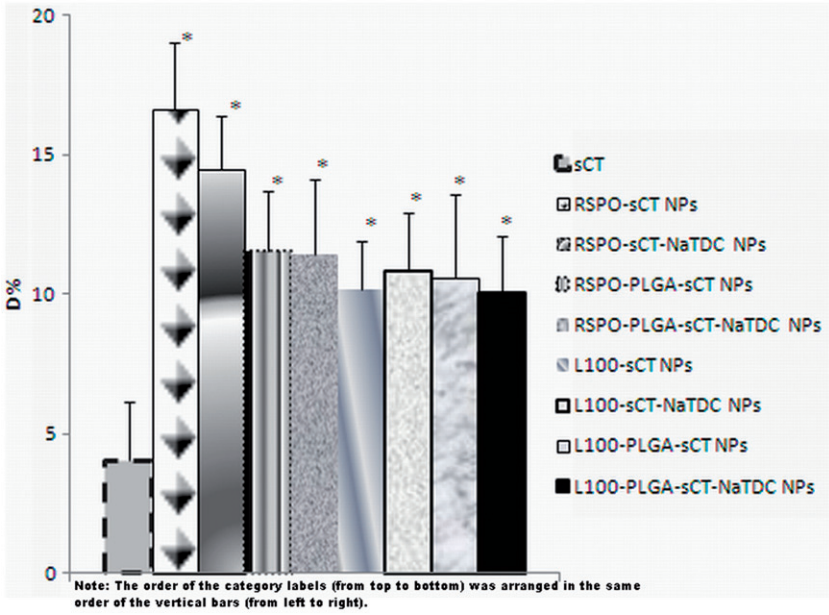

Figure 4. Comparison of *D*% of sCT solution and sCT-loaded nanoparticles after oral administration at doses of 100 μg/rat (weighing 200 g) (mean ± SD; $n = 6$ rats/group; Mann–Whitney *U*-test, $p < 0.05$ compared to the sCT solution). Note: The order of the category labels (from top to bottom) was arranged in the same order of the vertical bars (from left to right).

obtained. A simple HPLC method was used for the quantitative determination of sCT in this study. This method has a short separation time and a low LOQ value. sCT was well separated from the matrix components with no interfering peaks in the relevant portion of the trace under the optimum conditions The intra-day accuracy of this method ranged from −6% to 0.40% and precision from 3.02% to 6.86%. The inter-day accuracy ranged from −9.12% to 3.05% and precision from 2.00% to 8.45% (Table 1). All these results indicate that precision and accuracy of the assay are satisfactory.

About 30% of the sCT was released from all nanoparticle formulations in acidic medium (pH 1.2). Similar results for Eudragit®L and Eudragit®RS nanoparticles were previously reported by Eerikäinen et al. (2004).

Under buffered conditions (pH 6.8), about 75% and 66% of the drugs were found after approximately 3 h, while 97% and 85% of the drugs were released from Eudragit®L100 and Eudragit®RSPO nanoparticles after 6 h. This effect was due to the pH-dependent and independent release behaviour of Eudragit®L100 and Eudragit®RSPO, respectively. Eudragit®L copolymer was ionized and soluble at pH 6.8 and showed a relatively rapid drug release. On the other hand, approximately 65% and 50% of the sCT were released from Eudragit®L100-PLGA and Eudragit®RSPO-PLGA nanoparticles within 3 h, respectively, and about 95% and 80% of the drugs were released from these nanoparticles after 24 h, due to the presence of PLGA in these formulations. Consequently, the properties of the polymer have a very important effect on the controlled drug release from the nanoparticles (Eerikäinen et al., 2004; Bilati et al., 2005; Cetin et al., 2010; Glowka et al., 2010).

Drug release from the Eudragit® nanoparticles (L100 as well as RSPO) containing NaTDC was faster than from the Eudragit® nanoparticles without NaTDC, most probably due to decrease in the particle size of nanoparticles following addition of bile acid ($p < 0.05$, Figure 2). Acharya et al. (2010) reported that drug release from polymeric systems depends on at least three factors, such as the surface area, diffusion coefficient and concentration gradient of the drug. They also suggested that control of particle size of the formulation also resulted in a more accurate estimation and a greater reproducibility of the drug release properties of a given formulation. Similarly, Dawes et al. (2009) reported that the decrease in particle size causes a higher rate of inclusion of water into the polymeric system in the release medium and thus a faster diffusion of the drug. In this study, the cumulative release from sCT-loaded Eudragit®-PLGA nanoparticles without NaTDC was slightly lower than that from Eudragit®-PLGA nanoparticles with NaTDC at every time point ($p > 0.05$; Figure 2).

The characteristics of the polymers, such as hydrophilicity, negative charge potential and the presence of hydrogen bonds, can promote the formation of mucoadhesive bonds, which in turn determine the retention at application and target sites. The surface free energy of the polymer should permit the sufficient wetting by the mucosal surface. Anionic and cationic polymers exhibit strong mucoadhesion (Patel et al., 2011). Mucus, which covers the mucous membranes, is a weak viscoelastic gel that contains about 95% water and about 5% other components (mainly glycoproteins with negative charge). The glycoproteins participate in the mucoadhesion process by interacting with some of the polymers in the aqueous phase (Sigurdsson et al., 2002).

In this study, we used two types of polymethacrylates. Eudragit®L100 is an anionic copolymer based on methacrylic acid and methyl methacrylate. The ratio of the

free carboxyl groups to the ester groups is approximately 1:1 in Eudragit®L100. In contrast, Eudragit® RSPO, an ammonio methacrylate copolymer, is a cationic polymer that contains quaternary ammonium groups in its structure. These polymers have been used in the formulation of dosage forms, most commonly in the preparation of matrix tablets, oral sustained-release forms and in tablet coatings, microparticles, and nanoparticles (Kim et al., 2002; Kuksal et al., 2006; Rowe et al., 2006; Devarajan and Sonavane, 2007). The mucoadhesive properties of anionic polymers are caused by the physical entanglement, hydrogen-bonding and van der Waal's interactions with mucus. These interactions are stronger than the electrical repulsion. On the other hand, cationic polymers interact with mucus largely through electrostatic forces (Shaji and Patole, 2008).

In our *in vivo* study, the normal control rats did not show any significant changes in plasma calcium levels over the duration of the experiment. In contrast, a significant difference was noted between *D*% values of the sCT solution and each nanoparticle formulation ($p < 0.05$), with the order of the hypocalcemic effect being Eudragit®RSPO-sCT* > Eudragit®RSPO-PLGA-sCT* > Eudragit®L100-sCT** = Eudragit®L100-PLGA-sCT** (*$p < 0.05$; **$p > 0.05$; Figure 4). Eudragit®L100-sCT protected sCT against enzymatic degradation ($p < 0.05$ compared to sCT solution). Eaimtrakarn et al. (2002) prepared a gastrointestinal mucoadhesive patch system (GI-MAPS) for the oral delivery of protein drugs using three different polymers (hydroxypropyl methylcellulose phthalate (HP-55®), Eudragit®L100 and S100) as surface coatings and reported that Eudragit L100 layer of film dissolved in the jejunum, and as a result, GI-MAPS adhered to the jejunum wall after oral administration to dogs. They also found that the Eudragit®L100 system provided a higher pharmacological availability for the model protein, compared to the other polymers, because in the jejunum wall, the hydrolytic enzyme activity is lower compared to the duodenum and the effect of intestinal contents is also less than that in the ileum. In another study of this group, Eaimtrakarn et al. (2001), they examined the effect of the surface layer of intestinal mucoadhesive film systems made of Eudragit® L100, S100 or HP-55® on the retention and transit properties after intraduodenal administration to rats. They found similar results and expressed that Eudragit® L100 layer of film dissolved and retained for approximately 2 h in the jejunum. The functions of this intestinal mucoadhesive film system were summarized as pH-dependent intestinal adhesion shows site specificity and the retention time of this film in adhesion site is at least 2 h. Similar results were reported by Takada and Ushirogawa (1991). Morishita et al. (1993) prepared insulin microspheres for enteral delivery using Eudragit®L100, Eudragit®S100 and a 1:1 mixture of these polymers. The highest hypoglycaemic effect was obtained with the Eudragit®L100 system and this finding was explained by the authors as a greater insulin release from the Eudragit®L100 microspheres in the jejunum to upper ileum, compared to release from Eudragit®S microspheres. Karn et al. (2011) prepared atenolol-loaded liposomes coated with chitosan, Carbopol 974P, Eudragit®L100 or Eudragit®S100. They reported that the Eudragits-coated liposomes containing atenolol remained attached to the intestinal mucosa for an adequate time, allowing prolonged absorption of atenolol.

In addition, Garcia Fuentes et al. (2005) investigated the interaction of poly(ethylene glycol) (PEG)-coated and chitosan-coated lipid nanoparticles with Caco-2 cells and determined the potential of these coated nanoparticles for oral application of sCT in rats. They reported that both the coated nanoparticles interact with Caco-2 cell monolayer. However, the chitosan-coated nanoparticles significantly interact with the tight junctions and enhance the paracellular transport of the drug released at the epithelial level compared to PEG-coated nanoparticles. These observations were explained by authors as follows: (1) the interaction of chitosan-coated nanoparticles with the mucus resulted in the diffusion of these nanoparticles through the mucus layer; (2) the site-specific delivery of sCT for prolonged time was ensured by these interactions, and as a result, a prolonged hypocalcemic effect was obtained and (3) the interaction of chitosan with the tight junctions might enhance the paracellular transport of the peptide released at the epithelial level. Sakuma et al. (1997) prepared nanoparticles using graft copolymers having a hydrophobic backbone and hydrophilic branches such as poly(methacrylic acid), poly(N-isopropylacrylamide), poly(N-vinylacetamide), poly(N-vinylacetamide-co-vinylamine) and poly(vinylamine) and evaluated them for the oral delivery of sCT in rat. They found that poly(methacrylic acid) macromonomer chains on the surfaces of nanoparticles affect the rate of sCT incorporation in nanoparticles and the absorption of sCT. Further, the significant absorption enhancement of sCT was obtained by using bioadhesive polymers (poly(methacrylic acid), poly(N-isopropylacrylamide)). Consequently, they explained that this absorption enhancement of sCT is due to the mucoadhesive properties of nanoparticles and the increase in the stability of sCT against digestive enzymes after its incorporation into the nanoparticles.

In our study, cationic nanoparticles, especially the Eudragit®RSPO-sCT nanoparticles, showed the most significant hypocalcemic effect when compared to the effects of sCT solution and anionic nanoparticles (for *D*%, $p < 0.05$; Figure 4). This effect was due to an ionic interaction between the negative charges of the mucus layer on the surface of the intestine and the positive charge of the Eudragit®RSPO nanoparticles, which imparted mucoadhesive properties to these nanoparticles. These nanoparticles also protected sCT from the hostile intestinal conditions. When peptide drugs with positive charges, such as calcitonin or desmopressin, are incorporated in anionic mucoadhesive polymers, these drugs strongly interact with the polymers. This interaction may prevent drug release and cause a significant reduction in the mucoadhesive properties of the polymers. Therefore, cationic or non-ionic mucoadhesive polymers should be used for cationic peptides or peptidomimetics (Varaprasad Reddy and Sudheer Babu, 2010).

We have obtained results that support previously published information and that show that the hypocalcemic effect of Eudragit®L100 nanoparticles was lower than that of Eudragit®RSPO-sCT nanoparticles. Lopedota et al. (2009) prepared and physicochemically characterized nanoparticles for the transmucosal administration of glutathione (GSH) by the combination of the mucoadhesive properties of Eudragit®RS 100 with the penetration-enhancing capability of cyclodextrins. They showed that Eudragit®RS 100 nanoparticles interacted efficiently with mucin and delivered GSH along transmucosal routes based on peculiar features of these formulations, although their encapsulation efficiencies were relatively low.

We also prepared the nanoparticles containing sCT and an absorption enhancing agent. In general, the mechanisms of intestinal mucous permeation by absorption enhancers include the following: (1) The absorption enhancers (such as surfactants, bile acid derivates) lead to membrane perturbation and increase the permeability of drugs via interactions of them with membrane lipid/protein (Sood and Panchagnula, 2001). (2) Bile salts, which physiologically open epithelial tight junctions, increase the paracellular absorption (Catalioto et al., 2008). In addition, especially conjugate bile salts as NaTDC form lipophilic ion-pair complexes with various organic cations and thus increase the permeability of the cations across biological membranes (Song et al., 2001, 2005). (3) Some absorption enhancers, such as diethyl maleate and salicylic acid, increase the disorder of membrane status by decrease in membrane non-protein thiol (Sood and Panchagnula, 2001). In our study, sCT and NaTDC were used at ratio of 1:1 (w/w). We found no statistically significant difference between the *D*% of each nanoparticle formulation with and without NaTDC ($p > 0.05$; Figure 4). This observation might be due to the used NaTDC amount and the ratio of drug:NaTDC and indicates that further research will need to investigate the optimum ratio of sCT to NaTDC for the enhancement of intestinal sCT absorption from the chosen nanoparticle formulation in terms of appropriate *in vitro* characteristics and hypocalcemic effect in this study. Similar results were reported by previous studies (Song et al., 2001, 2005; Jung et al., 2009). Song et al. (2005) showed that using proliposomes containing sCT and sodium taurodeoxycholate enhanced the intestinal absorption of sCT. They previously determined the optimum concentration of sodium taurodeoxycholate using Caco-2 cells and thus used the various sCT:NaTDC ratios (300 μg/mL of sCT in 0%, 0.02%, 0.05%, 0.07%, 0.1%, 0.2%, w/v of NaTDC) and calculated the Merit index by measuring the transepithelial electrical resistance values for the evaluation of the integrity of the cell monolayers. The highest Merit index was obtained at an NaTDC concentration of 0.1% (w/v) and chosen as the ratio 0.75:2.5 (sCT:NaTDC, w/w%). They reported that the increase in permeability of sCT might be due to the formation of ion-pair between sCT with bile salts and result in an increase in the passive diffusion of sCT molecules across biological membranes. Furthermore, sCT and bile acid-loaded PLGA nanocarriers were prepared by Jung et al. (2009). They used 6 mg of sCT and various amounts of bile acid (0.75, 1.5, 3.75 and 7.5 mg) for preparation of nanocarriers and these formulations were administered to rats at a dose of 1250 μg of oral sCT. The most appropriate hypocalcemic effect was obtained using 1.5 mg of bile acid. However, when bile acid was used in the amount < 3.75 mg, the decrease in plasma Ca concentration was noticeably deteriorated. They explained that this finding is due to poor EE. Song et al. (2001) reported similar results. They prepared the ion-pair complexation of sodium taurodeoxycholate and tributylmethylammonium (an organic cation). They found that the uptake rate of this complex shows a concentration dependence uptake, and the ratio of bile acid:organic cation plays an important role.

Conclusion

In conclusion, all the nanoparticle formulations used in this study improved the oral absorption of sCT in rats. Oral administration of sCT-loaded nanoparticles led to a statistically significant decrease in plasma calcium level compared to administration of sCT solution. The sCT-loaded Eudragit®RSPO nanoparticles, due to their mucoadhesive properties, showed an important hypocalcemic effect for at least 24 h, compared to the other formulations, when administered to rats. No significant difference was found between nanoparticle formulations with and without NaTDC used at a single concentration. The impacts of NaTDC on the oral absorption of sCT encapsulated in Eudragit®RSPO nanoparticles should be further examined using different concentrations of NaTDC or using new absorption enhancers and also, the stability of chosen nanoparticle formulations in GIT fluids and their cytotoxic effects may be evaluated.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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