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Improved intestinal delivery of salmon calcitonin by water-in-oil microemulsions

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ABSTRACT

Therapeutic peptides are highly potent and specific in their functions, but difficulties in their oral administration require parallel development of viable delivery systems to improve their oral bioavailability. The objective of this study was to explore the feasibility of water-in-oil (w/o) microemulsions for improving the absorption of intraduodenally administered salmon calcitonin (sCT). The w/o microemulsions were prepared from medium chain triglyceride, Tween 80 and Span 80 or soybean phosphatidylcholine, propylene glycol and phosphate saline, and characterized by particle size and *in vitro* physical stability under dilution with different physiologically relevant diluents. The effects of addition of polymers such as hydroxypropylmethylcellulose and Carbomer into aqueous phase on the properties of microemulsions were assessed. sCT was efficiently encapsulated into microemulsions with nanoscaled diameter ranged from about 6 to 134 nm. As expected from the non-ionic nature of the investigated microemulsions, the physical stability, evaluated by visual inspection, the particle size and leakage percent under dilution, was found to be unaffected by pH and/or ionic strength of diluents and it was opposite for the microemulsions with ionic components. In addition, the dilution extent had a pronounced effect on the physical stability of the diluted microemulsions. The effect of polymers added into aqueous phase of the microemulsions on the absorption of the drug entrapped in microemulsions with different components was investigated. The optimized microemulsions were shown to generate substantial enhancement (up to 4-fold) of relative pharmacological activity of sCT with regard to the control solution of the drug. This indicated that the w/o microemulsions could offer the potential to significantly improve intestinal absorption of sCT.

1. Introduction

With the development of biotechnology, protein and peptide drugs are becoming more important in medication due to their high selectivity and their ability to provide effective action (Frokjaer and Otzen, 2005). Unfortunately, most of these drugs have considerably low bioavailability by oral administration owing to their high susceptibility to proteolytic degradation by gastrointestinal enzymes, poor membrane permeability of gastrointestinal mucosa and short half-life *in vivo* (Pauletti et al., 1996). Despite the efforts dedicated over the last decades towards making the oral administration of peptides and proteins feasible, the actual fact is that this objective still remains a challenge. Nevertheless, the success of the formulations undergoing clinical trials (Clement et al., 2004; Mehta, 2004) offers an optimistic prospect towards reaching this objective.

Many drug nanocarriers, such as liposomes (Kisel et al., 2001; Song et al., 2002), micelles (Silva-Cunha et al., 1998; Zhang et al., 2010), and microemulsions (Cheng et al., 2008), were proved to improve the gastrointestinal absorption of peptide and protein drugs to some extent. Amongst these nanocarriers, microemulsions were attracted much interest in recent years due to their prominent advantages, including forming spontaneously, ease of manufacturing, thermodynamic stability, protective effect against intestinal enzymes, and high dispersion property (Jadhav et al., 2006; Spernath and Aserin, 2006). Since oil-in-water (o/w) microemulsions are intended to improve the oral bioavailability of hydrophobic drugs, including the hydrophobic peptide drugs such as cyclosporine A (Gao et al., 1998), the development of water-in-oil (w/o) microemulsions to enhance the oral absorption of hydrophilic peptide drugs was attracted attention (Constantinides et al., 1994; Prasad et al., 2003; Watnasirichaikul et al., 2002). It was reported that w/o microemulsions could protect peptides and proteins from proteolysis or acidic degradation, and thus enhance their absorption in the gastrointestinal tract (Cilek et al., 2005). In addition, the presence and difference of surfactant and oil, for example, lecithin and medium chain triglyceride, served to increase the membrane permeability, thereby increasing drug uptake (Constantinides et al., 1994). However, there is no document on w/o microemulsion systems for hydrophilic salmon calcitonin (sCT) up to date.

sCT, which is a 3500-Da polypeptide with low membrane permeability and unstable nature in gastrointestinal tract, has a physiological role in the regulation of calcium homeostasis and is a potent inhibitor of osteoclastic bone resorption. Its therapeutic uses

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are the long-term treatment of Paget's disease and the short-term relief of some hypercalcemia conditions in clinic. The currently available treatment is limited to either intramuscular or subcutaneous administration, which compromises patient comfort (Tanko et al., 2004). A nasal spray is also available, but has limited use due to low bioavailability. It has been reported that the bioavailability of sCT solution was less than 0.1% following intra-duodenal, -colonic and -ileac administration in rats and dogs (Hee Lee et al., 2000; Lee et al., 2000; Sinko et al., 1995). The oral absorption of sCT may be enhanced by using suitable drug carriers (Lee and Sinko, 2000; Prego et al., 2006; Thirawong et al., 2008). These studies clearly emphasize the possibilities in enhancing the absorption of sCT in the presence of different absorption enhancers or in drug carrier systems in the oral route of administration due to the fact that a drug carrier can for example be employed to target transport carrier proteins or to open tight junctions between the epithelial cells (Hamman et al., 2005; Sarciaux et al., 1995).

In the present study, a novel w/o microemulsion for intestinal delivery of sCT was developed. We first characterized the phase behaviors of pseudo-ternary system containing medium chain triglyceride as the oil phase, phosphate buffered saline (PBS, pH 4.0) as the aqueous phase, Tween 80 as the main surfactant combined with other additives, such as Span 80 and soybean phosphatidylcholine as the adjuvant surfactants, propylene glycol as the cosurfactant. Then, the drug was encapsulated into microemulsions followed by characterization of their physicochemical properties such as particle size and physical stability under dilution. At last, the ability to enhance the intestinal absorption of sCT was investigated by determining the decrease in serum calcemic levels following intraduodenal administration to rats.

2. Materials and methods

2.1. Materials

Salmon calcitonin (sCT) was obtained from Shanghai Yanchang Biochemical Development Co. Ltd. (Shanghai, China) and stored in a deep freezer (−20 °C) before use. Medium chain triglyceride (MCT) was provided by Tieling Beiya Pharmaceutical Scybean Oil Co., Ltd. (Tieling, China). Tween 80 (HLB = 15) and Span 80 (HLB = 4.3) were provided by Farco Chemical Supplies (Hong Kong, China). Soybean phosphatidylcholin (SPC) (HLB = 3) was purchased from Shanghai Dongshang Industries Ltd. (Shanghai, China). Hydroxypropylmethylcellulose (HPMC) k15M and Carbopol 980 (CP) were obtained from BF Goodrich (USA) and Dow Chemical Company (USA), respectively. Phthalocyanine o-cresol integration ketone (OCPC), 8-hydroxyquinoline (8-HQ) and diethylamide were supplied by Sigma (Beijing, China). All other chemicals and reagents were of analytical grade or better.

Sprague-Dawley rats 200 ± 20 g were obtained from Animals Center of Peking University Health Science Center. All animals were provided with standard food and water ad libitum, and exposed to alternating 12-h periods of light and darkness. Temperature and relative humidity were maintained at 25 °C and 50%, respectively. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Peking University.

2.2. Preparation of phase diagram and formulation of w/o microemulsions

The pseudo-ternary phase diagram of oil (MCT), surfactant (Tween 80, SPC or Span 80), cosurfactant (propylene glycol (PG)), and PBS (pH 4.0) was developed using saline titration method to obtain the concentration ranges that can result in large existence area of w/o microemulsion. Briefly, homogeneous mixture of oil, surfactant and cosurfactant in fixed mass ratio was titrated with PBS under magnetic stirring at ambient temperature. The samples were equilibrated for 20 min before being assessed visually and determined as being clear and transparent w/o microemulsions, or crude emulsions. The physical states were represented on a pseudo-ternary phase diagram with one axis representing PBS, one representing oil and the third representing the mixture of surfactant and cosurfactant. The influence of mass ratio of surfactant to cosurfactant on the area of w/o microemulsion region was investigated on the pseudo-ternary phase diagram. All compounds used in this study were pharmaceutically acceptable and biocompatible.

Once the w/o microemulsion region was identified, a series of microemulsions were prepared simply through mixing the components with varying ratio of oil, surfactants, cosurfactant, and sCT solution in PBS (pH 4.0). Briefly, MCT, Tween 80, SPC or Span 80, and PG were accurately weighed into glass vials, adding the required mass of sCT solution. The components were then mixed by gentle stirring and vortex mixing until homogeneous mixture formed. The mixture was sealed in a glass vial and stored at room temperature until used. In addition, carbomer and hydroxy-propyl methyl cellulose were added into aqueous phase, respectively, in order to inhibit phase inversion.

2.3. Physical characterization of microemulsions

The viscosity of microemulsions was monitored by a rotational NDJ-1 viscometer (Shanghai, China). For measuring refractive index, a WAY-2S ABBE Refractometer (Hangzhou Chincan Trading Co., Ltd., China) was used. Introducing the determined viscosity and refractive index into the Nano-ZS workstation program (Nano-ZS, Malvern Instruments Inc.), the average particle size and size distribution of microemulsion droplets were determined by dynamic light scattering (DLS). All DLS measurements were performed with a scattering angle of 90° at 25 °C after diluting the microemulsions to an appropriate volume with oil phase. The results were the mean values of three samples.

2.4. Determination of content of sCT loaded in w/o microemulsions

The content of sCT in w/o microemulsions was determined by the method of HPLC. Briefly, sCT-loaded microemulsions were dissolved in methanol and sCT content in microemulsions was determined on a Shimadzu series HPLC system (Shimadzu LC-10AT, Kyoto, Japan) equipped with a UV detector (Shimadzu SPD-10A) and reversed phase column (ODS C18, 5 μm, 4.6 mm × 250 mm, Agilent, USA). The mobile phase consisted of 0.40% tetramethyl ammonium hydroxide–water:acetonitrile, 9:1 (v/v) for A solution, and 0.36% tetramethyl ammonium hydroxide–water:acetonitrile, 2:3 (v/v) for B solution. The gradient sequence was: 35–57% B from 0 to 21 min, 35% B from 20.01 to 30 min. And the flow rate was 1.0 mL/min. The detection wavelength was 220 nm. The column temperature was set at 40 °C.

2.5. Evaluation of physical stability of the microemulsions under different dilution

The physical stability of microemulsions under dilution was evaluated by visual inspection, droplet size and leakage percent (LP) of sCT measurements as reported previously with little modification (Constantinides and Yiv, 1995). Briefly, w/o microemulsions were diluted 200-fold and 500-fold with different physiologically relevant diluent solutions such as simulated gastric fluid (SGF) or simulated intestinal fluid (SIF), normal saline, deionized water, respectively, and then incubated at 37 °C under moderate stirring.

After 1 h-incubation period, the mean particle size of the resultant emulsion was determined by DLS. In the meantime, the emulsion was ultrafiltered and centrifuged at 8000 rpm for 10 min, the content of sCT in the lower aqueous phase was determined by HPLC method. The leakage percent (LP) of sCT from w/o microemulsions after dilution was then calculated based on the following equation:

$$\text{LP}\ \ (\%) = \frac{\text{mass of sCT in the lower aqueous phase}}{\text{total mass of sCT loaded in microemulsions}} \times 100.$$

By visual inspection criteria, stable systems were identified as those free of any physical changes, such as phase separation, flocculation and/or precipitation.

2.6. Hypocalcemia efficacy assessment

Administrations. 40 normal male Sprague-Dawley rats weighing 200 ± 20 g were equally divided into eight groups (five each) and fasted overnight but allowed access to water ad libitum prior to the experiments. The eight groups of rats were anesthetized with chloral hydrate at a dose of 300 mg/kg and intraduodenally administered (i.d.) with physiological saline, sCT solution in pH 4.0 PBS, sCT solution in pH 4.0 PBS with 1% CP and the sCT microemulsions at a dose of 275 μg/kg, respectively, after small surgery on duodenum. Muscle and skin of the main incision were sutured carefully. Animals were kept conscious during the experiments.

Sampling and determination. 0.2 mL of blood sample was periodically withdrawn from orbital venous plexus of the rats after administration. The blood samples were immediately put in the tubes, and then centrifuged at 8000 rpm for 10 min at room temperature. The serums were collected and stored at −20 °C until analysis. The absorption of sCT was evaluated by monitoring the hypocalcemic efficacy (Chen et al., 2009). Serum calcium levels were measured using a colorimetric calcium assay by UV spectrophotometer (Agilent 8453, Agilent Technologies, UK), which was based on the o-cresolphthalein complexation method as previously described (Qian et al., 1994). The area under the serum calcium concentration–time curve over 24 h ($AUC_{0-24\,h}$) was calculated according to the linear trapezoidal rule. Total calcium decreases (*D*%) in serum were calculated using a modification of the method described by Hirai et al. (1981) as follows:

$$D\% = \left[\frac{AUC_{control,0-24\,h} - AUC_{test,0-24\,h}}{AUC_{control,0-24\,h}}\right] \times 100.$$

The relative pharmacological activity (RPA) of various sCT formulations was calculated after measuring the area above the serum calcium levels versus time curves obtained after intraduodenal administration of sCT formulations using the following equation:

$$\text{RPA} = \frac{AAC_{test}}{AAC_{control}}$$

where AAC_{test} is the area above the serum calcium levels versus time curve resulting from microemulsion formulations and $AAC_{control}$ is the area above the serum calcium levels versus time curve resulting from sCT solution in PBS.

2.7. Statistical analysis

All data were expressed as mean standard deviation (S.D.) unless particularly outlined. The statistical significance of differences amongst more than two groups was determined by one-way ANOVA by the software SPSS 13.0. Values of $p < 0.05$ and $p < 0.01$ were considered to be significant and highly significant.

3. Results

3.1. Optimization of microemulsion composition and formulation of w/o microemulsions

The pseudo-ternary phase diagrams were constructed for MCT/Tween 80–SPC/PG/PBS and MCT/Tween 80–Span 80/PG/PBS systems (Fig. 1) to determine regions of microemulsion formation. In the present study, only the boundary between the single and multi-phase region was identified. As seen from Fig. 1, the profile was similar for the two systems. The regions to the left of the boundary lines were transparent w/o microemulsion regions with lower viscosity, and to the right turbid emulsion regions. In addition, it was observed that the area of microemulsion region increased with the increase of the emulsifier concentration in formulation, which was consistent with previous report (Li et al., 2005). In conclusion, promising w/o microemulsions were developed in this study into which water soluble drugs could be incorporated. Based on the principle of higher solubilization ability and reduced use of surfactant, the optimal formulations ME1 and ME3 listed in Table 1, in which sCT was incorporated 0.10 and 0.060 mg/g, respectively, were therefore selected for use in subsequent studies. Furthermore, HPMC and CP used as absorption enhancer and stabilizing agent were added into the aqueous phase of ME1 and ME3 to formu-

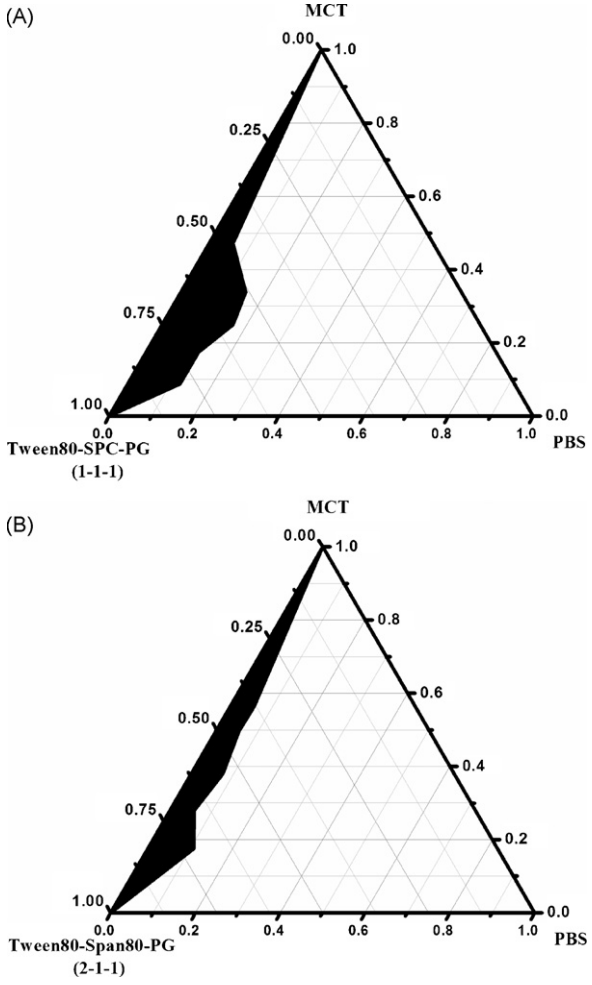

Fig. 1. Pseudo-ternary phase diagrams of medium chain triglyceride (MCT)/Tween 80–soybean phosphatidylcholine (SPC) (1:1)/propylene glycol (PG)/PBS (A) and MCT/Tween 80–Span 80 (2:1)/PG/PBS (B) with w/o microemulsion area (shaded). The ratio (K_m) of emulsifier (Tween 80–SPC and Tween 80–Span 80) to co-emulsifier (PG): (A) 2:1; (B) 3:1 (w/w).

Table 1
Formulations compositions (w/w) and mean particle size of the studied microemulsions ($n = 3$).

Formulation	ME1	ME2	ME3	ME4	ME5
Tween 80:SPC	1:1	1:1	–	–	–
Tween 80:Span 80	–	–	2:1	2:1	2:1
K_m	2:1	2:1	3:1	3:1	3:1
S_m: MCT	6:4	6:4	7:3	7:3	7:3
Aqueous phase	PBS (pH 4.0)	PBS with 1% CP	PBS (pH 4.0)	PBS with 1% CP	PBS with 2% HPMC–0.5% CP
Particle size (nm)	6.0 ± 0.6	9.9 ± 0.4	65.2 ± 2.8	133.5 ± 1.2	90.6 ± 2.5

late ME2, ME4 and ME5 (Table 1), respectively, which were also evaluated in the subsequent studies.

3.2. Particle size of w/o microemulsions

Table 1 summarizes the particle size of the prepared sCT-loaded w/o microemulsions determined by DLS. The mean diameter of the microemulsions ranged from about 6 nm to 134 nm, which suggested that the prepared microemulsions might be favorable to the drug absorption by the intestine with respect to the particle size. Noticeably, the ME1-related microemulsion systems had considerably smaller size compared with the ME3-related systems.

3.3. Physical stability of microemulsions upon dilution

The prepared w/o microemulsion formulations were diluted with excess of different physiologically relevant diluent solutions to evaluate their *in vivo* physical stability. Factors affecting particle size and leakage percent (LP), such as the dilution extent and diluents, were investigated. Fig. 2 summarizes the particle size and leakage percent upon 500-fold dilution of ME1 and ME4 using physiologically relevant aqueous solutions (diluents) with different pH and/or ionic strength. No significant difference in particle size (Fig. 2A) and leakage percent (Fig. 2B) between two diluents with different pH and/or ionic strength was observed for ME4 after dilution, suggesting that at least for ME4, neither the particle size nor the leakage percent was indeed significantly affected by pH and/or ionic strength of the diluents, which was in agreement with the previous report (Constantinides and Yiv, 1995). Additionally, phase separation, flocculation and/or precipitation were not observed. On the contrary, there could be seen significant difference in particle size (Fig. 2A) and leakage percent (Fig. 2B) for ME1 between two diluents with different pH and/or ionic strength, implying that at least for ME1, both the particle size and the leakage percent were significantly affected by pH and/or ionic strength of the diluents.

Effect of the dilution extent by SIF on the particle size and the leakage percent of the inverted microemulsions was also evaluated. As was seen from Fig. 3, upon 200-fold and 500-fold dilution, ME1 and ME2 produced particles with smaller mean size (<67 nm) compared with others (Fig. 3A1 and A2), and ME1 and ME2 after different dilution were transparent by visual inspection. Moreover, the dilution extent did not significantly affect the particle size (Fig. 3A2) and leakage percent (Fig. 3B2) of ME1, whereas the significant increase of the particle size and leakage percent was found for ME2 as the increase of dilution extent (Fig. 3A2 and B2). As for ME3, ME4 and ME5, the similar phenomenon to ME2 was also observed. In addition, the inverted microemulsions ME3, ME4 and ME5 after different dilution were translucent by visual inspection. Noticeably, at 200-fold dilution, ME4 produced particles with a mean diameter 133.9 ± 1.0 nm and LP was the lowest (44.1 ± 5.1%) amongst these inverted microemulsions (Fig. 3B1). The mean diameter of the emulsions upon a higher degree of 500-fold dilution of ME4 was 146.8 ± 2.6 nm, and LP increased up to 62.7 ± 0.6% (Fig. 3 B1) but was the lowest. This suggested the formation of heterogeneous coarse o/w emulsion and/or multiple (w/o/w) emulsion.

Based on these results of LP and nanoscaled particle size at different extent of dilution, it was concluded that ME4 exhibited the best physical stability upon dilution amongst the prepared inverted microemulsions although the system was not completely satisfied, and ME1 was the next in order, whereas ME5 displayed the worst.

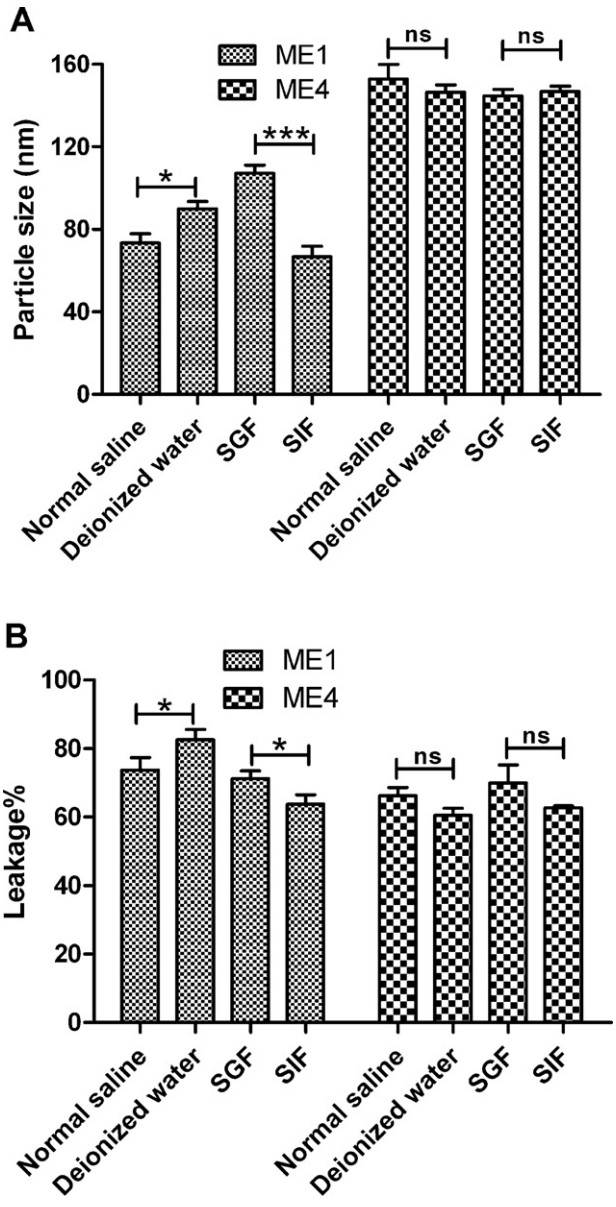

Fig. 2. Effect of diluents on the particle size (A) and leakage percent (LP) (B) of the microemulsions at 500-fold dilution at 37 °C ($n = 3$). SGF: simulated gastric fluid; SIF: simulated intestinal fluid. ns: $p > 0.05$; $^{*}p < 0.05$; $^{***}p < 0.001$.

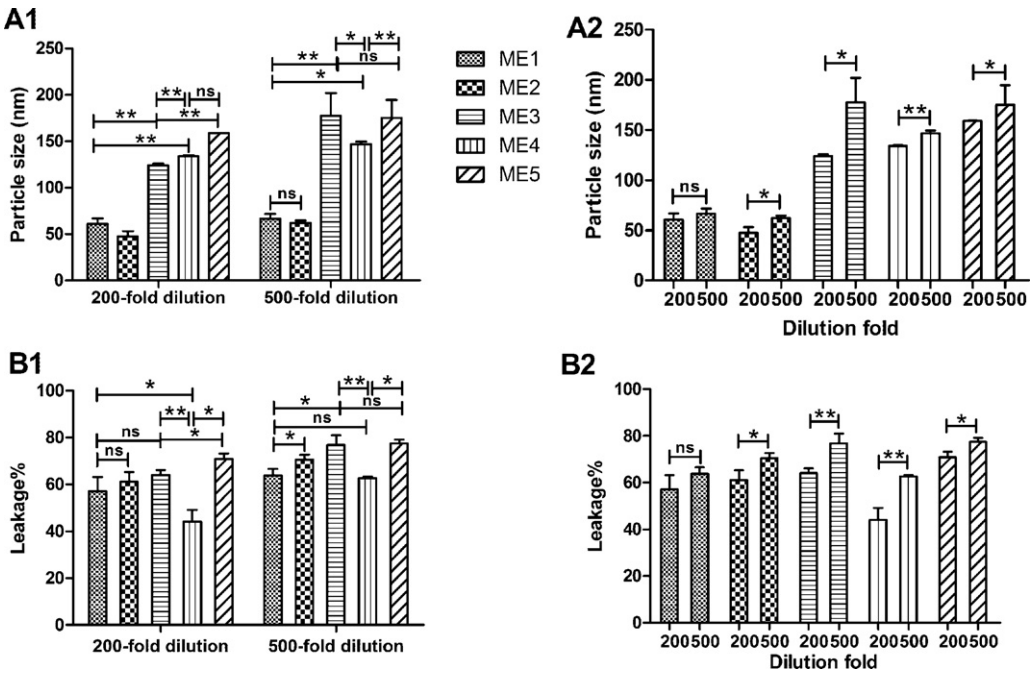

Fig. 3. Effect of the dilution extent on the particle size (A) and leakage percent (LP) (B) of the microemulsions using simulated intestinal fluid (SIF) at 37 °C ($n = 3$). ME1: MCT/Tween 80–SPC/PG/PBS; ME2: MCT/Tween 80–SPC/PG/PBS with 1% Carbopol 980 (CP), ME3: MCT/Tween 80–Span 80/PG/PBS; ME4: MCT/Tween 80–Span 80/PG/PBS with 1% Carbopol 980 (CP); ME5: MCT/Tween 80–Span 80/PG/PBS with 2% HPMC–0.5% Carbopol 980 (CP). ns: $p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$.

3.4. Hypocalcemic efficacy

To examine the influence of w/o microemulsions on the absorption of sCT, the hypocalcemic efficacies of sCT solution in pH 4.0 PBS, sCT-loaded inverted microemulsions were estimated by intraduodenal administration to rats at a single dose of 275 μg/kg. Normal saline was also intraduodenally administered as control. Figs. 4–7 show the mean serum calcium concentrations after administration of test formulations to the duodenum regions. Rats in the saline control group did not show a significant change in serum calcium level (Fig. 4); levels only fluctuated in the range 99–111% of the initial value within 24 h after intraduodenal administration. Although the *D*% value of sCT solution in PBS was only 5.2 ± 1.5% (Table 2), the clear hypocalcemic effect could be observed within the first 8 h following the administration into the duodenum, it significantly decreased the serum calcium levels compared to the saline control (Fig. 4A). This hypocalcemic profile was further and significantly improved by use of ME1 ($p < 0.01$) as shown in Fig. 4B, and the effect was sustained and did not reach to the base-line levels after 24 h administration. Unexpectedly, no significant difference was observed between ME3 and sCT solution in PBS ($p > 0.05$). ME1 and ME3 were found to have *D*% value of 18.7 ± 2.6% and 10.4 ± 4.9% (Table 2), respectively. The RPA calculated from Fig. 4B was 3.59 and 1.99 for ME1 and ME3 (Table 2), respectively.

Next, the absorption enhancement of Carbopol 980 (CP) on sCT solution was assessed. As shown in Fig. 5, the hypocalcemic profile of sCT solution in PBS together with 1% CP without formulating it into microemulsions was similar to that of sCT solution in PBS ($p > 0.05$). The intraduodenal administration of sCT solution in PBS together with 1% CP yielded comparable *D*% value (5.8 ± 1.2%) to sCT solution in PBS (5.2 ± 1.5%) (Fig. 5 and Table 2) and the RPA was only 1.11, which was in accordance with the early report (Baluom et al., 1997). This indicated that the stability of sCT was more predominant for hypocalcemic efficacies after intraduodenal administration as compared to the permeability of sCT to intestine.

Finally, the effect of addition of adhesive polymers CP and HPMC into microemulsions on the hypocalcemic efficacies of sCT-loaded microemulsions was assessed by intraduodenal administering them into rats. Unexpectedly, ME2 was found to have *D*% values of 12.0 ± 2.7% (Table 2) which was significantly lower than that of ME1 (18.7 ± 2.6%) ($p < 0.05$), indicating that ME2 failed to promote the duodenal sCT absorption compared with ME1 although the RPA of sCT was 2.31 for ME2 compared with sCT solution in PBS (Fig. 6 and Table 2). By contrast, the reduction in serum calcium levels was more profound (about 78.5% of calcium basal level at 6 h for ME4, as compared to 77.9% of calcium basal level at 2 h for ME3) and prolonged up to at least 11 h in the case of ME4 (Fig. 7). At 24 h after ME4 dosing, the serum calcium level was still 81.9% of initial calcium level. The hypocalcemic response was significantly improved by ME4 compared with ME3 ($p < 0.05$), and the RPA was improved even more by ME4 (RPA = 4.15). However, the

Table 2
The hypocalcemic efficacy and the relative pharmacological activity (RPA) of sCT after intraduodenal administration of various sCT formulations to rats at a single dose of 275 μg/kg ($n = 5$).

Formulation	Total Ca decrease *D*%	RPA*
sCT solution in pH 4.0 PBS	5.2 ± 1.5	–
sCT solution in pH 4.0 PBS with 1%CP	5.8 ± 1.2[a]	1.11
ME1	18.7 ± 2.6[b]	3.59
ME2	12.0 ± 2.7[b,d]	2.31
ME3	10.4 ± 4.9[a,d]	1.99
ME4	21.6 ± 4.2[b,e,f]	4.15
ME5	9.1 ± 2.2[c,g,h]	1.75

[a] $p > 0.05$ vs. intraduodenal administration of sCT solution in PBS.
[b] $p < 0.01$ vs. intraduodenal administration of sCT solution in PBS.
[c] $p < 0.05$ vs. intraduodenal administration of sCT solution in PBS.
[d] $p < 0.05$ vs. intraduodenal administration of ME1.
[e] $p > 0.05$ vs. intraduodenal administration of ME1.
[f] $p < 0.05$ vs. intraduodenal administration of ME3.
[g] $p > 0.05$ vs. intraduodenal administration of ME3.
[h] $p < 0.01$ vs. intraduodenal administration of ME4.
* The RPA was calculated by comparing the area above the serum Ca level curves (AAC) over 24 h of the formulations to the AAC obtained after the administration of the same dose of sCT solution in PBS.

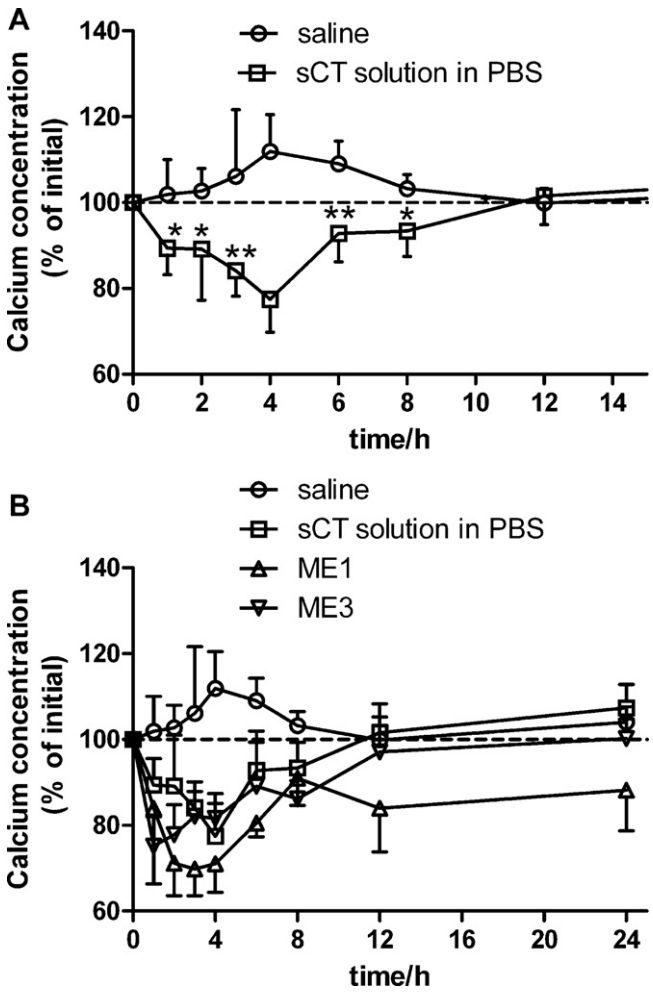

Fig. 4. Serum calcium level profiles after intraduodenal administration of sCT solution in PBS, ME1 and ME3 to rats, respectively, at a single dose of 275 μg/kg ($n = 5$). ME1: MCT/Tween80-SPC/PG/PBS; ME3: MCT/Tween 80–Span 80/PG/PBS. $^{*}p < 0.05$ vs. intraduodenal administration of saline. $^{**}p < 0.01$ vs. intraduodenal administration of saline.

hypocalcemic efficacy of ME5 was remarkably more poor than that of ME4 ($p < 0.01$) and comparable to that of ME3 ($p > 0.05$) (Fig. 7), indicating that HPMC present in microemulsions was unfavorable compared with CP for ME4, which was similar to that reported by

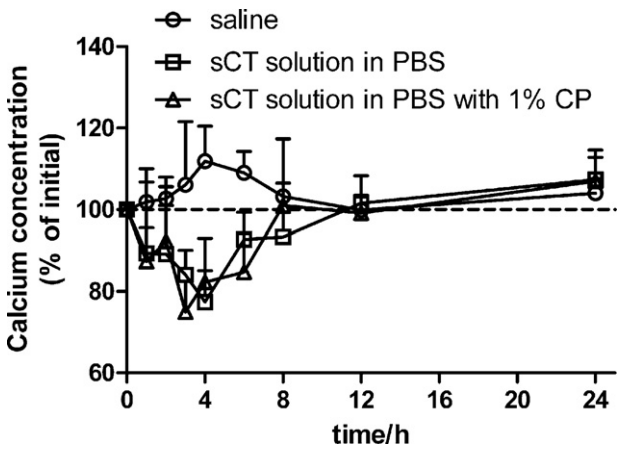

Fig. 5. Serum calcium level profiles after intraduodenal administration of sCT solution in PBS and sCT solution in PBS with 1% Carbopol 980 (CP) to rats, respectively, at a single dose of 275 μg/kg ($n = 5$).

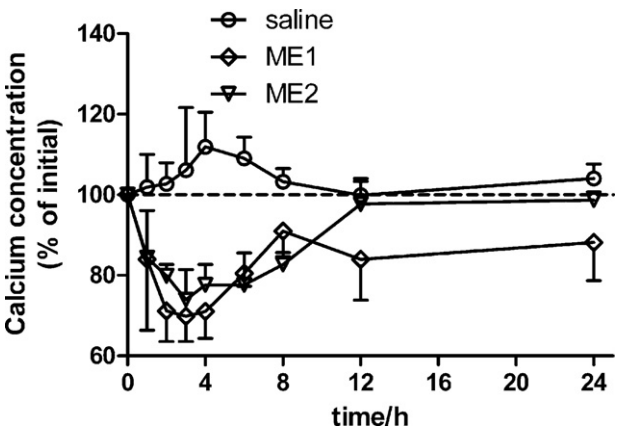

Fig. 6. Serum calcium level profiles after intraduodenal administration of sCT-loaded microemulsions (ME1 and ME2) to rats at a single dose of 275 μg/kg ($n = 5$). ME1: MCT/Tween 80–SPC/PG/PBS; ME2: MCT/Tween 80–SPC/PG/PBS with 1% Carbopol 980 (CP).

Ilan et al. (1996). Taken together, ME4 seemed to be an optimal formulation for intraduodenal delivery of sCT.

4. Discussion

The w/o microemulsion system has been proposed as the feasible alternative to oral administration of hydrophilic protein and peptide drugs owing to the protection of the drugs against enzymatic hydrolysis and acidic degradation, as well as the potential for largely enhanced absorption by absorption enhancers/surfactants included in the system. Nevertheless, there is no effective *in vitro* evaluation method to optimize the w/o microemulsion formulation exhibiting better behavior *in vivo* up to now. Herein five sCT-loaded w/o microemulsion formulations were developed and examined for their ability to improve the intestinal absorption of sCT, and the relation of the physical stability of the formulations upon dilution to the pharmacological activity of sCT loaded in the formulations was also assessed.

In the case of the emulsion system, as there are many components, it was reported that several factors may influence the pharmacological effect of the final formulation (Constantinides et al., 1994, 1996). These factors may also affect many other characteristics including *in vitro* and *in vivo* physical stability and physical

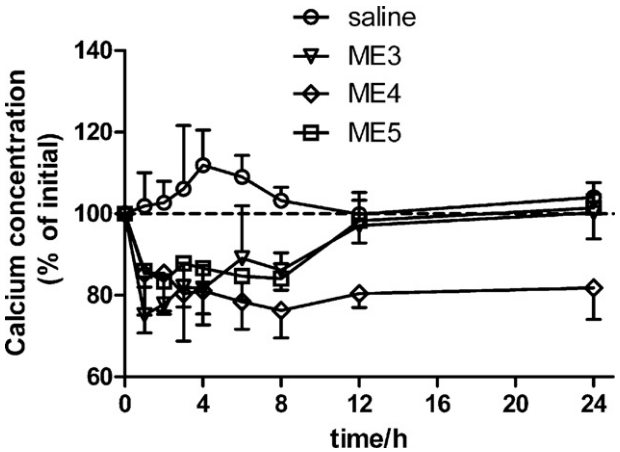

Fig. 7. Serum calcium level profiles after intraduodenal administration of sCT-loaded microemulsions (ME3, ME4 and ME5) to rats at a single dose of 275 μg/kg ($n = 5$). ME3: MCT/Tween 80–Span 80/PG/PBS; ME4: MCT/Tween 80–Span 80/PG/PBS with 1% Carbopol 980 (CP); ME5: MCT/Tween 80–Span 80/PG/PBS with 2% HPMC–0.5% Carbopol 980 (CP).

properties such as the droplet size and the drug leakage percent of the formulation. As for w/o microemulsions with thermodynamic stability, the *in vitro* physical stability is favorable, however, the *in vivo* physical stability is unsatisfied in general owing to phase inversion. Upon dilution with excess aqueous phase, w/o microemulsions are inverted into o/w emulsions/microemulsions and/or multiple w/o/w emulsions with a number of liquid crystalline phases being proposed as possible intermediates during this phase inversion process (Attwood and Florence, 1983), drastic physical changes might have therefore occurred, including changes in particle size and entrapment efficiency of drugs. Taking into account that these nanostructures were developed as carriers for oral administration of peptides, the evaluation of their *in vivo* physical stability was conducted by measuring the particle size and leakage percent of the drug after dilution with excess of aqueous phase with different pH and/or ionic strength. Factors affecting particle size and leakage percent (LP), such as the dilution extent and diluents, were investigated. As shown in Fig. 2, ME1 with an ionic component, such as amphoterics component SPC (Table 1), was sensitive to pH and/or ionic strength changes of diluents, whereas ME4 consisting of non-ionic components (oils and surfactants), such as MCT, Tween 80 and Span 80, and PG (Table 1), was not. In addition, the extent of dilution had a pronounced effect on particle size (Fig. 3A2) and leakage percent (Fig. 3B2) upon dilution of all the prepared microemulsions except ME1. In a general trend, the higher the dilution extent, the bigger the particle size and the higher the leakage percent. Overall, for ME3-related system, the addition of 1% CP in aqueous phase (produced ME4) significantly improved the phase inversion stability ($p < 0.01$ for LP), whereas the presence of HPMC (ME5) could not affect the stability ($p > 0.05$ for LP), which might be due to the stabilization effect of CP on the interfacial film formed by emulsifiers (Liu et al., 2008; Petrovic et al., 2009). On the contrary, for ME1-related system, the addition of 1% CP in aqueous phase (produced ME2) was not beneficial to the phase inversion stability, which was in accordance with the effect of pH on LP of ME1 presented in Fig. 2B. This might be attributed to the dominant fact that the presence of CP lowered the pH value of the aqueous phase, which led to the impairment of the emulsification of SPC (Grit and Crommelin, 1993; Grit et al., 1993; Werling et al., 2008), an amphoteric surfactant. The studies with respect to further improving the phase inversion stability of inverted microemulsions will be carried out.

The absorption enhancing effects of the inverted microemulsions on sCT were evaluated by intraduodenally administering these formulations into anesthetized rats and assessing hypocalcemic response. No significant difference ($p > 0.05$) in the hypocalcemic efficacy was observed between ME3 and sCT solution in PBS (Fig. 4B and Table 2), indicating that ME3 could not enhance the intestinal absorption of sCT compared to sCT solution. On the contrary, there was a highly significant difference ($p < 0.01$) in the hypocalcemic efficacy between ME1 and sCT solution in PBS (Fig. 4B and Table 2). We considered that this seemed to be attributed to the increasing permeability of the intestinal wall to the peptide by putative absorption enhancing agents, i.e. medium chain length fatty acid lipid components present in the microemulsion systems (Lundin et al., 1997; Yamamoto et al., 1997). More importantly, the absorption enhancement effect of ME1 system probably related to the increase of the stability of sCT in the intestinal tract as suggested by Cunha et al. (1997), which was due to the consistent trend of the hypocalcemic efficacy with that of the aforementioned physical stability under dilution for ME1 and ME3 (Fig. 3).

It was reported that the GI absorption of proteinaceous drugs could be improved in the presence of crosslinked acrylic acid derivatives, such as Carbomer (Baluom et al., 1997). In the case of our experiment, it appeared that ME2, formed by adding 1% CP into the aqueous phase of ME1, even significantly decreased the intestinal sCT absorption compared with ME1 ($p < 0.05$) although it highly significantly decreased the serum calcium levels compared to sCT solution in PBS ($p < 0.01$). Interestingly, by comparison with ME3, ME4 significantly increased the intestinal sCT absorption ($p < 0.05$), whereas ME5 did not exhibit the anticipative behavior ($p > 0.05$). In addition, the RPA value of ME4 was bigger than that of ME1 although there was no significant difference in the hypocalcemic efficacy between ME1 and ME4 (Table 2). It was worth noting that these results also related well with the aforementioned facts on the phase inversion stability of microemulsions under dilution (Fig. 3). The more stable the microemulsions under dilution, the better the absorption enhancement effect of the microemulsions. It appeared, therefore, that the formulation of the w/o microemulsions could be optimized by evaluating their phase inversion stability under dilution. In conclusion, ME4, a w/o microemulsion containing CP, was demonstrated to be useful carriers for enhancing sCT absorption via the intestinal tract. Nevertheless, further work is required to determine whether dilution on oral administration may reduce the reported absorption enhancing effects.

5. Conclusion

The w/o microemulsions with a particle size from about 6 nm to 134 nm were developed to effectively encapsulate sCT, a highly hydrophilic polypeptide. The data collected in the present study demonstrated that the sCT-loaded microemulsions exhibited phase inversion and a certain amount of the drug leaked out upon dilution with excess of the dispersed phase. Compared with the control solution, an obviously enhanced hypocalcemic efficacy was observed in rats after intraduodenal administration of the microemulsions. In addition, the presence of 1% CP in aqueous phase of the microemulsion had disparate effect on the absorption of the drug entrapped in microemulsions with different components, and addition of HPMC into aqueous phase of microemulsions was unfavorable although the mechanism was still uncertain. In general, the hypocalcemic efficacy of the prepared sCT-loaded microemulsions was affected by microemulsion components, and this relationship closely corresponded to their physical stability under dilution. Taken together, the w/o microemulsions would be a promising nanocarrier for improving the absorption of sCT by intraduodenal administration.

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