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Preliminary evaluation of a novel oral delivery system for rhPTH1-34: In vitro and in vivo

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ABSTRACT

rhPTH1-34 is clinically used for osteoporosis treatment. However, this peptide drug has no oral bioavailability because of proteolysis and low membrane permeability in gastrointestinal gut. This study explored the possibility of absorption enhancement for rhPTH1-34 through the oral delivery of the microemulsion. The microemulsion (85:15, oil/water) consisting of Labrasol, Crodamol GTCC, Solutol® HS 15, D-α-tocopheryl acetate (6:2:1:1, w/w) and saline water was developed and characterized, including particle size, morphology, drug loading efficiency and permeability, stability and pharmacokinetics. The microemulsion showed high drug loading efficiency (83%) and permeability, and significantly higher resistance to proteolysis in vitro study. The relative oral bioavailability was 5.4% and 12.0% when delivered to gastric and ileum. Besides, osteoporosis rats were induced and treated with oral rhPTH1-34 microemulsion (0.05 mg/kg), injection (0.01 mg/kg) and vehicle, respectively, for 8 weeks. The proximal tibia bone mineral content and density in oral rats (0.188 ± 0.008 g, 0.283 ± 0.014 g/cm^2) was significantly increased compared to the control rats (0.169 ± 0.006 g, 0.266 ± 0.011 g/cm^2), reaching to the sham rats. And the proximal tibia microstructure of oral rats was improved greatly, approaching sham level too. These findings revealed that oral microemulsion may represent an effective oral delivery system for rhPTH1-34.

1. Introduction

Osteoporosis is a systemic disorder characterized by decreased bone mass and the micro-architectural deterioration of bone tissue, leading to bone fragility and increased susceptibility to fractures of the hip, spine and wrist. Osteoporosis is a major and growing public health problem, especially considering the increasing elderly population (Rizzoli et al., 2001). Osteoporosis affects an estimated 75 million people in Europe, the United States and Japan combined. More than 100 million Chinese are affected by osteoporosis (Dobnig, 2004; Lindsay et al., 1997). Women are more susceptible to osteoporosis than men because of their reduced oestrogen synthesis following menopause. In addition, oestrogen deficiency, aging, medication, diet and lack of exercise can all lead to osteoporosis.

Within the past few years, several antiresorptive therapies have been introduced, such as bisphosphonates, hormone replacement therapy, selective oestrogen receptor modulators and calcitonin (Brixen et al., 2004). All of these drugs reduce bone loss and decrease fracture risk by inhibiting the process of resorption of osteoclasts, thus a lack of anabolic properties. In 2002, Forteo (generic name: teriparatide) was developed by Eli Lilly to stimulate bone formation. It contains teriparatide, a recombinant 1-34 N-terminal fragment of endogenous human parathyroid hormone (rhPTH1-34) (Deal and Gideon, 2003). The mechanism of action of PTH1-34 is distinct from that of the antiresorptive drugs; it is an anabolic agent that can stimulate both osteoclasts and osteoblasts. When administrated daily at a low dosage, PTH1-34 stimulates osteoblasts to a greater extent than osteoclasts, resulting in a net increase in bone mass and bone quality (Riggs et al., 1998; Stephan et al., 2007; Zeng et al., 1997). Unfortunately, the full therapeutic and commercial potential of Forteo has not been realised because of the difficulty of daily injections. Efforts have been made to discover alternative methods of delivering PTH. Amongst various routes of drug delivery, the oral route is perhaps the most preferred by both physicians and patients. Oral delivery of rhPTH1-34 represents a major challenge because of acid-induced hydrolysis in the stomach, enzymatic degradation in the gastrointestinal tract and poor membrane permeability. Under normal conditions, a negligible amount of intact rhPTH1-34 is absorbed through the gastrointestinal tract (Goldberg and Gomez-Orellana, 2003; Woodley, 1994).

We have developed an effective oral rhPTH1-34 microemulsion. In this formulation, rhPTH1-34 was solubilised in an amphiphilic medium to overcome enzymatic degradation and the poor permeability of the gastrointestinal tract. In addition, all of the inactive

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ingredients used are well tolerated, have GRAS (Generally Recognized as Safe) status and are accepted for oral delivery.

The aim of this investigation was to evaluate the feasibility of this novel microemulsion as a carrier for the oral delivery of PTH1-34. The formulation was evaluated by various physicochemical parameters, such as particle size, micrographic state, PTH1-34 encapsulation efficiency and drug stability. In addition, the potential efficacy of orally administered PTH1-34 was studied in both healthy and ovariectomized SD rats.

2. Materials and methods

2.1. Materials

rhPTH1-34 (teriparatide acetate) was purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China). Labrasol® was supplied by Gattefosse China Trading Co. Ltd. Crodamol GTCC, D-α-tocopheryl acetate and Solutol® HS 15 were purchased from Beijing Fengli Jingqiu Commerce and Trade Co. Ltd. (Beijing, China). Sodium pentobarbital, pepsin and trypsin were purchased from Sigma. All other chemical reagents were of high purity or HPLC grade.

2.2. Animals

Male and female Sprague Dawley (SD) rats were supplied by the Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China. They were housed in a light- and temperature-controlled room. All animal experiments were approved by the Institutional Animal Ethics Committee of Tsinghua University.

2.3. Cell culture

Caco-2 cells were purchased from Beijing Union Medical College Hospital. Cells were grown on 10 cm-plastic culture disks (Corning) in DMEM supplemented with 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acid solution, 20% (v/v) fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. For transport studies, Caco-2 cells (2.5×10^5 cells/cm^2) were seeded on polycarbonate membranes in a 12-well Transwell. Basolateral and apical sides received 1.5 mL and 0.5 mL culture medium, respectively.

2.4. rhPTH1-34 microemulsion preparation

The rhPTH1-34 formulation was prepared according to the patent (Ma and Zheng, 2011). Briefly, a weighed amount of rhPTH1-34 was dissolved in NaCl solution (0.9%, *w/v*), resulting in solution A. Labrasol (3 g), Crodamol GTCC (1 g), Solutol® HS 15 (0.5 g) and D-α-tocopheryl acetate (0.5 g) were mixed and then magnetically stirred to form a homogenous solution B. Samples were prepared by adding solution A to solution B drop by drop, and the mixture was mixed quickly to form a clear, homogenous PTH1-34-loaded formulation in which the mass ratio of solution A to solution B was 15: 85.

2.5. Size and morphology of the microemulsion

After a 50-fold dilution with distilled ionised water, samples were directly analysed by photon correlation spectroscopy (Zetasizer 3000HS, Malvern Instruments, UK) for particle size. Their morphology was characterized by transmission electron microscopy (TEM) analysis (H-7650; Hitachi, Kyoto, Japan). As indicated in an earlier study (Tagne et al., 2008), after a 50-fold dilution, the samples were applied to mesh copper grids, negatively stained with 10% phosphotungstic acid, and dried for 30 min for TEM analysis.

2.6. Drug encapsulation efficiency of the microemulsion

A fixed amount of rhPTH1-34 microemulsion was diluted to 60 μg/mL with NaCl solution, and 500 μL of the solution was transferred to the sample reservoir of a Nanosep® device with a 10 K MWCO (molecular weight cut-off) (Pall Life Sciences, Michigan, USA). The Nanosep was centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant and the filtrate were diluted appropriately, and the amount of drug in both phases was determined by HPLC. The encapsulation efficiency (EE) was calculated according to the equation:

$$\mathrm{EE} = \frac{W_\mathrm{i} - W_\mathrm{f}}{W_\mathrm{i} \times 100\%},$$

where W_i (in mg/mL) is the amount of initial rhPTH1-34 added and W_f is the amount of rhPTH1-34 in the filtrate receiver of the Nanosep® device.

2.7. Stability of rhPTH1-34 in microemulsion

The physical stability of the formulation stored at 4 °C was evaluated by periodic visual inspection for the presence of macroscopic phase separation, as shown by cloudiness or the formation of two distinct layers. Besides, size distribution and TEM images of the formulation stored at 4 °C were detected after 6 months. Enzymatic stability was assessed with pepsin (1:3000) and trypsin (1:125) individually. An enzyme solution containing 32.0 mg pepsin was prepared by dissolving it in 10.0 mL 0.08 M HCL. The trypsin solution was prepared by adding 5.0 mg trypsin to 10.0 mL Tris buffer (50 mM, pH 8.0). To each Eppendorf tube containing 120 μL pepsin solution, 120 μL of 1.0 mg/mL rhPTH1-34 microemulsion or rhPTH1-34 solution (dissolved in 0.08 M HCl and Tris buffer for pepsin and trypsin solution, respectively) was added. The solution was incubated at 37 °C. At designated time intervals (0, 5, 15, 30, 45, 60, 75, 90 and 120 min), the Eppendorf tubes were withdrawn, and the reaction was stopped immediately by adding 240 μL of 0.1 M NaOH (0.5% TFA to the trypsin solution). The samples were cooled and stored at 4 °C for later RP-HPLC analysis.

2.8. In vitro permeability study

The in vitro permeability of rhPTH1-34 was investigated as described previously (Sha et al., 2005). Caco-2 cells were cultured in a 12-well transwell for 21 days for the rhPTH1-34 permeability study. Complete culture medium was removed from both the apical and basolateral compartments and the monolayer was preincubated with 0.5 mL of pre-warmed HBSS for 30 min at 37 °C. After the preincubation, the media was removed from both compartments. The pristine rhPTH1-34 or rhPTH1-34 microemulsion diluted in HBSS (0.5 mL) was added into the apical side of the monolayer and blank HBSS (1.5 mL) was added to the basolateral side. Samples (0.2 mL) were removed from the basolateral side at given time intervals and blank HBSS (0.2 mL) was added to the basolateral side after sampling. rhPTH1-34 concentration in these samples was analysed by HPLC. Apparent permeability coefficients (Papp) of rhPTH1-34 were calculated according to the following equation:

$$P_\mathrm{app} = \frac{dQ/dt}{AC_0}$$

where dQ/dt indicates linear appearance rate of mass in the basolateral sides (g/mL/s); C_0 indicates initial concentration of rhPTH1-34 in apical side (g/mL); and A indicates surface area (cm^2).

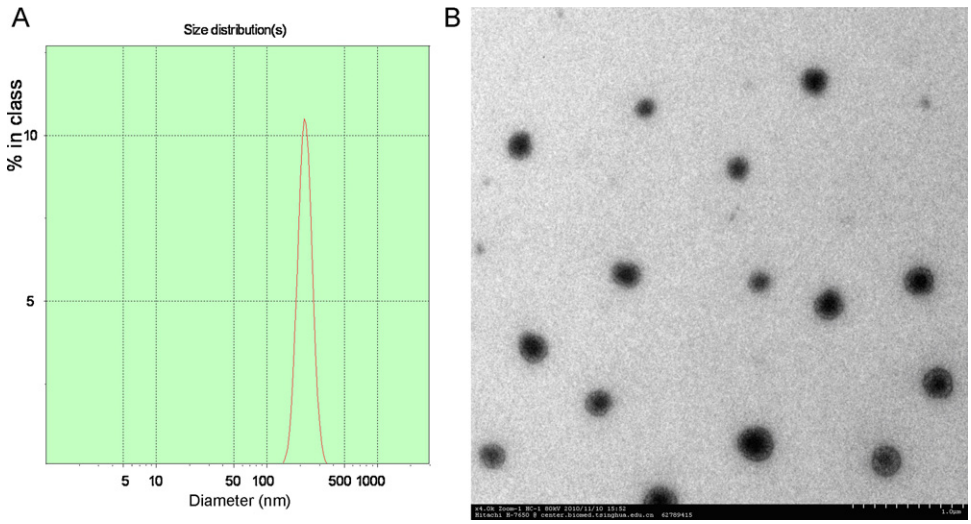

Fig. 1. Characterization of oral rhPTH1-34 microemulsion. (A) Photon correlation spectroscopy analysis of rhPTH1-34 microemulsion. (B) Transmission electron microscope picture of rhPTH1-34 microemulsion. Scale Bar = 1.0 μm.

2.9. Pharmacokinetics evaluation

Male Sprague Dawley rats weighing 180–220 g were fasted overnight and anaesthetised by an i.p. injection of pentobarbital (30–40 mg/kg) before drug administration. For ileum delivery, rats were restrained in a supine position on a board. A small midline incision was made in the abdomen, the ileum was identified, and the dose formulations were administered by syringe. The muscle and skin were then immediately sutured. For gastric delivery, the rats were treated by oral gavage. The rhPTH1-34 microemulsion was prepared as described above. Animals were administrated 0.05 mg/kg rhPTH1-34 microemulsion by ileum ($n = 7$) and gastric ($n = 10$) delivery. Rats ($n = 6$) were subcutaneously injected with 0.01 mg/kg rhPTH1-34 as a control. After treatment, blood samples were collected from the tail vein at 15, 30, 45, 60, 75, 90, and 105 min. Each blood sample was centrifuged at 1000 rpm for 5 min. The serum was harvested (60 μL) and stored −20 °C until being analysed by a PTH (1-34) High Sensitivity EIA kit (ALPCO Diagnostics, Salem, NH).

2.10. Osteoporosis model and efficacy study

As in previous osteoporosis rat model studies (Lasota and Danowska-Klonowska, 2004), ovariectomies were made by two dorsa-lateral incisions approximately 2 cm above the ovaries. The skin of one side was cut with sharp dissecting scissors together with the dorsal muscles, and the peritoneal cavity was thus accessed. The ovary was found surrounded by a variable amount of fat. Both the blood vessels and the fallopian tube near the ovary were ligated with cords, the connection between the fallopian tube and the ovary was cut, and the ovary was removed. Muscles and skins were sutured with a catgut. The other ovary was removed by the same method. Sham rats were subjected to the same protocol, but instead of removing the ovaries, a small clump of fat was taken out.

Ovariectomized SD rats were acclimated for 6 weeks following the ovariectomy. Then vehicle, subcutaneous native rhPTH1-34 injections (0.01 mg/kg), or the oral rhPTH1-34 formulation (0.05 mg/kg) was administered daily following an overnight fast. After treatment for 8 weeks, 6 days a week, the left tibias were collected, wrapped with gauze soaked in saline water, and stored at −20 °C for detection. Proximal tibia bone mineral density (BMD) and bone mineral content (BMC) were measured by DEXA (dual energy X-ray absorptiometry) directly. Then the left tibia was decalcified in formic acid for more than 24 h, and the proximal tibia was fixed and placed in decalcification solution for 6 h. Decalcified proximal tibias were washed with distilled water, dehydrated, embedded in paraffin, and longitudinally sectioned at a thickness of 3 μm. To reveal the microstructure clearly, bone sections were stained with HE staining prior to microscopy.

2.11. Statistical analysis

The pharmacokinetic parameters were determined by WinNonlin software and expressed as the mean ± standard error (S.E.). A paired *t*-test was performed to analyse the differences in BMC and BMD between the treatment groups. We considered a *p*-value of less than 0.05 to be statistically significant.

3. Results

3.1. Microemulsion characterization

As presented in Fig. 1 A, the particle size in the rhPTH1-34 formulation ranged from 140 to 350 nm (with an average of 220 nm). The homogenously distributed and nanoparticulate nature of the oral rhPTH1-34 formulation dispersion particle was further confirmed by TEM studies (Fig. 1B). TEM images also revealed the spherical and uniform nature of the formulation. The images also revealed the core (dark center) of the microemulsion particles and their shell structure. The drug encapsulation efficiency of microemulsified oral rhPTH1-34 was determined according to the formula described in the Methods section. The results indicated a drug encapsulation efficiency of 83%.

3.2. Stability of the microemulsion

The oral rhPTH1-34 microemulsion continued to be transparent and uniform after 6 months when stored at 4 °C. No cloudiness or stratification was observed. As shown by size distribution data (Fig. 2A) and TEM images (Fig. 2B), the particle size and the morphology of our formulation basically remains unchanged after 6 months of storage when compared with that of the microemulsion formulated immediately. Furthermore, the oral microemulsion was able to prevent the protease degradation of rhPTH1-34. Studies have confirmed that rhPTH1-34 can be totally degraded by gastrointestinal proteases in a very short time (Werle et al., 2006).

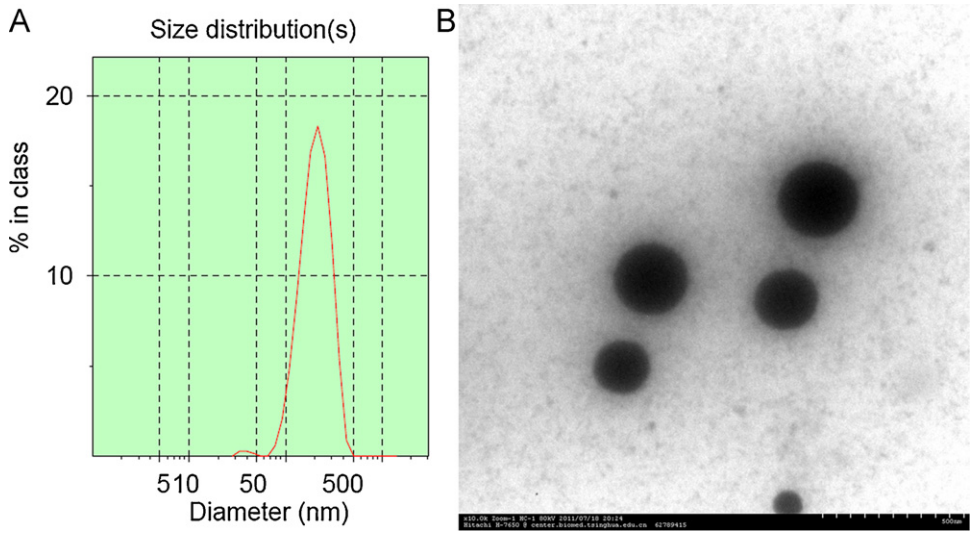

Fig. 2. Charcaterization of oral rhPTH1-34 microemulsion after 6-month storage at 4 °C. (A) Photon correlation spectroscopy analysis of rhPTH1-34 microemulsion. (B) Transmission electron microscopic picture of rhPTH1-34 microemulsion. Scale Bar = 500 nm.

As indicated in Fig. 3, 83% and 64% of intact rhPTH1-34 remained undigested after 5 min of enzyme hydrolysis by pepsin (Fig. 3A) and trypsin (Fig. 3B), respectively. With the extension of pepsin degradation time, there was still a certain amount of rhPTH1-34 protected by the formulation. Also, the formulation can protect rhPTH1-34 from degradation by trypsin. However, without the auxiliary components, rhPTH1-34 can be degraded almost completely within 5 min. Therefore, the oral microemulsion delivery system can protect rhPTH1-34 from degradation by proteases in the gastrointestinal gut.

3.3. *In vitro permeability study*

Caco-2 cell monolayer were widely used as an intestinal epithelial model to study the ability of microemulsion to increase the paracellular transport of rhPTH1-34. As depicted in Fig. 4, both 0.25% (v/v) and 0.5% (v/v) microemulsion are demonstrated to significantly increase the transport of rhPTH1-34 when compared to the rhPTH1-34 control. 0.25% and 0.5% microemulsion could increase the permeability of rhPTH1-34 by 5-fold and 7-fold after 120 min, respectively. Comparably, a prominent increase in the transport of rhPTH1-34 across the Caco-2 monolayer was observed in the 0.5% microemulsion. Treatment with pristine rhPTH1-34 without microemulsion was found to have very minor rhPTH1-34 transport, implying that rhPTH1-34 poorly penetrates the Caco-2 monolayer. In general, the membrane permeability of pristine rhPTH1-34 was low, but microemulsion could increase rhPTH1-34 permeability greatly.

3.4. *Pharmacokinetics evaluation*

As indicated in Fig. 5, the orally delivered rhPTH1-34 formulation was rapidly absorbed and reached its maximum serum concentration within 15 min. Nearly all of the pharmacokinetic parameters of the ileum delivery were comparable to those of s.c. injection, with the exception of C_{max} (136.0 vs. 217.6 pg/mL), as shown in Table 1. The half-life ($T_{1/2}$) was measured to be 16.1 min for gastric delivery and 12.0 min for ileum delivery. In the s.c. group, the $T_{1/2}$ was 12.5 min, and it was close to the $T_{1/2}$ for ileum delivery. The prolonged $T_{1/2}$ for gastric administration may result from early absorption in the upper tract. In terms of the AUC of the s.c. injection, the relative bioavailabilities (BA) of the oral rhPTH1-34 microemulsion were 5.4% and 12.0% for gastric and ileum

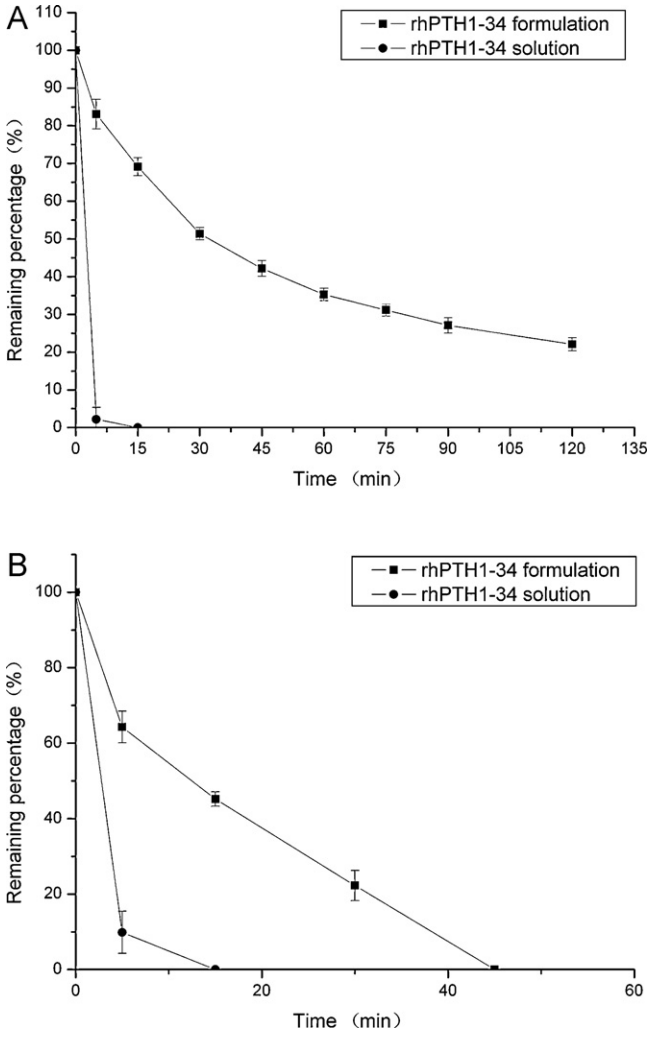

Fig. 3. Degradation of rhPTH1-34 by proteases. (A) Degradation of rhPTH1-34 by pepsin. (B) Degradation of rhPTH1-34 by trypsin. Values represent the mean ± S.E. $n = 3$.

Table 1
Summary results of pharmacokinetic parameters after administration of oral rhPTH1-34 nanoemulsion via a gastric or ileum delivery and rhPTH1-34 injection in SD rats (mean S.E).

Route of drug delivery	Dose (mg/kg)	$T_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	C_{max} (pg/mL)	T_{max} (min)	AUC (min*pg/mL)	BA
Gastric	0.05	5.8 ± 1.4	16.1 ± 2.3	48.4 ± 1.8	13.3 ± 1.2	1995.0 ± 79.4	5.4%
Ileum	0.05	5.1 ± 1.3	12.0 ± 1.7	136.0 ± 5.4	11.0 ± 1.0	4439.8 ± 150.2	12.0%
Injection	0.01	5.3 ± 1.3	12.5 ± 1.7	217.6 ± 8.0	11.4 ± 0.9	7384.6 ± 242.3	100.0%

$T_{1/2\alpha}$: absorption half-life; $T_{1/2\beta}$: elimination half-life; C_{max}: the peak plasma concentration; T_{max}: the time required for the drug concentration to reach the peak; AUC: area under the curve, representing the integral of the plasma drug concentration after it is administered; BA: bioavailability, calculated as (mean AUC oral/mean AUC s.c.) × (dose s.c./dose oral) × 100.

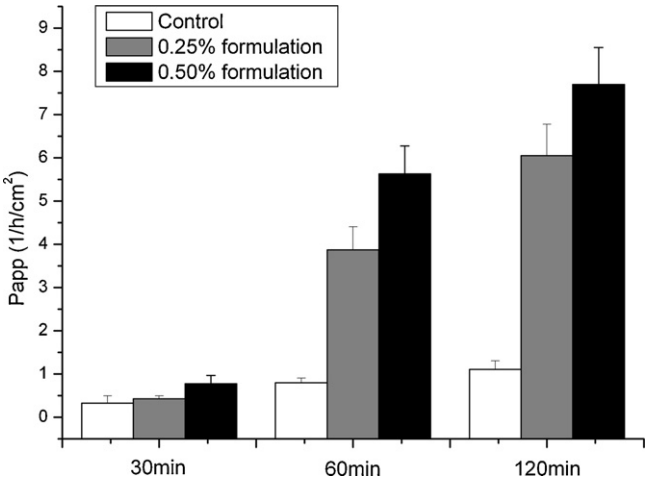

Fig. 4. Effect of microemulsion on the transport of rhPTH1-34 across the Caco-2 cell monolayer. Papp is apparent permeability coefficients. For each group, rhPTH1-34 concentration in the apical side was 3.5 mg/mL. For the control, no microemulsion but rhPTH1-34 was added in the apical side. Values represent the mean ± S.E. $n = 3$.

delivery, respectively. It could be concluded that the ileum delivery of the microemulsion was much better than gastric administration. However, due to the lack of an enteric-coated capsule for rat administration, the oral efficacy study of the rhPTH microemulsion must be delivered by the gastric tract (by oral gavage), which led to a loss of approximately half of the AUC compared to that of the ileum delivery.

As indicated in a previous study (Leone-Bay et al., 2001), rhPTH1-34 is a peptide, and direct oral administration of rhPTH1-34 powder showed no absorption in rats. In the pharmacokinetics study, we have demonstrated that an oral rhPTH1-34 microemulsion leads to better bioavailability by gastric and ileum administration. The increased bioavailability exhibited by the microemulsion may be attributed to inhibited protease degradation and enhanced absorption in the gastrointestinal tract.

3.5. BMC/BMD and microstructure

The potential clinical application of an orally administered formulation for rhPTH1-34 was evaluated in ovariectomized SD rats. As shown in Fig. 6, we observed a significant increase in left proximal tibia BMC and BMD in the oral (0.05 mg/kg) and injection (0.01 mg/kg) groups compared with the control group (OVA animals without any treatment). Compared with the control group, the proximal tibia BMC and BMD increased by 11.0% and 6.4% and by 24.3% and 15.7% in the oral (0.05 mg/kg) and injection (0.01 mg/kg) group, respectively, after 8 weeks of treatment. Although the increase in BMC and BMD in the oral microemulsion-treated rats was less than that in rats given injections, the oral rhPTH1-34 microemulsion can restore the BMC and BMD in ovariectomized rats to levels much closer to those of the sham rats. In injection-treated rats, the BMC and BMD were higher than in the sham control. In addition, the total tibia bone mineral density and content in both the oral and injection groups increased significantly compared to ovariectomized rats.

Moreover, rats given oral rhPTH1-34 microemulsions showed a restored trabecular bone structure, increasing both the volume and connectivity of the trabeculae, as indicated in Fig. 7. Compared to the control group, the oral group greatly improved its cancellous bone mass, and the oral bone micro-architecture was restored to a baseline level. There was increment of the cortical bone

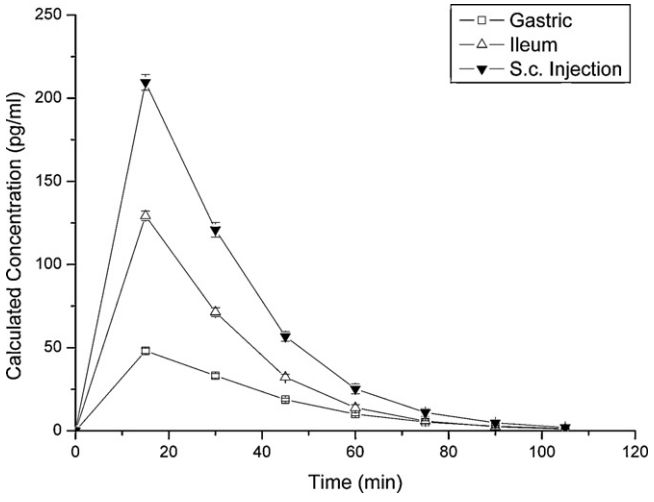

Fig. 5. The serum concentration–time profiles of rhPTH1-34 in normal rats. Rats were treated with a single delivery of the microemulsion via gastric delivery (0.05 mg/kg) or ileum delivery (0.05 mg/kg) and of a s.c. injection (0.01 mg/kg). Values represent the mean ± S.E.

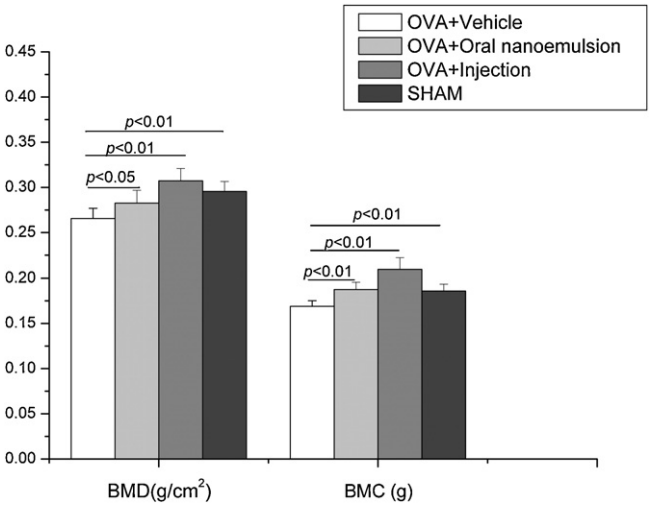

Fig. 6. Dual energy X-ray absorptiometry analysis of left proximal tibia BMD and BMC in rats. Sham rats were subjected to surgery without ovary removal as a control. Values represent the mean ± S.D.

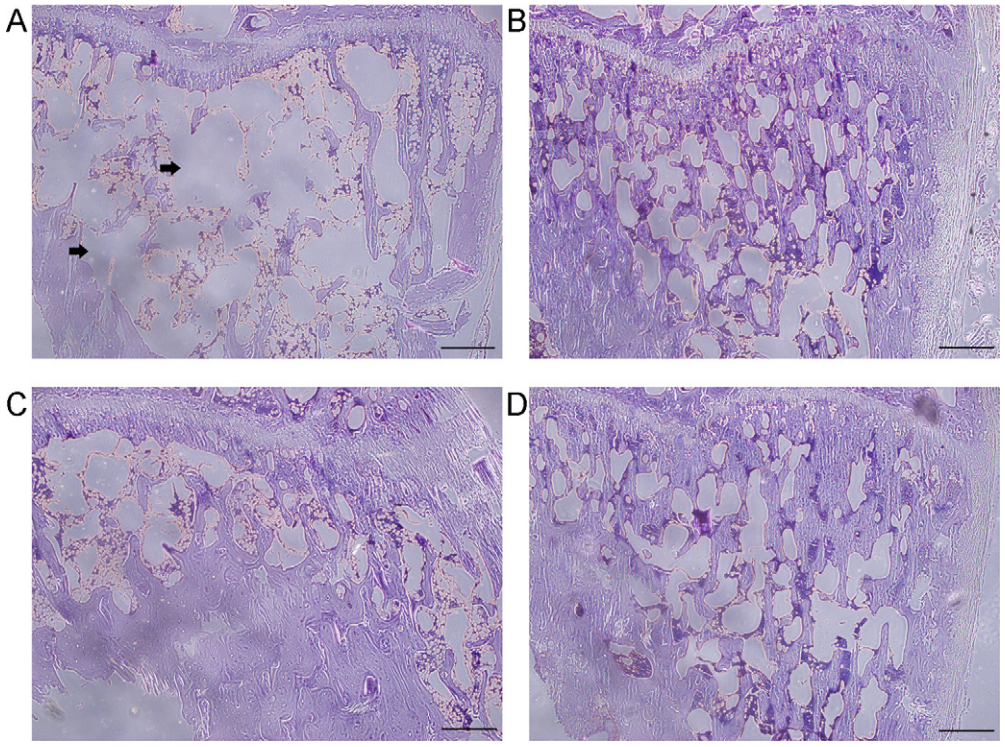

Fig. 7. Longitudinal section of proximal tibia metaphysis by HE staining. (A) Vehicle-treated OVA rats. (B) 0.05 mg/kg oral rhPTH1-34 microemulsion was administered to OVA rats. (C) 0.01 mg/kg rhPTH1-34 injection was administered to OVA rats. (D) Sham rats. All rats were treated 6 days per week for 8 weeks. Note the reduced mass of blank stained bone (arrows), indicative of cancellous osteopenia in the vehicle-treated OVA rats. Treatment of OVA rats with the oral rhPTH1-34 microemulsion significantly increased cancellous bone mass after 8 weeks of treatment to the level of the sham rats. rhPTH1-34 injection-treated rats showed increased cancellous bone formation as well, but the cortical bone mass increase was more obvious. Scale bar = 200 μm.

formation in oral rats as well. Additionally, improvements were observed in OVA rats given rhPTH1-34 injections. A clear improvement in cortical bone thickness was observed in rats administered with injections. In contrast to the oral rhPTH1-34 microemulsion, the injection seemed to increase the formation of cortical bone more than that of trabecular bone in ovariectomized rats. Because the BMD and microstructure returned to baseline levels in oral rats, the oral microemulsion delivery system was demonstrated to be effective in improving bone quality in ovariectomized rats.

4. Discussion

It has been proved that rhPTH1-34 can improve bone quality via stimulation of osteoblasts, instead of inhibition of osteoclasts like antiresorptive drugs(Reszka and Rodan, 2004). Antiresorptive drugs work well as preventive therapies, and moderately increase bone mineral density about 5% over 3-year treatment, but they are not effective enough for patients with established osteoporosis, whose bone mass can loss up to 20% (Chesnut et al., 2000; Riggs et al., 1981; Siris, 2000). Therefore, rhPTH1-34, as an anabolic agent that can increase bone formation actively, is a better choice for osteoporosis patients. However, the protein drug, rhPTH1-34, fails to oral delivery because of low membrane permeability and unstable nature in harsh gastric juice. The available rhPTH1-34 formulation needs daily subcutaneous injection for more than 2 years. Thus, it is expected that a suitable oral pharmaceutical formulation for rhPTH1-34 should be developed to resist proteolysis and increase intestinal absorption.

The microemulsion system consisited of water, non-ionic surfactants and co-surfactants, is in extensive use for its ability to form microemulsion as well as good biological acceptance (Alany et al., 2000). In microemulsion, water-soluble drug is solubilized and mainly encapsulated in water-phase core (Wu et al., 2001). Therefore, it is essential to first characterize microstructure of microemulsion formulation. Many experimental methods, like photon correlation spectroscopy (PCS) (Constantinides et al., 1994), NMR (Kreilgaard et al., 2000) and transmission electron microscopy (TEM) (Zhang and Chan, 2003), have been used to detect microstructure of microemulsion. In this study, particle size and morphology were analysed respectively by PCS and TEM. The TEM result demonstrated a core and coated-shell microstructure for microemulsion and that rhPTH1-34 was encapsulated in the dark core. Besides, HPLC analysis showed that the microemulsion delivery system had high encapsulation efficiency for rhPTH1-34.

The main barriers for macromolecular drug, rhPTH1-34, are instability in gastrointestinal juices and low intestinal permeability. Surfactants were selected to overcome restrictions for rhPTH1-34 oral delivery, including ameliorating stability and increasing intestinal permeability. In this rhPTH1-34 formulation, microemulsion delivery system was developed with Solutol® HS 15, Labrasol, Crodamol GTCC and D-α-tocopheryl acetate which are all pharmaceutically acceptable and commercially available components. Besides, those surfactants have been demonstrated to be good absorption enhancers. Solutol® HS 15 has been demonstrated to increase coenzyme Q10 bioavailability five-fold with Witepsol H35 and Lauroglycol FCC (Nepal et al., 2010). Both the Labrasol and GTCC (medium chain triglycerides) have been confirmed to significantly increase the bioavailability of heparin (Emanuele and Fareed, 1987; Hoffart et al., 2006), insulin (Eaimtrakarn et al., 2002; Watnasirichaikul et al., 2002), and other hydrophilic macromolecules. In our study, the great ability to resist pepsin and trypsin degradation of rhPTH1-34 was confirmed by the in vitro study for microemulsion. In the in vitro membrane permeability study, a significant increase of permeability was demonstrated for rhPTH1-34 encapsulated in microemulsion. In addition, a great enhancement of absorption bioavailability was illustrated in the normal rats after

ileum and gastric delivery of rhPTH1-34 microemulsion compared to rhPTH1-34 solution. Therefore, we assumed that microemulsion can improve rhPTH1-34 stability and increase permeability in gastrointestinal gut to promote rhPTH1-34 absorption. Until now, few oral rhPTH1-34 formulations have been reported. One oral rhPTH1-34 formulation (John et al., 2009; Leone-Bay et al., 2001), with 4-MOAC as enhancer, was demonstrated to have a bioavailability of 5% in rats, 2.1% in monkeys, and 0.3–0.4% in postmenopausal women. In our study, the bioavailability of oral rhPTH1-34 microemulsion was up to 12.0% in rats, when administered by ileum tract, which is 2.5 fold higher than that of previous study.

Furthermore, we designed to study whether rhPTH1-34 encapsulated in microemulsion had the pharmaceutical efficacy after oral administration of rhPTH1-34 microemulsion in osteoporosis rats. The osteoporosis rat model was induced by ovariectomy and administrated with rhPTH1-34 microemulsion by gavage for 8 weeks. Our results have demonstrated that the prepared rhPTH1-34 microemulsion greatly improved bone quality for the osteoporosis rats. First, BMC and BMD in left proximal tibia were detected to increase significantly in oral ovariectomized rats when compared to that of the vehicle-treated ovariectomized rats. Moreover, oral microemulsion restored BMC and BMD very close to the sham rat which is equivalent to the normal rat. Second, micro-architecture of left proximal tibia was observed. In the vehicle-treated control ovariectomized rats, most trabecular bone and cortical bone in proximal tibia was destroyed because of ovariectomy. In case of ovariectomized rats treated by oral delivery of rhPTH1-34 microemulsion, we have observed that the oral microemulsion restored the structure of trabecular bone by increasing both the volume and connectivity of the trabeculae. Also cortical bone in proximal tibia has been restored. Both are approaching the sham rats. These results are consistent with early studies on rhPTH1-34 (Hodsman et al., 2000; Jiang et al., 2003; Mashiba et al., 1995). Thus, hydrophilic bioactive macromolecule, rhPTH1-34 encapsulated in oral microemulsion can greatly ameliorate bone quality for osteoporosis. Besides, microemulsion restored the proximal tibia BMD in ovariectomized rats close to the sham level at dose of 0.05 mg/kg rhPTH1-34 after 8-week treatment. However, the previous rhPTH1-34 formulation was reported to achieve that at a much higher dose of rhPTH1-34 (Leone-Bay et al., 2001). Above all, compared to the previous oral formulation, this novel microemulsion delivery system has advantages both in bioavailability and pharmaceutical efficacy.

The microemulsions have been demonstrated to promote hydrophilic macromolecule absorption in this study and previous works (Constantinides and Scalart, 1997; Constantinides et al., 1994; Prasad et al., 2003). However, the mechanism by which microemulsions enhance peptide absorption in intestinal wall was not well understood. Thus, future works are needed to investigate absorption mechanism of rhPTH1-34 encapsulated in microemulsion. Besides, though obvious side effects, like death and abnormal behavior, weren't observed during long-term efficacy investigation, studies in animals are still needed to further confirm microemulsion safety.

5. Conclusions

In the present study, we have demonstrated that rhPTH1-34 can be effectively encapsulated in oral microemulsion delivery system. And microemulsion can greatly resist gastrointestinal protease degradation of rhPTH1-34 in vitro study. Also, rhPTH1-34 membrane permeability was enhanced greatly in the microemulsion. Pharmacokinetics evaluation in healthy rats showed that this newly formulated oral delivery system can inhibit proteolysis and enhance rhPTH1-34 absorption after gastric and ileum delivery. Besides, relative bioavailability through ileum delivery is much higher than reported oral rhPTH1-34 formulations. Compared with the control rats, a significantly increase in proximal tibia BMC and BMD were confirmed in osteoporosis rat model after oral delivery of rhPTH1-34 microemulsion. Moreover, obviously improvement in bone micro-architecture was observed in oral rats. In conclusion, our study has demonstrated that this newly formulated microemulsion represents an effective delivery system for rhPTH1-34. Also, it shows promise for treating osteoporosis with rhPTH1-34 orally. Additional studies and refinement appear warranted to enhance the use of this novel delivery system to develop a potential agent for osteoporosis in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.08.029.

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