Coated minispheres of salmon calcitonin target rat intestinal regions to achieve systemic bioavailability: comparison between intestinal instillation and oral gavage

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Abstract

Achieving oral peptide delivery is an elusive challenge. Emulsion-based minispheres of salmon calcitonin (sCT) were synthesized using single multiple pill (SmPill®) technology incorporating the permeation enhancers (PEs): sodium taurodeoxycholate (NaTDC), sodium caprate (C_{10}), or coco-glucoside (CG), or the pH acidifier, citric acid (CA). Minispheres were coated with an outer layer of Eudragit® L30 D-55 (designed for jejunal release) or Surelease®/Pectin (designed for colonic release). The process was mild and *in vitro* biological activity of sCT was retained upon release from minispheres stored up to 4 months. *In vitro* release profiles suggested that sCT was released from minispheres by diffusion through coatings due to swelling of gelatin and the polymeric matrix upon contact with PBS at pH 6.8. X-ray analysis confirmed that coated minispheres dissolved at the intended intestinal region of rats following oral gavage. Uncoated minispheres at a dose of ~2000 I.U. sCT/kg were administered to rats by intra-jejunal (i.j.) or intra-colonic (i.c.) instillation and caused hypocalcaemia. Notable sCT absolute bioavailability (*F*) values were: 5.5% from minispheres containing NaTDC (i.j), 17.3% with CG (i.c.) and 18.2 % with C_{10} (i.c.). Coated minispheres administered by oral gavage at threefold higher doses also induced hypocalcaemia. A highly competitive *F* value of 2.7% was obtained for orally-administered sCT-minispheres containing CG (45 μmol/kg) and coated with Eudragit®. In conclusion, the SmPill® technology is a potential dosage form for several peptides when formulated with PEs and coated for regional delivery. PK data from instillations over-estimates oral bioavailability and poorly predicts rank ordering of formulations .

Key words: Salmon calcitonin, oral bioavailability, oil and water emulsions, intestinal permeation enhancers, oral peptide and protein delivery

1 Introduction

The therapeutic peptide pipeline has expanded substantially in the last 30 years and new injectable peptides are constantly emerging [1]. Developing oral delivery systems for peptides is important in order to improve patient compliance and, in the case of selected anti-diabetic peptides, to provide a more physiological route of delivery than injection. However, most peptides have unfavorable physicochemical properties for oral delivery including large molecular weight (MW), high hydrophilicity, a tendency to aggregate, susceptibility to pancreatic serine proteases, and low intestinal permeability [2]. Salmon calcitonin (sCT, MW 3432), is a benchmark peptide to test any technology for oral delivery as it encompasses all five problems [2]. It is a second line anti-resorptive treatment for osteoporosis, as well as for Paget's disease and hypercalcemia, and is marketed in injectable and nasal formats [3]. Its commercial viability for long term use in post-menopausal women has been questioned recently due to possible links to cancer, and regulatory agencies have now recommended only short term use given its relatively narrow risk-to-benefit profile [4]. The most advanced oral sCT formulation, TBRIA™ (Tarsa Therapeutics, PA, USA), contains the peptide in vesicles protected by citric acid (CA), which is released in the duodenum following erosion of a pH-dependent methacrylate-based polymeric coating. This formulation completed Phase III [5] and an New Drug Application was submitted to the FDA in 2015.

Drugs from delayed-release formulations of small molecules are released following passage through the stomach (e.g. Depakote (AbbVie, IL, USA) [6]), whereas extended-release formulations deliver the drug partially after ingestion and then the remainder over an extended time-frame (e.g. Embeda® (Pfizer, NY, USA) [7]). Targeted release of drugs to selected regions of the GI tract can be achieved by coating solid dosages with enteric, time-dependent, or pH-dependent materials. The most common site for systemic delivery is to target the small intestine using enteric coated solid dosage forms with pH-dependent polymers including poly(meth)acrylates: e.g. Eudragit® (Evonik Rohm GmbH, Germany) [8], and cellulose-based ones: e.g. hydroxypropylmethylcellulose, acetate succinate and polyvinyl derivatives [9]. On the other hand, colonic drug delivery has attracted interest primarily for local delivery of small molecules to treat ulcerative colitis and colorectal cancer [10]. Similar to the small intestine, colonic delivery can be achieved by coating solid dosage forms with enteric polymers, but they

require sufficient thickness to prevent premature release and are subject to high intra-subject variability. Budesonide MMX® (multi-matrix system, Valeant, Bridgewater, NJ, USA) is a time- and pH-dependent delivery system consisting of enteric coated (Eudragit® L) pellets with a rate-limiting polymer containing budesonide to permit extended release in the colon, which was recently approved for colitis patients by the FDA [11]. Other colonic targeting strategies include covalent linkage of a drug with a carrier (e.g. azo-, cyclodextrin-, glycoside-, glucuronide- conjugates), or delivery of intact drug embedded in biodegradable matrices /hydrogels, microemulsions, bioadhesive polymers, or in multiparticulates [12].

We used an emulsion-based coated minisphere technology, the single multiple pill (SmPill®, Sigmoid Pharma Ltd., Ireland), to test oral delivery of sCT with a view to providing data that could lead to establishment of a platform technology to release peptides in intestinal regions for systemic delivery. To our knowledge, there are no examples of a platform technology that can be modified with selected coatings and permeation enhancers (PEs) designed both to release a peptide in two different intestinal regions and to promote its subsequent permeation. Sodium caprate (C_{10}) and coco-glucoside (CG) were included in sCT-SmPill® as PEs [13, 14]. Both molecules have a safe profile [15, 16] and history of use in man either in a rectal suppository (C_{10}), or in personal care products (CG) [13, 17]. The third molecule selected as a potential PE for sCT was sodium taurodeoxycholate (NaTDC) [18], while citric acid (CA) was selected on the basis of a primary mechanism of action to protect sCT from serine proteases [19]. In previous work, sCT-SmPill® minispheres containing C_{10}, CG, and NaTDC were efficacious and improved absolute bioavailability (*F*) of sCT following rat intra-jejunal (i.j.) and intra-colonic (i.c.) instillations [2]. The objectives of this work were firstly to develop suitable coatings for sCT-SmPill® minispheres and then to use X-ray imaging to confirm delivery to rat jejunum and to rat colon following oral administration. Secondly, we compared the associated pharmacokinetic (PK) and pharmacodynamic (PD) profiles for sCT delivered from minisphere formulations either (a) when instilled to jejunal or colonic regions in uncoated formats, or (b) when coated versions were administered by gavage.

2 Material and methods

Sodium caprate (C_{10}) (PubChem CID: 4457968), citric acid (CA) (CID: 311), and sodium taurodeoxycholate (NaTDC) (CID: 56840807), D-sorbitol (CID: 5780) and gelatin were obtained from Sigma-Aldrich, Ireland. Coco-glucoside (CG), was supplied as Plantacare® 818 UP (Cognis, Germany). Media, buffers and supplements were obtained from GIBCO®, Ireland. Synthetic sCT (CID 16129616) was purchased from Polypeptide Laboratories (Copenhagen, Denmark); it had an average activity of 5100 I.U./mg. Transcutol HP® (CID: 8146), Kolliphor EL® (CID: 5849927), and Miglyol 810® (no CID) were obtained from Gattefossé (France), BASF (Germany), and Sasol (UK) respectively. $BaSO_4$ (Bariogel®) was purchased from Cristália SA (Brazil). Opadry® white and Surelease®/Pectin were purchased from Colorcon (UK); Eudragit® L30-D55 was from Evonik (UK). The Parameter™ cAMP ELISA was obtained from R&D Systems (UK).

2.1 SmPill® minisphere preparation with PEs

Minispheres were loaded with 60% (w/w) barium sulphate ($BaSO_4$) to enable X-ray detection. Minispheres were prepared by the methods of [2,21], but using only an aqueous phase. Formulations were prepared at 65°C by mixing milliQ® water, gelatin, sorbitol, followed by addition of an oral suspension of 1 g/ml $BaSO_4$. The gelatin melted and a clear solution was formed; samples were then equilibrated without stirring at 65°C for 1h. The solution was then extruded into cold Miglyol 810® to form minispheres. Minispheres were kept in Miglyol 810® at 2-8°C for 30 min, separated with a sieve and allowed to dry at 2-8°C for 7 days. Additional minisphere prototypes loaded with sCT were prepared using a water-in-oil emulsion method [2]. The theoretical percentage of each component in four dried minisphere formulation prototypes is shown (Table 1). The typical diameter of dried minispheres was confirmed as 1–2 mm [2].

Table 1. Ratios by weight of components in four sCT-SmPill® minisphere prototypes.

Component	1. CA	2. NaTDC	3. C_{10}	4. CG
sCT	2.1	2.2	2.2	2.1
Transcutol HP®	19.8	14.9	16.4	14.2
Kolliphor EL®	8.5	6.4	7.0	6.1
Miglyol 810®	6.5	4.9	5.3	4.6

D-Sorbitol	5.4	4.0	3.9	3.2
Gelatin	51.4	42.0	40.1	38.7
CA	6.3	-	-	-
NaTDC	-	25.6	-	-
C10	-	-	25.1	-
CG	-	-	-	31.2

2.2 Coating of SmPill® minispheres

A two-step coating was the approach chosen for coating minispheres, with the first layer was made of Opadry® White in each case in order to protect the construct from moisture. The second layer was composed of either Eudragit® L30 D-55 (Eudragit®) to promote pH-dependent jejunal release [21], or a mixture of Surelease®/Pectin (ratio of 98:2) designed for colonic release [22]. Coating was carried out using bottom spray Wurster coating equipment (MFL.01 Bench top laboratory fluid bed unit, Freund-Vector Corporation, USA). Dried minispheres were weighed and dispensed in the Wurster tube, and the coating agent was then sprayed through the nozzle resulting in a spray pattern concurrent with air feed. Minispheres were accelerated in the spray and dried due to the hot air while continuing in an upward path, before falling back into the tube for respraying. Minispheres were allowed to dry inside the tube for 5 min. Minispheres coated with Eudragit® were further cured for 1 h at 40°C, while minispheres coated with Surelease®/Pectin were cured at 50°C, both yielding solid spheres with diameters of 1-2 mm. Table S.1 shows the parameters for minisphere-coated prototypes.

Table S. 1. Parameters used for coating SmPill® minispheres.

	Opadry® White	**Eudragit® L30 D-55**	**Surelease®/Pectin (98:2)**
Pump rate (g/min)	0.2	0.2	0.3
Nozzle air (psig)	25.8	25.8	25.8
Airflow (LPM)	197	197	197
Inlet Temperature (°C)	57.8 - 60	37.5 - 40	57.9 - 60
Exhaust Temperature (°C)	37.2 - 40	28.0 - 30	37.8 - 40

$BaSO_4$-SmPill® minispheres were prepared and coated with a first layer of Opadry® White, and split into two groups. Half were secondarily-coated with Eudragit® and the other half with Surelease®/Pectin. The final theoretical weight gain for each $BaSO_4$ formulation was respectively 8.2% Opadry White + 5.8% Eudragit® and 8.2% Opadry White + 11.5% Surelease®/Pectin. Precise concentrations of polymers added to the sCT-SmPill® minisphere surfaces to give a coating percentage gain are described (Table S. 2).

Table S. 2. Coatings of sCT- SmPill® minisphere prototypes.

Formulation	**Coating**
SmPill®-CA (j)	7.5% Opadry® White and 7.3% Eudragit®
SmPill®-NaTDC (j)	8.2% Opadry® White and 7.5% Eudragit®
SmPill®-CG (j)	7.9% Opadry® White and 7.0% Eudragit®
SmPill®-C10 (c)	7.0% Opadry® White and 10.8% Surelease®/Pectin
SmPill®-CG (c)	7.9% Opadry® White and 11.0% Surelease®/Pectin

(j) jejunum; (c) colon.

2.3 Radiological experiments

Rat *in vivo* experiments were approved by the Federal University of Rio Grande do Sul animal Ethics Committee (CEUA UFRGS) under project number 19969 and the Hospital de Clínicas de Porto Alegre animal Ethics Committee (CEUA HCPA) under protocol number 10,001. A restricted feed regimen was carried Out. The process consisted of feeding restricted to 4 h per day over 14 days. At day 1, rats had food access withdrawn at 1 pm. From day 2 to day 14, food was given to animals at 9 am and withdrawn at 1 pm; water was allowed *at libitum*. On day 15, each of six rats per group were first orally dosed with 1 ml of the iodine-based contrast agent, Ultravist® 300 (Schering AG, Germany) 20 min prior to minisphere administration. Three coated $BaSO_4$-SmPill® minispheres were given by oral gavage at 9 am to each rat using a syringe fitted with 1.5 mm diameter flexible tubing. Each minisphere was administered separately without water to avoid possible premature dissolution. Food was allowed 2 h after the beginning of experiments and water was allowed throughout. Anaesthesia was induced before each X-ray imaging process with isoflurane using a vaporising unit at a rate of 4 L/min mixed with 1 L/min O_2, and it was maintained by reducing the rate of isoflurane to 2 L/min. Optimal imaging conditions were achieved with X-ray beams

(Mediroll, Hungary) of 4 mAs and 44 kV. Anaesthetized rats were placed on a kappa board which is transparent to X-rays up to 5 cm above the film; the rat mouth and nose were fitted into a mask through which they continued to receive isoflurane at 1.5% concentration during imaging. X-rays were carried out at 30, 60, 120, 180 and 240 min after oral administration of minispheres coated with Eudragit®, and at 30, 60, 180, 240, 300, 360, 420 and 480 min for minispheres coated with Surelease®/Pectin. Between consecutive X-ray radiographs, rats were returned to their cages and allowed free access to water; food was allowed after 2 h of initial dosage. Rats were euthanized at the end of the experiment in a CO_2 chamber.

2.4 Analytical characterization of sCT-SmPill® coated minispheres

sCT-SmPill® formulations were analysed for loading by a content assay and for drug release by dissolution. For the content assay, a solution of 0.1 mg sCT/ml was used as a standard. Thus, a corresponding amount of minispheres were weighed out to 10 ml volumetric flasks to obtain a theoretical final concentration of 0.1 mg sCT/ml in solution. 5 ml of a 50:50 mixture of acetonitrile (ACN) and water was added to each flask and minispheres were sonicated for approximately 1 h at room temperature. After minispheres were completely dissolved, 5 ml of methanol (MeOH) was added to each flask. Solutions were filtered through a 0.45 µm filter and transferred to a vial. With regard to dissolution. Dissolution experiments were carried out in a dissolution tester (Vankel Industries, Inc), following the USP basket method and a standard solution containing 0.02 mg sCT/ml of was used as a reference. In order to use fewer minispheres, each vessel was only filled with 200 ml of media and sampling was made manually with a 1 ml pipette. Tests were carried out for 2 h in HCl (pH 1.0 M) to simulate the acidic stomach environment, and then medium was fully replaced by PBS (adjusted pH 6.8), in which dissolution was carried for further 6 h (for Eudragit® coating) or for 22 h (for Surelease®/Pectin coating). After sampling, solutions were filtered through a 0.45 µm filter and transferred to a vial. Analysis was carried out using the validated HPLC method for sCT [23]. The data was fitted to the empirical Weibull equation and to mathematical dependent models (Baker-Londsdale, Korsmeyer-Peppas, Hixson-Crowell, Higuchi and First order) [24] using the software SigmaPlot® (Systat Software Inc) version 12.5. With respect to HPLC analysis, a simplified validation of the method for the analysis of sCT released from minispheres was carried by means of

linearity, accuracy, precision, LOQ, LOD, range and specificity, based on [25]. The HPLC system consisted of a Waters Alliance e2695 separations module and a Waters 2489 UV/visible detector with Empower 2 software for data acquisition (Waters, Ireland). Separation of sCT was performed on a C_{18} reversed phase Kinetic column (3 x 100 mm ID, 2.6 µm particle size, 100 Å pore diameter, Phenomenex) under controlled temperature at 25°C, injecting 25 µl sample for assay or 70 µl for dissolution analysis. The flow rate was set at 0.4 ml/min and the linear gradient used was as follows (where A = 0.1% (v/v) TFA in water and B = 0.1% (v/v) TFA in acetonitrile): t =0 min, A:B (75:25, v/v); t = 0–5 min, A:B (75:25, v/v); t = 5–15 min, A:B (60:40, v/v); t = 15–17 min, A:B (60:40, v/v), t = 17–21 min, A:B (75:25, v/v), t = 21–23 min, A:B (75:25, v/v). The UV response was monitored at 215 nm.

2.5 sCT in vitro cyclic AMP bioactivity assay in T47D cells

T47D human breast cancer cells (passage 19 to 25) were maintained in RPMI-1640 culture medium containing 10% foetal calf serum, 1% Pen-Strip, and insulin (0.2 IU/ml). Cells were seeded on 24 well tissue culture plates at an initial density of 2.5×10^5 cells/well and incubated in humidified 37°C incubator with 5 % CO_2 in air for 24 hours. Media was removed and 0.5 ml of PBS pH 7.4 supplemented with the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, 0.2 mM) was added, plates were incubated at 37°C, 5 % CO_2 for 30 min. After removing PBS from pre-incubation, 1 ml of each treatment was added to well, and cells were incubated again at 37°C, 5 % CO_2 for 20 min. Cells were removed by adding 150 µl of trypsin to each well, and further 150µl of media to deactivate trypsin after cells had lifted off. Cells were transferred to Eppendorf tubes and centrifuged for 10 min at 10.000 rpm at 4°C. The pellet was then washed three times with cold PBS, and resuspended in 500µl of cell lysis buffer (from R&D cAMP kit). A freeze/thaw cycle was repeated twice to ensure cell lysis.

The theoretical loading of the four prototype formulations was approximately 2 mg sCT/100 mg minispheres (Table 1), and individual calculations were carried for prototypes in order to allow the same concentration of minisphere-released sCT to be exposed to T47D cells. Dilutions were carried in PBS supplemented with 0.2 mM IBMX to obtain a final concentration of 10 nM sCT to be incubated with cells. Native

sCT solution was prepared in PBS supplemented with 0.2 mM IBMX and added to T47D cells as a positive control. 0.2 mM IBMX in PBS was used as a control for basal levels of cAMP. Intracellular cAMP was analysed by a specific EIA (Parameter™ cAMP, R&D systems UK) following the manufacturer's protocol.

2.6 Stability of sCT-SmPill® coated minispheres at 5°C

sCT-SmPill® formulations were kept at 5°C ±3°C over 4 months and analysed each month by HPLC and by *in vitro* bioactivity in order to access the stability of sCT included in the formulations. With respect to HPLC analysis, a simplified validation of the method for the analysis of sCT released from minispheres was carried by means of linearity, accuracy, precision, LOQ, LOD, range and specificity, based on [26]. The HPLC system consisted of a Waters Alliance e2695 separations module and a Waters 2489 UV/visible detector with Empower 2 software for data acquisition (Waters, Ireland). Separation of sCT was performed on a C_{18} reversed phase Kinetic column (3 x 100 mm ID, 2.6 µm particle size, 100 Å pore diameter, Phenomenex) under controlled temperature at 25°C, injecting 25 µl sample for assay or 70 µl for dissolution analysis. The flow rate was set at 0.4 ml/min and the linear gradient used was as follows (where A = 0.1% (v/v) TFA in water and B = 0.1% (v/v) TFA in acetonitrile): t =0 min, A:B (75:25, v/v); t = 0–5 min, A:B (75:25, v/v); t = 5–15 min, A:B (60:40, v/v); t = 15–17 min, A:B (60:40, v/v), t = 17–21 min, A:B (75:25, v/v), t = 21–23 min, A:B (75:25, v/v). The UV response was monitored at 215 nm.

2.7 Rat intra-intestinal instillations

Male Wistar rats (Biotério Central UFRGS, Brazil) weighing 280-340g were housed under controlled environmental conditions with a 12:12 h light/dark cycle. Anaesthesia was induced as described above. Rats were euthanized at the end of experiments by overdosing with 0.5 ml of sodium thiopental. Absorption studies were carried out by instilling uncoated sCT-SmPill® minispheres by the i.j. or i.c. routes at dose levels of ~2000 I.U. (~390µg) sCT/kg, according to [2], with 6 rats per group Lower numbers of minispheres were instilled into each segment in the current study due to the higher loading achieved here compared to our previous instillation study [2].

2.8 Oral delivery of SmPill® minispheres to rats

~6000 I.U. (1.2 mg) sCT/kg rat was orally administered to rats which had undergone the training for restricted feeding. Six rats were used for each experimental group. At pre-determined time points, light anesthesia was induced as described above for approximately 2 min in order to carry out blood sampling. After sampling, rats were returned to cages to recover from anesthesia. For rats dosed orally with minispheres, blood samples were taken via retro-orbital vein puncture at 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0 and 8.0 h, .ie. 350 µl was taken at the first 6 time points, and 200 µl taken for the remaining three. Samples were centrifuged (6500 g, 5 min, 4ºC). The top plasma layer was transferred into fresh tubes and kept at -20ºC before analysis. Rats were euthanized at the end of experiments in a CO_2 chamber.

2.9 Serum calcium and sCT analysis

Serum samples were thawed, vortexed and centrifuged at 5,000 g at 4ºC for 5 min to remove excess lipid layers. 60-100 µl serum samples were transferred to micro-tubes fitted with 85 mm tubes. In some cases, 0.9% NaCl was used as a diluent to expand the sample sizes when less than 60 µl serum was available. A fully automated Labmax 240 clinical chemical analyser (Labtest Diagnóstica SA, Brazil) was used for total calcium measurement (Reagent reference number: 95, Labtest Diagnóstica SA, Brazil). sCT was measured in rat serum using an extraction-free sCT ELISA (Cat. S-1155, Peninsula Laboratories, USA) with a detection limit of 25 ng/ml [2]. Absolute biovailability (*F*) was calculated in order to compare the the value (estimated from the area under the curve, AUC) obtained from extravascular delivery of sCT-SmPill® with the value following intravenous (i.v.) dosing of sCT [26]. The relative *F* measured the bioavailability of sCT-SmPill® formulation compared to the bioavailability of native sCT delivered by the same extravascular route.

2.10 Statistics

Statistical analysis was carried out using Prism-5® software (GraphPad®, USA). Unpaired Student's t-tests, ANOVA one-way repeated measurements, or two-way ANOVA with Dunnett's post-test were used as stated. Results are expressed as the mean ± SEM. A significant difference was considered to be present if $P<0.05$.

3. Results

3.1 Radiological experiments with barium-loaded minispheres in rats

Three minispheres coated with either Opadry® white and Eudragit® or with Opadry® white and Surelease®/Pectin were orally administered to rats on day 15 following the feeding protocol. For both formulations there was low inter-rat variability in terms of stomach- emptying time for the three minispheres. Five out of six rats in each group had no minispheres in the stomach after 2 h. Rats administered three Eudragit®-coated minispheres emptied them from the stomach after 1 h, and these minispheres dissolved only after they reached the small intestine, confirming jejunal delivery (Fig. 1).

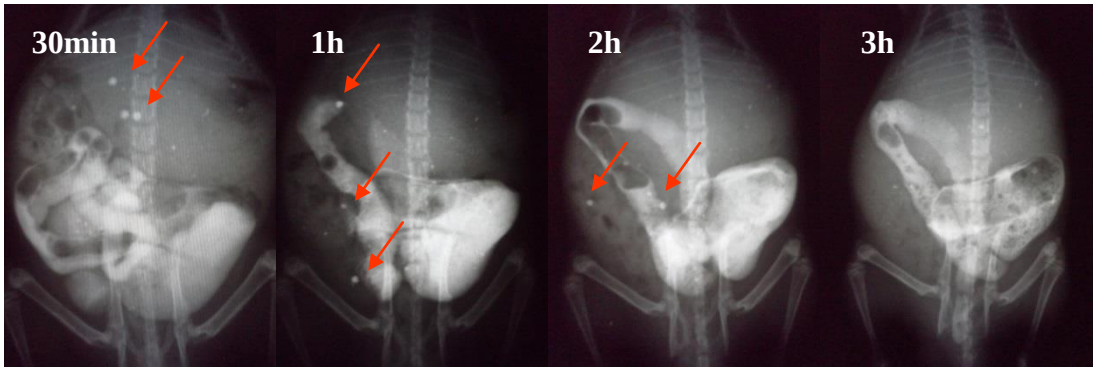
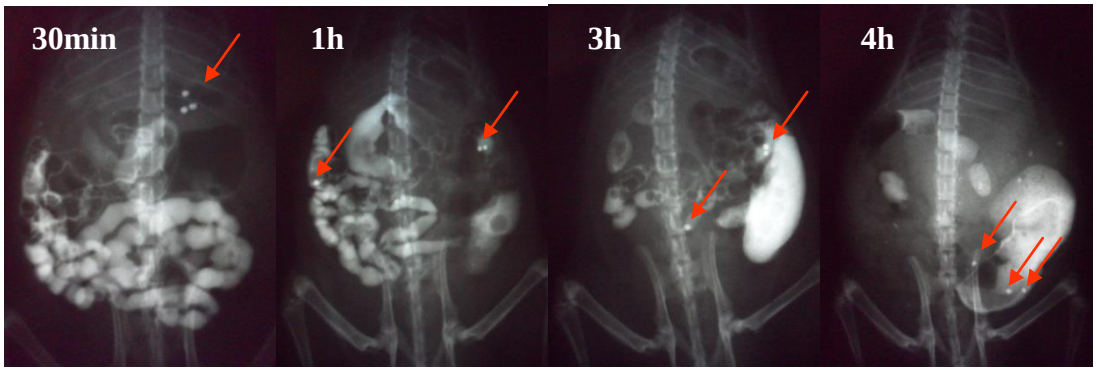


Fig. 1. Representative X-ray images of the GI tract of a rat showing the movement of three $BaSO_4$–loaded minispheres coated with Opadry® white and Eudragit®. 30 min after gavage: three minispheres in the stomach; 1 h: three in the small intestine; 2 h: two in the small intestine; 3 h: none detected in the small intestine.

Rats which received three Surelease®/Pectin-coated minispheres also showed consistency regarding gastric emptying. These minispheres achieved colonic delivery and dissolved at ~7 h (Fig. 2).



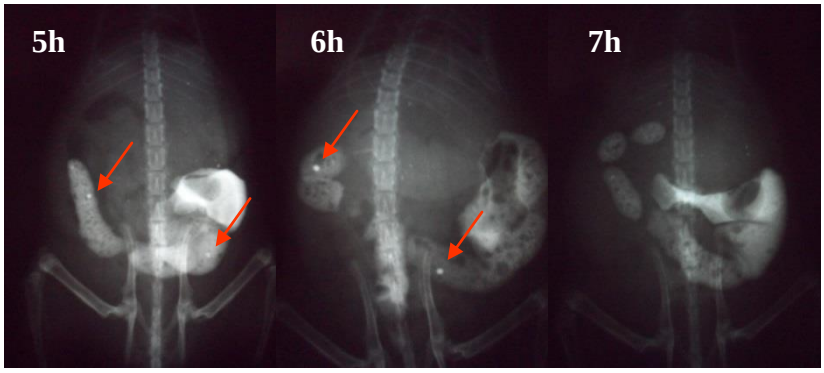


Fig. 2. Representative X-ray images of the GI tract of a rat showing the movement of the three $BaSO_4$–loaded minispheres coated with Opadry® white and Surelease®/Pectin. 30 min after gavage: three minispheres in the stomach; 1 h: two minispheres in the stomach and one in the small intestine; 3 h: one minisphere in the small intestine and two at the entrance of the caecum; 4 h: two minispheres in the caecum and one in the entrance of the colon; 5 h: one minisphere in the caecum and one in the colon; 6 h: two minispheres in the colon; 7h: no minispheres detected in colon.

The X-ray data therefore suggests that minispheres coated with Opadry® white and either Eudragit® or Surelease®/Pectin can be orally administered to rats and will dissolve in rat jejunum or colon respectively and may be suitable for achieving regional sCT release.

3.2 Analytical characterization of sCT-SmPill® coated minispheres

The theoretical concentration of sCT in minisphere prototypes was slightly different from the concentration assayed by HPLC. This was probably due to variability in the formulation process associated with some sCT degradation. sCT-SmPill® was prepared by adding a solution of sCT, prepared just before mixing to the previously-formed emulsion, and then a theoretical concentration of sCT per gram of minispheres was calculated. All sCT-SmPill® minispheres were analysed for loading with same standard sCT solution of 0.1 mg/ml as a reference. Thus, a corresponding amount of minispheres were weighed out to obtain a theoretical final concentration of 0.1 mg/ml of sCT in solution (according to the theoretical potency described in Table 1). However, in the case of formulations containing CG and C_{10}, the actual sCT concentrations were > 0.1 mg/ml. Table S. 3 shows concentrations of sCT for each sCT-SmPill® prototype before and after final coatings with either Eudragit® or Surelease®/Pectin. Coated minispheres

had higher sCT concentrations compared to their respective uncoated core. Therefore, it is likely that coatings provide protection to the sCT present in the core, in addition to the protection already provided by gelatin [2].

Table S. 3. Loading of sCT in SmPill® formulations with selected PEs for uncoated and coated minispheres designed for jejunal or colonic release. Results are given as the mean theoretical concentration of sCT (%), n = 3 independent formulations.

Formulation	**sCT Assay**	
	Mean %	**RSD%**
SmPill®-CA (uncoated)	81.0	0.3
SmPill®-CG (uncoated)	106.7	5.3
SmPill®-NaTDC (uncoated)	89.4	10.0
SmPill®-C_{10} (uncoated)	93.3	2.5
SmPill®-CA (i.j. coated)	90.8	1.2
SmPill®-CG (i.j., coated)	115.4	0.4
SmPill®-NaTDC (i.j., coated)	98.0	2.4
SmPill®-CG (i.c., coated)	121.3	2.6
SmPill®-C10 (i.c., coated)	103.9	0.6

* % RSD: Relative standard deviation = (SD/Mean)*100. Initial coatings in each case were Opadry® white. i.j. (Jejunum; Eudragit®); i.c. (colon; Surelease®/Pectin).

Dissolution of minispheres was first assessed in HCl (pH 1,0) for 2 h and then the minispheres were transferred to a modified PBS (pH 6.8) in order to simulate the pH change from dosages passing from the stomach to the small/large intestine. Minispheres with Eudragit® coatings remained intact at acid pH, whereas they released all sCT within 3.5 h at pH 6.8 (Fig. 3A), confirming the potential of this coating to release sCT in jejunum. Similarly, minispheres coated with Surelease®/Pectin released no sCT at pH 1.0, but released all the sCT following a 24 h at pH 6.8 (Fig. 3B), thereby demonstrating delayed release and potential to release in the colon (Table 3).

For formulations coated with Eudragit®, the lag time (t_0) for release was approximately 2.4 h to release 50% of sCT. Eudragit®-coated minispheres loaded with CG began however, to release more quickly according to the rate parameter (k) (Table 2). Regarding formulations designed for colonic delivery, times (t_0) to release 50% of sCT were determined as 4.6 and 7.2 h respectively for minispheres containing C_{10} and CG and coated with the same surface coating weight of Surelease®/Pectin, indicating that

the minisphere core is likely to contribute to the delayed release of sCT. According to X-ray imaging, Surelease®/Pectin-coated minispheres reached and dissolved in rat colon at ~7 h. The β parameter characterizes the curve according to the shape; for SmPill®-NaTDC (j) and SmPill®-CG (c), β was < 1 characteristic of parabolic curves. For the remaining formulations, β was > 1, characteristic of sigmoidal curves [27]. Fraction dissolved values (D %) in Table 2 were in accordance with analytical loading data.

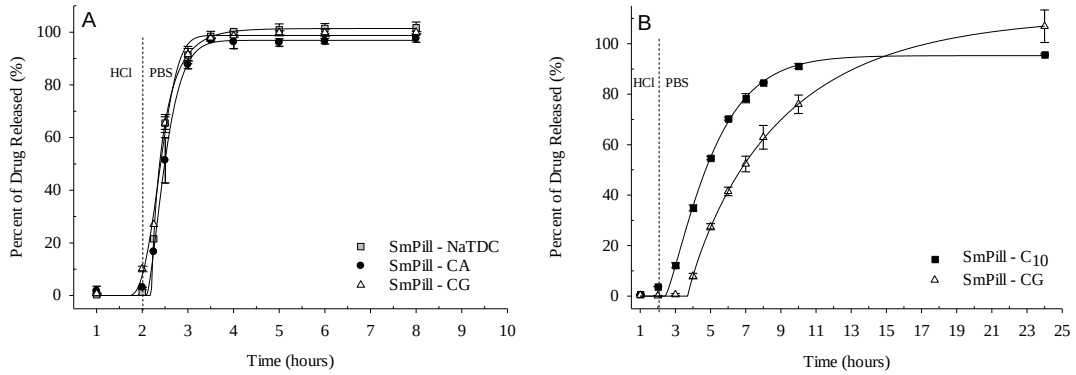


Fig. 3. Dissolution profiles of sCT-SmPill® minispheres with coatings designed for (A) jejunal release (SmPill®-NaTDC, SmPill®-CG, SmPill®-CA) and (B) colonic release (SmPill®-C_{10}, SmPill®-CG). Release was carried for 2 h at pH 1.0 and for 6 h at pH 6.8 (A) or for 22 h at pH 6..8 (B). n=2 for each time point, results are mean ± SD. The Weibull function for each formulation was fitted.

Table 2. Parameters obtained by fitting minisphere sCT release profiles to Weibull equation.

Formulation	**t_0 (min)**	**k**	**β**	**D (%)**	**r^2**
SmPill®-CA (j)	2.48(0.01)	0.495(0.06)	1.45(0.23)	96.91(0.78)	0.99
SmPill®-CG (j)	2.39(0.02)	0.086(0.02)	2.55(1.04)	98.76(1.37)	0.99
SmPill®-NaTDC (j)	2.38(0.00)	0.287(0.01)	0.82(0.03)	101.40(0.32)	0.99
SmPill®-CG (c)	7.25(0.06)	5.341(0.03)	0.89(0.02)	111.00(1.00)	0.99
SmPill®-C_{10} (c)	4.59(0.07)	2.908(0.02)	1.23(0.01)	95.27(1.57)	0.99

t_0: dissolution lag-time; k: rate parameter; β: shape parameter; D: fraction dissolved as $t\rightarrow\infty$. j (Jejunum; Eudragit®); c (colon; Surelease®/Pectin). Numbers in brackets (SEM).

Weibull fitting is an empirical model, so it only estimates dissolution kinetics [27]. Five model-dependent approaches were chosen to compare release profiles of sCT from minispheres. Only selected portions of the sCT-SmPill® dissolution data were used,

depending on the formulation. The objective in selecting data points was to include only data which represented the dynamics of the dissolution process, discarding time points at plateau regions. The procedure to compare two dissolution profiles by a model-dependent approach relied on correlation coefficients. The Korsmeyer-Peppas model presented r^2 values close to 0.9 (Table 3) for all dissolutions profiles.

Table 3. Correlation coefficients (r^2) obtained by fitting sCT-SmPill® release profiles to Baker-Londsdale, Korsmeyer-Peppas, Hixson-Crowell, Higuchi and First order mathematical models.

Formulation	Baker-Londsdale	Korsmeyer-Peppas	Hixson-Crowell	Higuchi	First order
SmPill®-CA (j)	0.223706	0.939786	0.382261	0.244878	0.359747
SmPill®-CG (j)	0.272224	0.922456	0.454609	0.303852	0.420412
SmPill® -NaTDC (j)	0.229409	0.883694	0.387453	0.254312	0.362252
SmPill®-CG (c)	0.499999	0.941460	0.763840	0.540438	0.732105
SmPill®-C10 (c)	0.602362	0.914152	0.845734	0.675845	0.801988

j (Jejunum; Eudragit®); c (colon; Surelease®/Pectin).

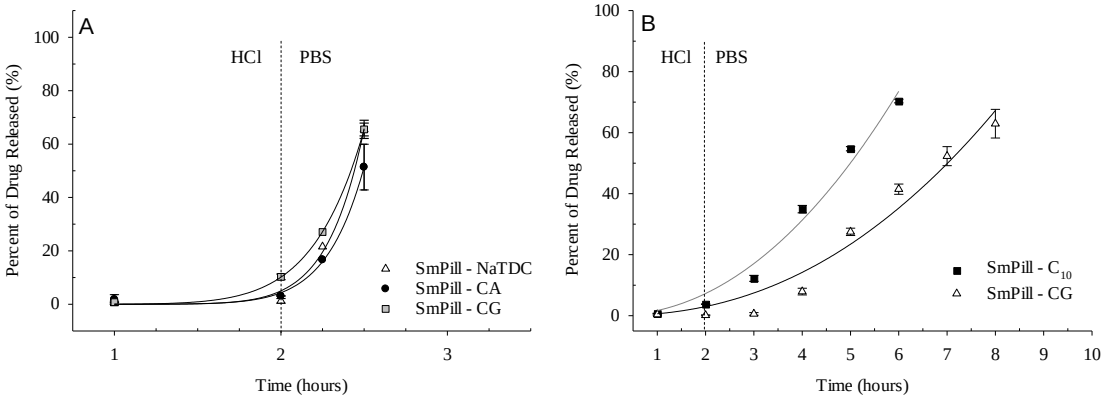
Korsmeyer-Peppas is a simple relationship which described drug release from polymeric systems which relates a fraction of drug released (M_t/M_∞) with the release rate constant (k) at a certain time (t) and the release exponent (n) [28]. In this model, the value of n characterizes the release mechanism of the drug. To calculate this value, only the portion of the release curve where $M_t/ M_\infty < 0.6$ was used [29]. Thus, new fitting of the data was carried out to obtain the parameters for the Korsmeyer-Peppas equation (Table 4, Suppl. Fig 1). Since $n > 0.85$, the mechanism which controlled the release of sCT from minispheres was the 'super case II transport' [30]. The dominant mechanism for sCT release from coated minispheres containing PEs was therefore diffusion through the coatings due to swelling of the gelatin and the polymeric matrix upon contact with dissolution media.

Table 4. Parameters (SEM) obtained by fitting release profiles to Korsmeyer-Peppas model.

Formulation	Korsmeyer-Peppas		
	k	n	r^2

SmPill®-CA (j)	0.019 (0.001)	11.120(0.085)	0.996887
SmPill®-CG (j)	0.031 (0.004)	8.357 (0.167)	0.999761
SmPill® -NaTDC (j)	0.020 (0.002)	11.530 (1.350)	0.993407
SmPill®-CG (c)	0.632 (0.000)	2.244 (0.000)	0.999999
SmPill®-C_{10} (c)	1.687 (0.715)	2.107 (0.252)	0.980186

j (Jejunum; Eudragit®); c (colon; Surelease®/Pectin). Numbers in brackets (SEM).



Suppl. Fig 1. Dissolution of sCT-SmPill® minispheres with coatings designed for (A) jejunal release (SmPill®-NaTDC, SmPill®-CG, SmPill®-CA) and (B) colonic release (SmPill®-C_{10}, SmPill®-CG). Release was carried out initially for 2 h in HCl pH 1,0 and then in PBS pH 6.8 for a further 8 h (A) or 22 h (B). n=2 for each time point, results are mean±SD. The Korsmeyer-Peppas function is plotted. j (Jejunum; Eudragit®); c (colon; Surelease®/Pectin).

3.3 Stability of sCT-SmPill® coated minispheres

sCT-SmPill® minispheres were kept at 5±3°C and monitored over 4 months in order to assess sCT stability. At each time point, minispheres were dissolved and released sCT was analysed by HPLC and by bioassay on T47D cells. The concentration of sCT in minispheres stored at 5°C had less than a 20% difference after 4 months for all formulations, except sCT-SmPill® CG (j), which had a 40% reduction after month 2 (Table 5A). This data suggests, that at the specific ratio between sCT and CG (1:14.9), there is some level of peptide degradation possibly caused by CG in the Eudragit®-coated formulation. Nevertheless, Surelease®/Pectin seems to prevent sCT degradation for the formulation containing CG. Although a clear pattern such as that obtained by HPLC analysis was not identified for T47D cells, sCT released from minispheres

retained > 85% of biological activity after 4 months at 5°C (Table 5B). Overall, both types of analysis indicated good sCT stability in coated minispheres. Although a clear pattern such as obtained by HPLC analysis was not identified for T47D cells, sCT released from minispheres retained > 85% of biological activity after 4 months at 4°C (Table 5B).

Table 5. Assay of sCT released from SmPill® minispheres over 4 months (M) at 4°C.

A. Formulation	**M 0**	**M 1**	**M 2**	**M 3**	**M 4**
SmPill®-CA (j)	90.8±1.2	90.8±1.1	93.5±0.2	84.4±1.0	80.3±0.6
SmPill®-CG (j)	115.4±0.4	108.4±0	75.9±8.3	74.8±4.3	73.6±5.2
SmPill®-NaTDC (j)	98.0±2.4	96.7±0.5	92.5±3.9	86.2±0.3	84.0±3.2
SmPill®-CG (c)	121.3±2.6	117.9±5.2	106.6±0.9	112.5±5.1	104.4±6.0
SmPill®-C_{10} (c)	103.9±0.6	98.3±0.3	96.8±0.6	88.2±0.5	84.5±2.9

B. Formulation	**M 0**	**M 1**	**M 2**	**M 3**	**M 4**
SmPill®-CA (j)	92.8±9.2	102.6±5.5	103.9±24.9	86.1±2.7	94.3±4.6
SmPill®-CG (j)	91.6±2.2	92.0±8.9	102.8±5.3	83.6±11.4	102.4±12.8
SmPill®-NaTDC (j)	97.0±12.5	99.5±10.7	108.7±14.0	90.2±10.7	94.1±6.8
SmPill®-CG (c)	108.4±10.2	90.0±2.8	99.8±7.0	84.8±8.6	93.0±2.9
SmPill®-C_{10} (c)	110.4±7.4	104.4±11.4	92.0±12.7	84.8±8.0	96.4±9.8

Results are percentage of the theoretical amount of sCT added to minispheres compared to standards and analysed by HPLC (A) or by in vitro bioassay (B). Concentrations of 10 nM of sCT were added on T47D cells in each case and were compared to native sCT to calculate percentage. Values are given as mean ± %RSD, n=2-3.

3.4 Intestinal instillations of uncoated sCT-SmPill® minispheres to rats

Intra-intestinal instillations of the uncoated sCT-SmPill® minispheres were carried out in order to obtain PK and PD profiles of formulations (Table 6, Fig.4). Minispheres were loaded with ~2% of sCT (w/w). The relative *F* of sCT-SmPill® minispheres to native sCT by i.j. instillation was 2.1%, 1.4% and 1.2% for minispheres containing NaTDC, CG, and CA respectively. Although the presence of NaTDC yielded the maximal AUC, hypocalcaemia was more pronounced for minispheres containing CG (Fig. 4). This may be related to the fact that sCT released from minispheres containing CG had a longer $t_{1/2}$ compared to those containing NaTDC (Table 6). Uncoated minispheres containing C_{10} and CG gave absolute *F* values of 18.2±1.8% and 17.3±1.9% respectively when instilled by the i.c. route, an increase of 2.6 and 2.5 fold

over native sCT. Hypocalcaemia proved that sCT delivered from each minisphere prototype was biologically active (Fig 4).

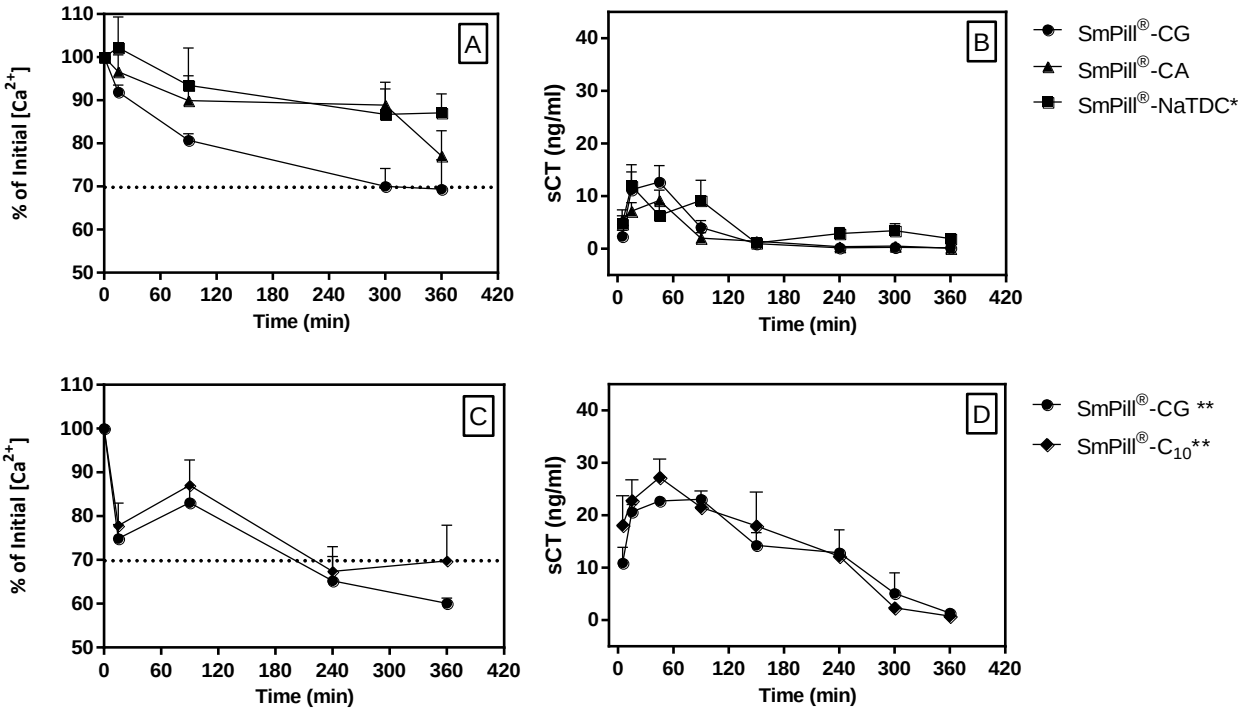


Fig. 4 (A) Serum calcium and (B) serum sCT concentrations after i.j. instillation of sCT-SmPill® minispheres containing ~2000 I.U. sCT/kg with either CG (15 µmol/kg), CA (6 µmol/kg) or NaTDC (9 µmol/kg). (C) Serum calcium and (D) serum sCT concentrations after i.c. instillation of minispheres containing ~2000 I.U. sCT/kg and CG (15 µmol/kg), CA (6 µmol/kg) or C_{10} (23 µmol/kg). Results are mean±SEM, n=5-6. *P < 0.05 and ** P < 0.01 compared to native sCT instilled by the same route, presented in Table 6 (one way ANOVA for *F* and $AUC_{0–360}$ min, post-Dunnett's test).

Table 6. PK parameters of instillations of uncoated minispheres over 6 h.

Sample	C_{max} (ng/ml)	T_{max} (min)	$t_{1/2}$ (min)	$AUC_{(0-\infty)}$ (min.ng/ml)	Absolute $F_{(0-\infty)}$* (%)
sCT (i.j.)	11.7±2.8	5	59.5±4.8	732±257	2.6±0.9
SmPill®-NaTDC (i.j.)	11.9±1.1	15	101.4±5.4	1548±268	5.5±1.0*
SmPill®-CG (i.j.)	12.7±2.6	45	252.2±20.0	1027±211	3.7±0.8
SmPill®-CA (i.j.)	8.2±2.2	45	51.7±6.7	747±101	2.7±0.4
sCT (i.c.)	26.1±5.6	5	42.8±3.2	1974 ±431	7.0±1.5
SmPill®-CG (i.c.)	23.0±1.2	90	38.7±3.5	4878±538	17.3±1.9**
SmPill®-C_{10} (i.c.)	27.2±2.6	45	79.7±7.3	5131±493	18.2±1.8**

sCT-SmPill® minispheres contained ~2000 I.U. sCT/kg co-formulated with NaTDC (9 µmol/kg), C_{10} (23 µmol/kg), CG (15 µmol/kg) or CA (6 µmol/kg). 2 ml saline/kg or 2000 I.U. sCT solution/kg, were dosed to controls. Absolute *F* of intra-jejunal (i.j.) or intra-colonic (i.c.) instillations was calculated relative to an i.v. dose of 800 I.U. sCT/kg, $AUC_{(0-\infty)}$ = 11261±900 min.ng/ml. *P < 0.05 and **P < 0.01 compared to native sCT instilled by the same route (one-way ANOVA for *F* and AUC_{0-360} min, post-Dunnett's test).

3.5 Oral gavage of sCT-SmPill® coated minispheres to rats

Selected prototype sCT-SmPill®-coated formulations administered orally to rats revealed oral *F* values ranging from 0.5±0.1 to 2.7±0.6%**,** much lower than for instillations. Rats receiving minispheres coated with Eudragit® presented, in general, higher C_{max} values compared to minispheres coated with Surelease®/Pectin (Fig. 5). Native sCT solution gave an absolute *F* of 0.9±0.1. T_{max} values were longer for all coated minispheres compared with native sCT (P<0.05, Table 7), likely due to the lag time for release of sCT. SmPill®-CG coated with Eudragit® increased serum sCT concentrations compared to native sCT (P<0.05). For sCT-SmPill® minispheres coated with Eudragit®, the T_{max} varied from 2.5 to 3 h, in accordance with the dissolution and X-ray imaging data. The maximum hypocalcaemic effect was observed at 2 h for minispheres containing CG and CA, and at 4 h for minispheres containing NaTDC. Here, no clear correlation between PD and PK data could be observed, and the only clear conclusion is that serum calcium decreases indicate that the sCT from formulations which entered rat circulation is biologically active.

The T_{max} values of sCT released from orally-administered minispheres coated with Surelease®/Pectin were 3 h and 5 h for formulations containing C_{10} and CG respectively (Table 7), suggesting successful colonic delivery and subsequent absorption of sCT. Overall, sCT serum concentrations were lower for colonically-released formulations than for jejunal (Fig. 5). sCT-SmPill®-C_{10} still yielded 1.7±0.5% *F* after i.c. release, but this was not statistically increased compared to native sCT. Even though formulations containing CG coated with Surelease®/Pectin showed 0.5±0.1% absolute *F*, all formulations led to reduction in serum calcium concentration.

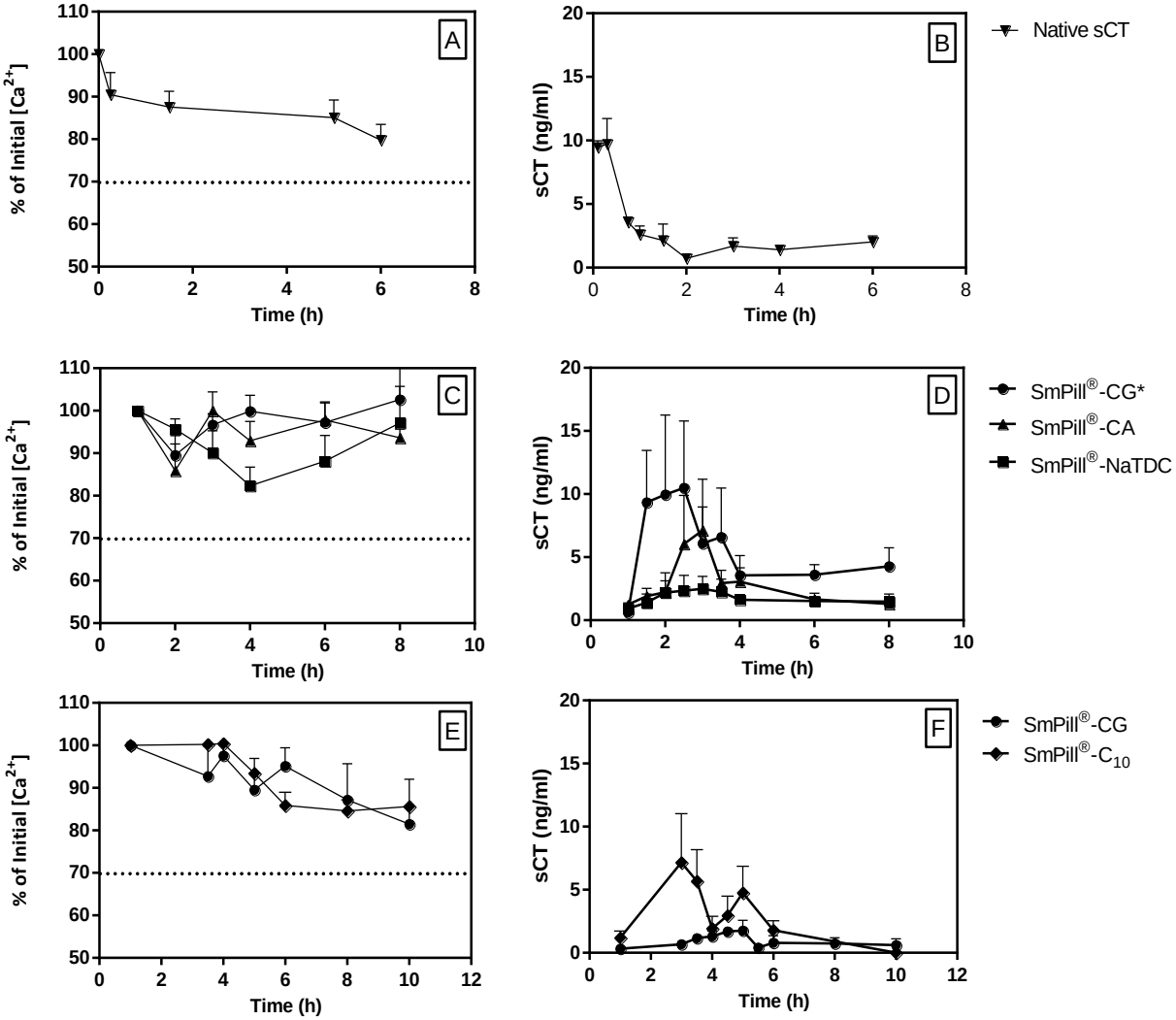


Fig. 5. (A) Serum calcium and (B) serum sCT concentrations after oral gavage of ~6000 I.U. sCT solution/kg. (C) Serum calcium and (D) serum sCT concentrations after oral administration of sCT-SmPill® minispheres coated with Eudragit® and containing ~6000 I.U. of SCT/kg combined with CA (18 μmol/kg), NaTDC (26 μmol/kg) or CG (45 μmol/kg). (E) Serum calcium and (F) serum sCT concentrations after oral administration of sCT-SmPill® minispheres coated with Surelease®/Pectin containing ~6000 I.U. sCT/kg and C_{10} (69 μmol/kg) or CG (45 μmol/kg). Results are mean ± SEM, n=5-6. *P < 0.05 compared to native sCT (one way ANOVA for *F* and $AUC_{0–360}$ min, post-Dunnett's test).

Table 7. PK parameters of oral administration native sCT or sCT-SmPill® coated minispheres to rats.

Sample **Oral delivery**	**C_{max}** **(ng/ml)**	**T_{max}** **(min)**	**$t_{1/2}$** **(min)**	**$AUC_{(0-\infty)}$** **(min.ng/ml)**	**Absolute $F_{(0-\infty)}$** **(%)**
sCT oral	10.0±2.8	20	132.2±6.3	781±94	0.9±0.1
SmPill®-NaTDC (j)	2.5±1.5	180	144.9±9.9	728±207	0.9±0.2
SmPill® -CG (j)	10.5±3.4	150	243.3±13.9	2239±466	2.7±0.6*
SmPill® -CA (j)	7.1±3.1	180	469.8±20.7	1131±308	1.4±0.4
SmPill® -C_{10} (c)	7.1±2.9	180	172.9±5.6	1389±415	1.7±0.5
SmPill®-CG (c)	1.7±1.4	300	147.9±11.4	374±69	0.5±0.1

Doses were ~6000 I.U. native sCT/kg, or sCT-SmPill® coated minispheres designed for jejunal (j; Eudragit®) or colonic (c; Surelease®/Pectin) release containing ~6000 I.U. sCT/kg combined with CA (18 μmol/kg), NaTDC (26 μmol/kg), C10 (69 μmol/kg) or CG (45 μmol/kg). * $P<0.05$ compared to native sCT (one-way ANOVA, post Dunnet's test). Absolute *F* was calculated relative to an i.v. dose of 800 I.U. sCT/kg, $AUC_{(0-\infty)}$ = 11261±900 min.ng/ml.

4. Discussion

In order to develop an effective formulation for oral sCT delivery, SmPill® minispheres were prepared including selected PEs or a pH-modifying agent (CA) and then coated for release in rat jejunum or colon. *In situ* instillation studies in rats confirmed that NaTDC, C_{10} and CG were effective as PEs in uncoated sCT- SmPill® minispheres for intestinal delivery of sCT either in rat jejunal or colonic segments. Appropriate coatings were used to control the release of sCT from minispheres following gavage. Firstly, X-ray was used to investigate the segments of the rat GI in which minispheres would dissolve. Techniques described on the literature for tracking of solid oral dosage forms in rodents include gamma scintigraphy [31], magnetic resonance imaging (MRI) [32], positron emission tomography (PET) [33], micro-computerized tomography (μCT) [34] and X-ray [35]. The latter was simple, cheap and offers simultaneous visualization of both capsule and the GI tract using contrast agents [35]. The passage of coated minispheres through the GI tract confirmed minisphere dissolution at the intended sites. This is a novel use of X-rays to track minisphere location and dissolution in rats for such a technology.

Gastric emptying issues are a major problem when working with oral solid dosage forms in rats and, along with the difficulty in making scaled-down formulations, these

may account for the low number of such studies. Enteric-coated gelatin capsules of approximately 2.5 mm diameter were emptied from rat stomach between 2 and 8 h, but there was also high variability [36]. With the help of the pro-kinetic, metoclopramide, those capsules exited rat stomach earlier and more consistently [36]. Others demonstrated that 14 days of a fixed feeding regimen to rats led to an accelerated solid gastric emptying due to presence of inter-digestive gastric contractions, and also due to an increase in their maximal amplitude [37]. Plasma ghrelin levels, which regulate the occurrence of stomach contractions in rats, reached its plasma C_{max} 30 min before feeding [37]. We hypothesised that a similar fixed-fed regimen would promote less variable gastric emptying of SmPill® minispheres from the rat stomach.

Here, X-ray imaging showed consistent gastric emptying of three minispheres in rats with the 4 h feeding regimen, and confirmed the suitability of the selected coatings for regional delivery of sCT to jejunum and colon respectively. Minispheres coated with Eudragit® L30 D55 started to dissolve and to release $BaSO_4$ as soon as the pH increased. According to *in vitro* dissolution tests, the lag time after increasing the pH from 1.0 to 6.8 is approximately 30 min to detect 50% of sCT released, which reinforces the hypothesis that this formulation is suitable for jejunal release. Surelease®/Pectin films combine the colon-specific degradation properties of pectin with the protective properties of the water-insoluble polymer, ethyl cellulose [38, 39]. Minispheres coated with Surelease®/Pectin were detected in the rat colon by X-ray imaging just before disintegrating, demonstrating the suitability of this formulation for colonic release of sCT. Even though rat colonic microflora is not able to digest pectin (unlike in man), pectin is still resistant to proteases active in the upper GI tract of both species [40]. In addition, pectin is retained in the coating layer absorbing surrounding water, and swells to form water channels and facilitates dissolution.

In vitro release of sCT from minispheres was modelled with the Korsmeyer-Peppas equation, yielding an apparent diffusional exponent (n) > 0.85, which represents a swelling-controlled release system [30]. In these systems, the drug is dispersed within a glassy polymer, gelatin, in the SmPill®. As the fluid enters gelatin, the glass transition of the gelatin is lowered allowing for relaxation of the macromolecular chains. sCT is able to diffuse out of the swollen area of the minisphere, but no diffusion occurs through

the glassy polymer phase. The rate of drug release in this model is controlled by the velocity and position of the front dividing the glassy and rubbery portion of the polymer. Various drug delivery system can be modelled with Korsmeyer-Peppas kinetic model, for example the release of chondroitin sulphate from the hydrophilic HPMC tablets [41], and theophylline released from wax matrix granules [42]. Overall, the Korsmeyer-Peppas model suggests that biological fluids induce swelling of coating layers and gelatin, channels are formed, which allow sCT to be released from minispheres. This provides mechanistic insight into how the minispheres may release sCT in biological media.

Evidence that the biological activity of sCT was retained after it was incorporated into SmPill® was indicated by the hypocalcaemia observed in rats after intra-intestinal instillations of uncoated minispheres. It has been repeatedly demonstrated, that reductions in serum calcium in response to sCT administered by i.v., intestinal instillations and the oral route in rats achieved a highly reproducible and maximum plateau of approximately 70% of basal level [2,43, 44]. Additional indications that the SmPill®-released sCT retained biological activity was obtained *in vitro* using the cAMP cell bioassay [45], which confirmed that the formulation process was mild [2]. This assay was also used in combination with HPLC assay to demonstrate four months stability for coated sCT-SmPill® minispheres kept at 5±3°C. This is an advantage for the SmPill® process, since most literature suggests that sCT in aqueous solution is labile [46]. Furthermore, it suggests that this mild and scalable technology may be suitable for other peptides in addition to sCT.

To compensate for dilution in the GI tract, oral sCT dose levels need to be much higher than that for intra-intestinal instillations [47]. Another reason to increase the dose for oral gavage to 6000 I.U./kg versus 2000 I.U./kg for instillations was because sCT metabolism in the small intestinal lumen is dependent upon concentration [48]. It was necessary to increase the concentration of sCT in contact with the intestinal epithelium, but without increasing the number of minispheres administered, which was impractical and could reduce gastric emptying. Jejunal (i.j.) and colonic (i.c.) instillation of coated sCT-SmPill® minispheres confirmed the similarity between PK and PD profiles of these formulations containing ~2% of sCT (w/w) with profiles obtained in previous work of

formulations containing 0.4% (w/w) of sCT [2]. As a corollary, the concentration of NaTDC, C_{10}, CG and CA combined with sCT had to be decreased in relation to previous formulations [2] in order to allow spherical minispheres to be formed. Oral delivery of three sCT-SmPill® minisphere prototypes resulted in an enhancement in bioavailability compared to native sCT solution given by oral gavage. The best relative *F* achieved for oral delivery of coated sCT-SmPill® minispheres was 2.7% ($P<0.05$) for SmPill®-CG (j) versus sCT solution. In addition, hypocalcaemia confirmed the biological activity of released sCT from each orally-administered minisphere prototype.

A plausible explanation for lower absolute *F* values by oral gavage compared to those from intra-intestinal instillations for equivalent minisphere, is that according to *in vitro* dissolution data, the total concentration of sCT released *in vivo* at the time of C_{max} on PK profiles was likely to be lower than 100% (at a dose of 6000 I.U. sCT/kg following oral gavage), and this will increase the variability of absorption. For jejunal release of sCT from SmPill® minispheres, the concentration of sCT available was likely to be close to 6000 I.U./rat, which may explain the better performance of these formulations *in vivo* than formulations designed for colonic delivery of sCT. Some have shown that *in vivo* release of minispheres coated with Surelease®/Pectin might be faster than *in vitro* [49]. However, for colonic minisphere formulations, *in vitro* dissolution profiles showed that there was a lag time of 5 - 7 h for 50% of sCT to be released, suggesting that not all sCT loaded in the minispheres was available at the time that C_{max} was reached. Additionally, it was observed that rats dosed with minispheres designed for colonic delivery excreted any food remaining in the GI tract at the end of experiments. This was probably because rats in a fixed-feeding regimen were fasted for almost 20 h prior to experiments, but food was then allowed within 2 h of dosage. This had no implication for jejunally-released minispheres, but it may have caused an interaction with colonic release.

In addition, there is a possibility that the restricted feed regimen used to rats orally dosed with SmPill® improved the gastric emptying of minispheres, but also may have increased the proteolytic enzymes activity. Digestive enzymes can adapt to a specific diet in terms of nutrient intake [50]. Moreover, circadian rhythms of specific digestive enzymes in the small intestine of rats were mainly affected by anticipatory period when

rats expect to be fed other than the food intake properly [51]. Anticipatory activity effect was also observed in rats included in a restricted feeding schedule of 2 h daily over 21 days. In our study, we did not analyse proteolytic enzymes profile of rats included in the fixed feed protocol. However, assuming that minispheres were administered at the time that rats expected to be fed, the anticipatory activity may have increased the proteolytic activity in the small intestine, leading to increased degradation of sCT dosed orally compared to intra-intestinal instillations. Further investigation is likely to be performed in order to verify enzyme activity using the specific fixed-feed protocol of this work**.** Finally, we envisage further gavage studies with refined minispheres: here, three large minispheres were administered by oral gavage per rat, whereas smaller diameter minispheres with lower loading could permit administration of higher numbers overall, perhaps paving the way for a multi-particulate delivery system.

In summary, the PK results indicated that systemic absorption of sCT was successfully achieved across jejunal and colonic segments from oral delivery of coated SmPill® minispheres to rats. Regarding formulations designed for jejunal release, the bioavailability data suggests that use of CG as a PE yielded a positive effect on sCT jejunal absorption. Whereas for coated minispheres intended for colonic release of sCT, C_{10} inclusion led to oral *F* of sCT (1.7±0.5%), but this was not statistically increased over control. It is important to note that marketed nasal versions of peptides including sCT are estimated to have absolute *F* of ~1% [52]. To our knowledge there are just a few examples in the literature showing successful oral delivery of sCT to rats for regional absorption using solid dosage forms. Enteric-coated capsules containing sCT and CA administered by gavage to rats achieved *F* ranging from just 1.2-1.8%, depending on the amount of CA added [53], similar to the 1.4% seen here for SmPill®-CA minispheres coated with Eudragit®. Furthermore, sCT entrapped in PHEA-graft-polymethacrylate aggregates, enabled a C_{max} of 58 ng/ml to be detected following oral gavage to in rats at similar dose levels to the current study [54]. Regarding studies with humans, absolute bioavailabilities ranging from 0.5 to 1.4% were achieved in a Phase I trial using a tablet containing sCT and a caprylic acid derivative (Eligen® technology, Emisphere, USA) [55]. Two other Phase I trials for enteric-coated preparations combining sCT with CA and either taurodeoxycholic acid or lauryl carnitine showed an

average absolute *F* of 0.03% and 0.38%, respectively [56]. The Eligen® carrier, 5-CNAC showed greater efficacy in suppression of bone resorption compared to a marketed nasal formulation, but it still failed the primary endpoint in Phase III trial for osteoporosis [57]. No oral formulation of sCT has yet been approved by the FDA.

5 Conclusions

Suitable coatings for sCT-SmPill® minispheres were developed in order to promote delayed release of sCT into either rat jejunum or colon, as confirmed by X-ray imaging. Stability studies indicated that sCT was stable over four months at 5°C when entrapped in minispheres. *In situ* instillations demonstrated that increased absorption of sCT solution and sCT from uncoated minispheres occurred in colon compared to jejunum. Among the PEs evaluated in coated minispheres by oral gavage, CG was the most effective in jejunally-delivering sCT compared to sCT solution, but considerable loss in PK was seen by oral gavage compared to regional instillations. Data comparing formulations administered by instillation indicate a large overestimation of bioavailability compared to oral gavage in rats, and moreover, that rank order of formulation cannot be accurately predicted from the former. Results from oral gavage of coated sCT-SmPill® minispheres suggested that the resulting PK was more predictable and had a higher relative *F* from formulations targeted to jejunum compared to colon. Levels of *F* achieved with sCT- SmPill® by oral gavage are on a par with reported values for sCT in other advanced oral formulations and have the potential to be improved. The results underline the potential of SmPill® technology for controlled release of sCT and other peptides in selected intestinal regions.

Acknowledgements and declarations This study was funded by Science Foundation Ireland Cluster (The Irish Drug Delivery Network) grant number 07-SRC-B1154. Sigmoid Pharmaceuticals contributed reagents, staff time, training and access for PhD student Tanira Aguirre at their research facility. MR and IC are employees of Sigmoid Pharma. We thank Mrs. Marta J. G. Cioato and Mrs Fabíola S. Meyer from *Unidade de Experimentação Animal* of *Hospital de Clínicas de Porto Alegre* for the facility and the structure to animal studies, and to Mr. Ernesto E. Bernardes for assistance with X-Ray imaging.

References

[1] A.L. Lewis, J. Richard, Challenges in the delivery of peptide drugs: an industry perspective., Ther. Deliv. 6 (2015) 149–63. doi:10.4155/tde.14.111.

[2] T.A.S. Aguirre, M. Rosa, I.S. Coulter, D.J. Brayden, In vitro and in vivo preclinical evaluation of a minisphere emulsion-based formulation (SmPill®) of salmon calcitonin, Eur. J. Pharm. Sci. 79 (2015) 102–111. doi:10.1016/j.ejps.2015.09.001.

[3] N. Binkley, M. Bolognese, A. Sidorowicz-Bialynicka, T. Vally, R. Trout, C. Miller, et al., A Phase 3 trial of the efficacy and safety of oral recombinant calcitonin: the Oral Calcitonin in Postmenopausal Osteoporosis (ORACAL) trial., J. Bone Miner. Res. 27 (2012) 1821–9. doi:10.1002/jbmr.1602.

[4] European Medicines Agency, European Medicines Agency recommends restricting use of trimetazidine-containing medicines. 2012., Eur. Med. Agency. (2012). http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2012/07/news_detail_001573.jsp&mid=WC0b01ac058004d5c1 (accessed December 15, 2015).

[5] N. Binkley, H. Bone, J.P. Gilligan, D.S. Krause, Efficacy and safety of oral recombinant calcitonin tablets in postmenopausal women with low bone mass and increased fracture risk: a randomized, placebo-controlled trial., Osteoporos. Int. 25 (2014) 2649–56. doi:10.1007/s00198-014-2796-0.

[6] Depakote (divalproex sodium) tablets - product label., (2015). http://www.accessdata.fda.gov/drugsatfda_docs/label/2015/018723s055lbl.pdf (accessed December 20, 2015).

[7] EMBEDA® - product label., (2014). http://www.accessdata.fda.gov/drugsatfda_docs/label/2014/022321s016lbl.pdf (accessed December 20, 2015).

[8] R.I. Mustafin, Interpolymer combinations of chemically complementary grades of Eudragit copolymers: A new direction in the design of peroral solid dosage forms of drug delivery systems with controlled release (review), Pharm. Chem. J. 45 (2011) 285–295. doi:10.1007/s11094-011-0618-7.

[9] N. Reix, P. Guhmann, W. Bietiger, M. Pinget, N. Jeandidier, S. Sigrist, Duodenum-specific drug delivery: in vivo assessment of a pharmaceutically developed enteric-coated capsule for a broad applicability in rat studies., Int. J. Pharm. 422 (2012) 338–40. doi:10.1016/j.ijpharm.2011.10.017.

[10] K. Keohane, M. Rosa, I.S. Coulter, B.T. Griffin, Enhanced colonic delivery of ciclosporin A self-emulsifying drug delivery system encapsulated in coated minispheres, Drug Dev. Ind. Pharm. In press (2015) 1–9. doi:10.3109/03639045.2015.1044905.

[11] S.P.L. Travis, S. Danese, L. Kupcinskas, O. Alexeeva, G. D'Haens, P.R. Gibson, et al., Once-daily budesonide MMX in active, mild-to-moderate ulcerative colitis: results from the randomised CORE II study., Gut. 304258 (2013) 1–9. doi:10.1136/gutjnl-2012-304258.

[12] S. Amidon, J.E. Brown, V.S. Dave, Colon-targeted oral drug delivery systems: design trends and approaches, AAPS PharmSciTech. 16 (2015) 731–741. doi:10.1208/s12249-015-0350-9.

[13] S. Maher, T.W. Leonard, J. Jacobsen, D.J. Brayden, Safety and efficacy of sodium caprate in promoting oral drug absorption: from in vitro to the clinic., Adv. Drug Deliv. Rev. 61 (2009) 1427–49. doi:10.1016/j.addr.2009.09.006.

[14] T. A. S. Aguirre, M. Rosa, S.S. Guterres, A.R. Pohlmann, I. Coulter, D.J. Brayden, Investigation of coco-glucoside as a novel intestinal permeation enhancer in rat models, Eur. J. Pharm. Biopharm. 88 (2014) 856–865. doi:10.1016/j.ejpb.2014.10.013.

[15] Food additives permitted for direct addition to food for human consumption, Sec. 172.863 Salts of fatty acids, (n.d.). http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.863 (accessed November 26, 2015).

[16] Agency Response Letter GRAS Notice No. GRN 000237, (n.d.). http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm153914.htm (accessed November 26, 2015).

[17] K. Petechin, C. Boyke, Cleaning composition with decyl and coco glucosides, US Patent 2013/0115205 A1, 2013.

[18] K.-H. Song, S.-J. Chung, C.-K. Shim, Enhanced intestinal absorption of salmon calcitonin (sCT) from proliposomes containing bile salts., J. Control. Release. 106 (2005) 298–308. doi:10.1016/j.jconrel.2005.05.016.

[19] S.H. Welling, F. Hubálek, J. Jacobsen, D.J. Brayden, U.L. Rahbek, S.T. Buckley, The role of citric acid in oral peptide and protein formulations : Relationship between calcium chelation and proteolysis inhibition, Eur. J. Pharm. Biopharm. 86 (2014) 544–551. doi:10.1016/j.ejpb.2013.12.017.

[20] I. Coulter, B. Mcdonald, V. Aversa, Composition comprising oil drops, WO2010/133609 A2, 2010.

[21] E.T. Cole, R.A. Scott, A.L. Connor, I.R. Wilding, H.U. Petereit, C. Schminke, et al., Enteric coated HPMC capsules designed to achieve intestinal targeting., Int. J. Pharm. 231 (2002) 83–95. http://www.ncbi.nlm.nih.gov/pubmed/11719017 (accessed June 4, 2013).

[22] S. Bourgeois, R. Harvey, E. Fattal, Polymer colon drug delivery systems and their application to peptides, proteins, and nucleic acids, Am. J. Drug Deliv. 3 (2005) 171–204. doi:10.2165/00137696-200503030-00003.

[23] T. Aguirre, Oral delivery of salmon calcitonin using a novel liquid emulsion drug delivery system (LEDDSTM), PhD Thesis. University College Dublin, 2013.

[24] J.E. Polli, G.S. Rekhi, L.L. Augsburger, V.P. Shah, Methods to compare dissolution profiles and a rationale for wide dissolution specifications for metoprolol tartrate tablets., J. Pharm. Sci. 86 (1997) 690–700. doi:10.1021/js960473x.

[25] ICH, Validation of analytical procedures: text and methodology Q2(R1), (2005). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf.

[26 J. Gabrielsson, D. Weiner, Pharmacokinetic and Pharmacodynamic Data Analysis: Concepts and Applications, 4th Ed., Swedish Pharmaceutical Press, 2000.

[27] P. Costa, J.M. Sousa Lobo, Modeling and comparison of dissolution profiles, Eur. J. Pharm. Sci. 13 (2001) 123–133. doi:10.1016/S0928-0987(01)00095-1.

[28] R.W. Korsmeyer, R. Gurny, E. Doelker, P. Buri, N. a. Peppas, Mechanisms of solute release from porous hydrophilic polymers, Int. J. Pharm. 15 (1983) 25–35. doi:10.1016/0378-5173(83)90064-9.

[29] S. Dash, P.N. Murthy, I. Nath, P. Chowdhury, Kinetic modeling on drug release from controlled drug delivery systems, 67 (2010) 217–223.

[30] P.L. Ritger, N.A. Peppas, A simple equation for description of solute release II. Fickian and anomalous release from swellable devices, J. Control. Release. 5 (1987) 37–42. doi:10.1016/0168-3659(87)90035-6.

[31] S. Haruta, K. Kawai, S. Jinnouchi, K.I. Ogawara, K. Higaki, S. Tamura, et al., Evaluation of absorption kinetics of orally administered theophylline in rats based on gastrointestinal transit monitoring by gamma scintigraphy., J. Pharm. Sci. 90 (2001) 464–73. http://www.ncbi.nlm.nih.gov/pubmed/11170036 (accessed June 16, 2013).

[32] V. Christmann, J. Rosenberg, J. Seega, C.M. Lehr, Simultaneous in vivo visualization and localization of solid oral dosage forms in the rat gastrointestinal tract by magnetic resonance imaging (MRI)., Pharm. Res. 14 (1997) 1066–72. doi:10.1023/A:1012161630481.

[33] E. Andreozzi, J.W. Seo, K. Ferrara, A. Louie, Novel method to label solid lipid nanoparticles with 64cu for positron emission tomography imaging., Bioconjug. Chem. 22 (2011) 808–18. doi:10.1021/bc100478k.

[34] N. Reix, P. Guhmann, W. Bietiger, M. Pinget, N. Jeandidier, S. Sigrist, Duodenum-specific drug delivery: in vivo assessment of a pharmaceutically developed enteric-coated capsule for a broad applicability in rat studies., Int. J. Pharm. 422 (2012) 338–40. doi:10.1016/j.ijpharm.2011.10.017.

[35] S. Saphier, A. Rosner, R. Brandeis, Y. Karton, Gastro intestinal tracking and gastric emptying of solid dosage forms in rats using X-ray imaging., Int. J. Pharm. 388 (2010) 190–5. doi:10.1016/j.ijpharm.2010.01.001.

[36] K. Albrecht, M. Greindl, C. Kremser, C. Wolf, P. Debbage, A. Bernkop-Schnürch, Comparative in vivo mucoadhesion studies of thiomer formulations using magnetic resonance imaging and fluorescence detection., J. Control. Release. 115 (2006) 78–84. doi:10.1016/j.jconrel.2006.06.023.

[37] J. Zheng, H. Ariga, H. Taniguchi, K. Ludwig, T. Takahashi, Ghrelin regulates gastric phase III-like contractions in freely moving conscious mice., Neurogastroenterol. Motil. 21 (2009) 78–84. doi:10.1111/j.1365-2982.2008.01179.x.

[38] A. Ghaffari, K. Navaee, M. Oskoui, K. Bayati, M. Rafiee-Tehrani, Preparation and characterization of free mixed-film of pectin/chitosan/Eudragit RS intended for sigmoidal drug delivery., Eur. J. Pharm. Biopharm. 67 (2007) 175–86. doi:10.1016/j.ejpb.2007.01.013.

[39] F. Sadeghi, J.L. Ford, A. Rajabi-Siahboomi, The influence of drug type on the release profiles from Surelease-coated pellets, Int. J. Pharm. 254 (2003) 123–135. doi:10.1016/S0378-5173(03)00003-6.

[40] G. Dongowski, H. Anger, Metabolism of pectin in the gastrointestinal tract, in: J. Visser, A.G.J. Voragem (Eds.), Pectins and Pectinases, Elsevier, 1995.

[41] A. Avachat, V. Kotwal, Design and evaluation of matrix-based controlled release tablets of diclofenac sodium and chondroitin sulphate., AAPS PharmSciTech. 8 (2007) E88. doi:10.1208/pt0804088.

[42] T. Hayashi, H. Kanbe, M. Okada, M. Suzuki, Y. Ikeda, Y. Onuki, et al., Formulation study and drug release mechanism of a new theophylline sustained-release preparation., Int. J. Pharm. 304 (2005) 91–101. doi:10.1016/j.ijpharm.2005.07.022.

[43] S.M. Ryan, X. Wang, G. Mantovani, C.T. Sayers, D.M. Haddleton, D.J. Brayden, Conjugation of salmon calcitonin to a combed-shaped end functionalized poly(poly(ethylene glycol) methyl ether methacrylate) yields a bioactive stable conjugate., J. Control. Release. 135 (2009) 51–9. doi:10.1016/j.jconrel.2008.12.014.

[44] S.M. Ryan, J.M. Frías, X. Wang, C.T. Sayers, D.M. Haddleton, D.J. Brayden, PK/PD modelling of comb-shaped PEGylated salmon calcitonin conjugates of differing molecular weights., J. Control. Release. 149 (2011) 126–32. doi:10.1016/j.jconrel.2010.10.004.

[45] W.-P. Cheng, C. Thompson, S.M. Ryan, T. Aguirre, L. Tetley, D.J. Brayden, In vitro and in vivo characterisation of a novel peptide delivery system: amphiphilic

polyelectrolyte-salmon calcitonin nanocomplexes., J. Control. Release. 147 (2010) 289–97. doi:10.1016/j.jconrel.2010.07.128.

[46] K.C, Lee, Y.J. Lee, H. M Song, C. J. Chun, P. P. DeLuca, .Degradation of synthetic salmon calcitonin in aqueous solution. Pharm. Res. 9 (1992) 1521-1523.

[47] S. Maher, D.J. Brayden, Overcoming poor permeability: translating permeation enhancers for oral peptide delivery, Drug Discov. Today Technol. 9 (2012) e113–e119. doi:10.1016/j.ddtec.2011.11.006.

[48] P. Sinko, Y. Lee, V. Makhey, G. Leesman, Biopharmaceutical approaches for developing and assessing oral peptide delivery strategies and systems: in vitro permeability and in vivo oral absorption of salmon, Pharm. Res. 16 (1999) 527–533. doi:10.1023/A:1018819012405.

[49] I.S. Ahmed, J.W. Ayres, Comparison of in vitro and in vivo performance of a colonic delivery system., Int. J. Pharm. 409 (2011) 169–77. doi:10.1016/j.ijpharm.2011.02.061.

[50] T. Corring, The adaptation of digestive enzymes to the diet: Its physiological significance., Reprod Nutr Dev. 20 (1980) 1217–35.

[51] M. Saito, E. Murakami, T. Nishida, Y. Fujisawa, M. Suda, Circadian rhythms of digestive enzymes in the small intestine of the rat II. Effects of fasting and refeeding., J. Biochem. 80 (1976) 563–8.

[52] M. Grant, A. Leone-Bay, Peptide therapeutics: it's all in the delivery., Ther. Deliv. 3 (2012) 981–96.

[53] L. Wu, G. Zhang, Q. Lu, Q. Sun, M. Wang, N. Li, et al., Evaluation of salmon calcitonin (sCT) enteric-coated capsule for enhanced absorption and GI tolerability in rats., Drug Dev. Ind. Pharm. 36 (2010) 362–70. doi:10.1080/03639040903173580.

[54] M. Licciardi, G. Pasut, G. Amato, C. Scialabba, A. Mero, M. Montopoli, et al., PHEA-graft-polymethacrylate supramolecular aggregates for protein oral delivery., Eur. J. Pharm. Biopharm. 84 (2013) 21–8. doi:10.1016/j.ejpb.2012.12.011.

[55] T. Buclin, M. Cosma Rochat, P. Burckhardt, M. Azria, M. Attinger,

Bioavailability and biological efficacy of a new oral formulation of salmon calcitonin in healthy volunteers., J. Bone Miner. Res. 17 (2002) 1478–85. doi:10.1359/jbmr.2002.17.8.1478.

[56] W. Stern, J. Gilligan, Oral peptide pharmaceutical products, US patent 6086918, 2000.

[57] M. a Karsdal, K. Henriksen, a C. Bay-Jensen, B. Molloy, M. Arnold, M.R. John, et al., Lessons learned from the development of oral calcitonin: the first tablet formulation of a protein in phase III clinical trials., J. Clin. Pharmacol. 51 (2011) 460–71. doi:10.1177/0091270010372625.

Tables 1-7

Table 2. Ratios by weight of components in four sCT-SmPill® minisphere prototypes.

Component	1. CA	2. NaTDC	3. C_{10}	4. CG
sCT	2.1	2.2	2.2	2.1
Transcutol HP®	19.8	14.9	16.4	14.2
Kolliphor EL®	8.5	6.4	7.0	6.1
Miglyol 810®	6.5	4.9	5.3	4.6
D-Sorbitol	5.4	4.0	3.9	3.2
Gelatin	51.4	42.0	40.1	38.7
CA	6.3	-	-	-
NaTDC	-	25.6	-	-
C10	-	-	25.1	-
CG	-	-	-	31.2

Table 2. Parameters obtained by fitting minisphere sCT release profiles to Weibull equation.

Formulation	t_0 (min)	k	β	D (%)	r^2
SmPill®-CA (j)	2.48(0.01)	0.495(0.06)	1.45(0.23)	96.91(0.78)	0.99
SmPill®-CG (j)	2.39(0.02)	0.086(0.02)	2.55(1.04)	98.76(1.37)	0.99
SmPill®-NaTDC (j)	2.38(0.00)	0.287(0.01)	0.82(0.03)	101.40(0.32)	0.99
SmPill®-CG (c)	7.25(0.06)	5.341(0.03)	0.89(0.02)	111.00(1.00)	0.99
SmPill®-C_{10} (c)	4.59(0.07)	2.908(0.02)	1.23(0.01)	95.27(1.57)	0.99

t_0: dissolution lag-time; k: rate parameter; β: shape parameter; D: fraction dissolved as $t\rightarrow\infty$. j (Jejunum; Eudragit®); c (colon; Surelease®/Pectin). Numbers in brackets (SEM).

Table 3. Correlation coefficients (r^2) obtained by fitting sCT-SmPill® release profiles to Baker-Londsdale, Korsmeyer-Peppas, Hixson-Crowell, Higuchi and First order mathematical models.

Formulation	Baker-Londsdale	Korsmeyer-Peppas	Hixson-Crowell	Higuchi	First order
SmPill®-CA (j)	0.223706	0.939786	0.382261	0.244878	0.359747
SmPill®-CG (j)	0.272224	0.922456	0.454609	0.303852	0.420412
SmPill® -NaTDC (j)	0.229409	0.883694	0.387453	0.254312	0.362252
SmPill®-CG (c)	0.499999	0.941460	0.763840	0.540438	0.732105
SmPill®-C10 (c)	0.602362	0.914152	0.845734	0.675845	0.801988

j (Jejunum; Eudragit®); c (colon; Surelease®/Pectin).

Table 4. Parameters (SEM) obtained by fitting release profiles to Korsmeyer-Peppas model.

Formulation	Korsmeyer-Peppas		
	k	**n**	**r^2**
SmPill®-CA (j)	0.019 (0.001)	11.120(0.085)	0.996887
SmPill®-CG (j)	0.031 (0.004)	8.357 (0.167)	0.999761
SmPill® -NaTDC (j)	0.020 (0.002)	11.530 (1.350)	0.993407
SmPill®-CG (c)	0.632 (0.000)	2.244 (0.000)	0.999999
SmPill®-C_{10} (c)	1.687 (0.715)	2.107 (0.252)	0.980186

j (Jejunum; Eudragit®); c (colon; Surelease®/Pectin). Numbers in brackets (SEM).

Table 5. Assay of sCT released from SmPill® minispheres over 4 months (M) at 4°C.

A. Formulation	**M 0**	**M 1**	**M 2**	**M 3**	**M 4**
SmPill®-CA (j)	90.8±1.2	90.8±1.1	93.5±0.2	84.4±1.0	80.3±0.6
SmPill®-CG (j)	115.4±0.4	108.4±0	75.9±8.3	74.8±4.3	73.6±5.2
SmPill®-NaTDC (j)	98.0±2.4	96.7±0.5	92.5±3.9	86.2±0.3	84.0±3.2
SmPill®-CG (c)	121.3±2.6	117.9±5.2	106.6±0.9	112.5±5.1	104.4±6.0
SmPill®-C_{10} (c)	103.9±0.6	98.3±0.3	96.8±0.6	88.2±0.5	84.5±2.9

B. Formulation	**M 0**	**M 1**	**M 2**	**M 3**	**M 4**
SmPill®-CA (j)	92.8±9.2	102.6±5.5	103.9±24.9	86.1±2.7	94.3±4.6
SmPill®-CG (j)	91.6±2.2	92.0±8.9	102.8±5.3	83.6±11.4	102.4±12.8
SmPill®-NaTDC (j)	97.0±12.5	99.5±10.7	108.7±14.0	90.2±10.7	94.1±6.8
SmPill®-CG (c)	108.4±10.2	90.0±2.8	99.8±7.0	84.8±8.6	93.0±2.9
SmPill®-C_{10} (c)	110.4±7.4	104.4±11.4	92.0±12.7	84.8±8.0	96.4±9.8

Results are percentage of the theoretical amount of sCT added to minispheres compared to standards and analysed by HPLC (A) or by in vitro bioassay (B). Concentrations of 10 nM of sCT were added on T47D cells in each case and were compared to native sCT to calculate percentage. Values are given as mean ± %RSD, n=2-3.

Table 6. PK parameters of instillations of uncoated minispheres over 6 h.

Sample	C_{max} (ng/ml)	T_{max} (min)	$t_{1/2}$ (min)	$AUC_{(0-\infty)}$ (min.ng/ml)	Absolute $F_{(0-\infty)}$* (%)
sCT (i.j.)	11.7±2.8	5	59.5±4.8	732±257	2.6±0.9
SmPill®-NaTDC (i.j.)	11.9±1.1	15	101.4±5.4	1548±268	5.5±1.0*
SmPill®-CG (i.j.)	12.7±2.6	45	252.2±20.0	1027±211	3.7±0.8
SmPill®-CA (i.j.)	8.2±2.2	45	51.7±6.7	747±101	2.7±0.4
sCT (i.c.)	26.1±5.6	5	42.8±3.2	1974 ±431	7.0±1.5
SmPill®-CG (i.c.)	23.0±1.2	90	38.7±3.5	4878±538	17.3±1.9**
SmPill®-C_{10} (i.c.)	27.2±2.6	45	79.7±7.3	5131±493	18.2±1.8**

sCT-SmPill® minispheres contained ~2000 I.U. sCT/kg co-formulated with NaTDC (9 µmol/kg), C_{10} (23 µmol/kg), CG (15 µmol/kg) or CA (6 µmol/kg). 2 ml saline/kg or 2000 I.U. sCT solution/kg, were dosed to controls. Absolute *F* of intra-jejunal (i.j.) or intra-colonic (i.c.) instillations was calculated relative to an i.v. dose of 800 I.U. sCT/kg, $AUC_{(0-\infty)}$ = 11261±900 min.ng/ml. *P < 0.05 and **P < 0.01 compared to native sCT instilled by the same route (one-way ANOVA for *F* and AUC_{0-360} min, post-Dunnett's test).

Table 7. PK parameters of oral administration native sCT or sCT-SmPill® coated minispheres to rats.

Sample Oral delivery	C_{max} (ng/ml)	T_{max} (min)	$t_{1/2}$ (min)	$AUC_{(0-\infty)}$ (min.ng/ml)	Absolute $F_{(0-\infty)}$ (%)
sCT oral	10.0±2.8	20	132.2±6.3	781±94	0.9±0.1
SmPill®-NaTDC (j)	2.5±1.5	180	144.9±9.9	728±207	0.9±0.2
SmPill® -CG (j)	10.5±3.4	150	243.3±13.9	2239±466	2.7±0.6*
SmPill® -CA (j)	7.1±3.1	180	469.8±20.7	1131±308	1.4±0.4
SmPill® -C_{10} (c)	7.1±2.9	180	172.9±5.6	1389±415	1.7±0.5
SmPill®-CG (c)	1.7±1.4	300	147.9±11.4	374±69	0.5±0.1

Doses were ~6000 I.U. native sCT/kg, or sCT-SmPill® coated minispheres designed for jejunal (j; Eudragit®) or colonic (c; Surelease®/Pectin) release containing ~6000 I.U. sCT/kg combined with CA (18 µmol/kg), NaTDC (26 µmol/kg), C10 (69 µmol/kg) or CG (45 µmol/kg). * P<0.05 compared to native sCT (one-way ANOVA, post Dunnet's test). Absolute *F* was calculated relative to an i.v. dose of 800 I.U. sCT/kg, $AUC_{(0-\infty)}$ = 11261±900 min.ng/ml.

Supplementary Tables

Table S. 1. Parameters used for coating SmPill® minispheres.

	Opadry® White	**Eudragit® L30 D-55**	**Surelease®/Pectin (98:2)**
Pump rate (g/min)	0.2	0.2	0.3
Nozzle air (psig)	25.8	25.8	25.8
Airflow (LPM)	197	197	197
Inlet Temperature (°C)	57.8 - 60	37.5 - 40	57.9 - 60
Exhaust Temperature (°C)	37.2 - 40	28.0 - 30	37.8 - 40

$BaSO_4$-SmPill® minispheres were prepared and coated with a first layer of Opadry® White, and split into two groups. Half were secondarily-coated with Eudragit® and the other half with Surelease®/Pectin. The final theoretical weight gain for each $BaSO_4$ formulation was respectively 8.2% Opadry White + 5.8% Eudragit® and 8.2% Opadry White + 11.5% Surelease®/Pectin. Precise concentrations of polymers added to the sCT-SmPill® minisphere surfaces to give a coating percentage gain are described (Table S. 2).

Table S. 2. Coatings of sCT- SmPill® minisphere prototypes.

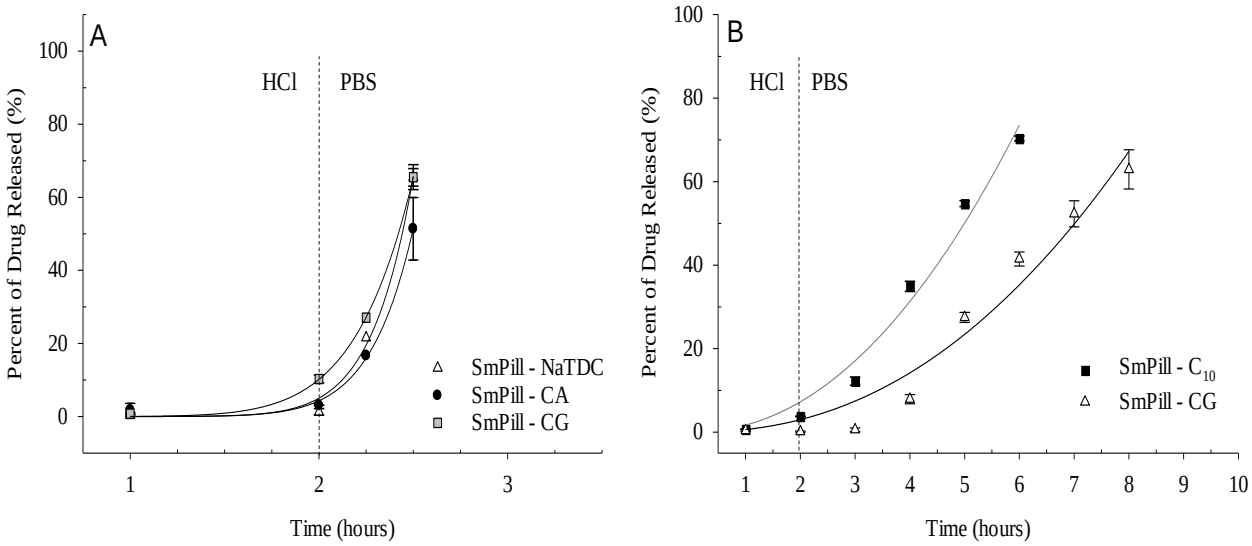
Formulation	**Coating**
SmPill®-CA (j)	7.5% Opadry® White and 7.3% Eudragit®
SmPill®-NaTDC (j)	8.2% Opadry® White and 7.5% Eudragit®
SmPill®-CG (j)	7.9% Opadry® White and 7.0% Eudragit®
SmPill®-C10 (c)	7.0% Opadry® White and 10.8% Surelease®/Pectin
SmPill®-CG (c)	7.9% Opadry® White and 11.0% Surelease®/Pectin

(j) jejunum; (c) colon.

Table S. 3. Loading of sCT in SmPill® formulations with selected PEs for uncoated and coated minispheres designed for jejunal or colonic release. Results are given as the mean theoretical concentration of sCT (%), n = 3 independent formulations.

Formulation	**sCT Assay**	
	Mean %	**RSD%**
SmPill®-CA (uncoated)	81.0	0.3
SmPill®-CG (uncoated)	106.7	5.3
SmPill®-NaTDC (uncoated)	89.4	10.0
SmPill®-C_{10} (uncoated)	93.3	2.5
SmPill®-CA (i.j. coated)	90.8	1.2
SmPill®-CG (i.j., coated)	115.4	0.4
SmPill®-NaTDC (i.j., coated)	98.0	2.4
SmPill®-CG (i.c., coated)	121.3	2.6
SmPill®-C10 (i.c., coated)	103.9	0.6

* % RSD: Relative standard deviation = (SD/Mean)*100. Initial coatings in each case were Opadry® white. i.j. (Jejunum; Eudragit®); i.c. (colon; Surelease®/Pectin).



Suppl. Fig. 1

Suppl. Fig 1. Dissolution of sCT-SmPill® minispheres with coatings designed for (A) jejunal release (SmPill®-NaTDC, SmPill®-CG, SmPill®-CA) and (B) colonic release (SmPill®-C_{10}, SmPill®-CG). Release was carried out initially for 2 h in 0.1M HCl pH 1,0 and then in PBS pH 6.8 for a further 8 h (A) or 22 h (B). n=2 for each time point, results are mean±SD. The Korsmeyer-Peppas function is plotted. j (Jejunum; Eudragit®); c (colon; Surelease®/Pectin).