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Investigation of polymeric nanoparticles as carriers of enalaprilat for oral administration

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Abstract

Enalaprilat is a typical angiotensin-converting enzyme inhibitor and is very poorly absorbed from the gastrointestinal tract. The aim of this study was to design and characterize poly-(lactide-co-glycolide) (PLGA) and polymethylmethacrylate (PMMA) nanoparticles containing enalaprilat and to evaluate the potential of these colloidal carriers for the transport of drugs through the intestinal mucosa. Nanoparticle dispersions were prepared by the emulsification–diffusion method and characterized according to particle size, zeta potential, entrapment efficiency and physical stability. Effective permeabilities through rat jejunum of enalaprilat in solution and in enalaprilat-loaded nanoparticles were compared using side-by-side diffusion chambers. The solubility of enalaprilat is very low in many acceptable organic solvents, but in benzyl alcohol is sufficient to enable the production of nanoparticles by the emulsification–diffusion process. The diameters of drug-loaded PMMA and PLGA nanoparticles were 297 and 204 nm, respectively. The concentration of the stabilizer polyvinyl alcohol (PVA) in dispersion has an influence on particle size but not on drug entrapment. The type of polymer has a decisive influence on drug content—7 and 13% for PMMA and PLGA nanoparticles, respectively. In vitro release studies show a biphasic release of enalaprilat from nanoparticle dispersions—fast in the first step and very slow in the second. The apparent permeability coefficient across rat jejunum of enalaprilat entapped in PLGA nanoparticles is not significantly improved compared with enalaprilat in solution.

Keywords: Nanoparticles; Poly-(lactide-co-glycolide); Ex vivo permeability; Entrapment efficiency; Oral drug delivery

1. Introduction

The most convenient route of application for pharmaceuticals is the peroral route. However, the intestinal epithelium may constitute a permeability barrier for the absorption of orally administered drugs. This problem stimulated a search for new strategies to overcome mucosal barriers. Among various approaches, the ability of colloidal systems (liposomes, nanoparticles, polymeric micelles) to cross the intestinal mucosa has been investigated extensively. Nanoparticles have been studied extensively as carriers for oral drug delivery, in order to improve the bioavailability of

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drugs with poor absorption characteristics (Florence et al., 1995), to deliver vaccine antigens to the gut-associated lymphoid tissues (Eldridge et al., 1990), to control the release of drugs, to reduce GI mucosal irritation and to ensure their stability in the GI tract (Sakuma et al., 1997). Despite controversial indications as to the extent and mechanism of transport of these colloidal carriers, there is now no dispute over the fact that particulate uptake does take place, especially via the M-cells of the Peyer's patches and the isolated follicles of the gut-associated lymphoid tissue (GALT), and also via the normal enterocytes. It is still uncertain, however, whether sufficient carrier particles are taken up to produce therapeutically effective levels of drug (Tobío et al., 1998). However, the improvement of drug absorption by nanoparticles can be also a consequence of increased stability of a drug in the GI tract and of the bioadhesion of nanoparticles to the GI mucosa (Sakuma et al., 1998). The residence time of drugs at the absorption site is increased by prolonging their GI transit time and the drug concentration in the vicinity of the epithelial cells raised due to the extended time of contact of the drug with the intestinal mucous layer (Sakuma et al., 1997).

The aim of our study was to investigate the effect of nanoparticles on the transport of a model drug through intestinal mucosa. Enalaprilat, a representative of the angiotensin-converting enzyme (ACE) inhibitors with low bioavailability after peroral administration, was chosen as the model drug to be incorporated into nanoparticles. Poly-(D,L-lactic-co-glycolic acid) 50/50 (PLGA) and methacrylic acid copolymer (PMMA) were used for preparing PLGA and PMMA nanoparticles. Nanoparticles were compared in terms of size, polydispersity, surface potential, encapsulation efficiency and drug release. The possibility of improving the GI absorption of enalaprilat by administering enalaprilat-loaded PLGA nanoparticles was also investigated. For this purpose the permeability through rat jejunum of enalaprilat in solution and in enalaprilat-loaded nanoparticles was evaluated using side-by-side diffusion chambers.

2. Materials and methods

2.1. Materials

Enalaprilat with molecular weight of 384.43 g/mol was supplied by KRKA (Novo mesto, Slovenia) (Fig. 1). D,L–PLGA 50/50 (lactic acid/glycolic acid) copolymer (Resomer RG 502) with molecular weight of 12000 g/mol was purchased from Boehringer (Ingelheim, Germany) and methacrylic acid copolymer Eudragit S100 was obtained from Röhm (Darmstadt, Germany). Polyvinyl alcohol (PVA) having a molecular weight of 27000 g/mol (Mowiol 4-98, Hoechst AG, Germany) was chosen as a stabilizing hydrocolloid. Benzyl alcohol was purchased from Merck (Germany).

2.2. Preparation of nanoparticles

A 10, 15 or 20% w/w aqueous solution of stabilizing hydrocolloid PVA (4 g) was added to a 5% w/w solution of polymer (PLGA or PMMA) in benzyl alcohol (2.1 g) with stirring for 5 min at 15 000 rpm with a rotor-stator homogenizer (Omni International, Gainesville, USA). A water-in-oil emulsion was first obtained, which then underwent a phase inversion on completing the addition of the aqueous colloidal solution. Since benzyl alcohol is miscible at a ratio of 1:25 (w/v) with water, enough water (66 g) was subsequently added to the emulsion in order to allow diffusion of the organic solvent into the water, leading to precipitation of the polymer and formation of nanoparticles (Leroux et al., 1995). In the case of drug-loaded nanoparticles, a predetermined amount of enalaprilat was first dissolved in the benzyl alcohol (2% w/v solution). Dispersions of nanoparticles were kept refrigerated at 8 °C without stirring. Nanoparticles used for permeability studies of enalaprilat across rat jejunum were isolated by lyophilization.

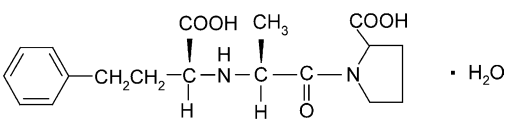

Fig. 1. Chemical structure of enalaprilat.

2.3. Lyophilization of nanoparticle dispersions

Enalaprilat free and enalaprilat-loaded PLGA nanoparticles were freeze-dried in the absence or presence of the lyoprotectant trehalose. Trehalose was added at a weight ratio of 1:1 or 1:2 (nanoparticles (NP): trehalose (T)). Samples were frozen at −70 °C and placed immediately in the freeze-drying chamber (Christ Beta 1-8K, Osterode, Germany). The first drying step was performed at −15 °C for 20 h. In the second, the temperature was increased to 15 °C for 10 h. Sample reconstitution was performed by adding Ringer solution to the dried nanoparticles with manual shaking.

2.4. Determination of drug content in nanoparticles

Since the amount of entrapped drug was determined by an indirect method, the influence of the preparation process on enalaprilat stability was checked. The aqueous solution of enalaprilat was stirred for 5 min at 15 000 rpm and its concentration measured before and after homogenisation. Since the concentrations were found to be comparable, it was concluded that the preparation procedure does not influence the stability of the drug.

The amount of entrapped drug in nanoparticles was determined by measuring the amount of drug in the water phase after filtering a nanoparticle dispersion through a 0.1-μm syringe filter. The clear filtrate was analysed by high performance liquid chromatography (HPLC), using a Hypersil ODS column (5 μm, 250 × 4 mm^2; BIA Separations d.o.o. Ljubljana, Slovenia) at 70 °C. The mobile phase was a mixture of solution A (phosphate buffer pH 2.0, 0.01 M) and solution B (acetonitrile) at a ratio of 60:40 (v/v). The detection wavelength was 210 nm and the flow rate 1 ml/min (Rotar, 2000). The retention time under these conditions was 3.65 min. The difference between the initial amount of drug and the non-entrapped fraction in the filtrate, as measured by HPLC, allowed the entrapment efficiency of the drug by nanoparticles to be determined. Entrapment efficiency is expressed both as drug content (%w/w) and drug entrapment (%w/w), as given by Eqs. (1) and (2), respectively. The mean value of three replicate determinations is reported.

$$\text{Drug content (\%w/w)} = \frac{(\text{total mass of drug used in preparation of nanoparticles} - \text{mass of drug in filtrate}) \times 100}{\text{mass of nanoparticles}} \quad (1)$$

$$\text{Drug entrapment (\%w/w)} = \frac{(\text{total mass of drug used in preparation of nanoparticles} - \text{mass of drug in filtrate}) \times 100}{\text{total mass of drug used in preparation of nanoparticles}} \quad (2)$$

2.5. Characterization of nanoparticles

The mean particle diameter of nanoparticles was estimated by photon correlation spectroscopy (PCS, Zetasizer 3000, Malvern, UK) at a fixed angle of 90°. Samples were diluted with dust-free water to give the recommended scattering intensity of 100 000 counts/s. Each value is the mean of five measurements of 120 s each, divided into ten sub-runs. The diameter is calculated from the autocorrelation function of the intensity of light scattered from particles, assuming a spherical form for the particles. For mean size calculation the cumulant algorithm, which takes into account only one population of particles, was used. The polydispersity index (*PI*) is a measure of dispersion homogeneity and ranges from 0 to 1. Values close to 0 indicate a homogeneous dispersion while those greater than 0.3 indicate high heterogeneity.

The particle charge was quantified as zeta potential by laser Doppler anemometry using a Zetasizer 3000. Samples were diluted with distilled water. Zeta potentials were calculated from the mean electrophoretic mobility by applying the Smoluchowski equation. The results are the mean of five determinations ± standard deviation.

2.6. In vitro release studies

In vitro release studies were performed by incubating a 5-ml dispersion of nanoparticles in 100 ml media (water, gastric fluid pH 1.2 or intestinal fluid pH 7.5, without enzymes). The dispersions were stirred continuously at room temperature. At preselected times, 5 ml of dispersion were withdrawn and filtered through a 0.1-μm filter. The filtrates were analysed for drug content by HPLC.

2.7. Determination of ex vivo permeability of enalaprilat across rat jejunum

The experiments were performed as described previously (Kristl and Tukker, 1998). Rat jejunum was obtained from male Wistar rats (250–320 g). Guidelines and legislative regulations on the use of animals for scientific purposes were followed. After rats were sacrificed, the small intestine was immediately excised and placed into ice-cold, bubbled (carbogen, 95:5 O_2/CO_2) Ringer buffer. The jejunum 20–30 cm distal from the piloric sphincter was used. The tissue was rinsed with ice-cold standard Ringer buffer to remove luminal content and cut into segments, which were opened along the mesenteric border and placed between the two EasyMount side-by-side diffusion chambers with an exposed tissue area of 1 cm^2 (Physiologic Instruments, USA). Care was taken to avoid visible Peyer's patches. During the experiment, the tissue was bathed on both sides with Ringer buffer containing 10 mM glucose at the serosal and 10 mM manitol at the mucosal side. The buffers were gassed with carbogen and thermostated at 37 °C. Tissue integrity was validated by measuring the permeability across the intestinal tissue of a paracellular transport marker, sodium fluorescein. After 20 min equilibration, enalaprilat in solution or enalaprilat-loaded nanoparticles dispersed in Ringer buffer containing fluorescein were added to the mucosal side. The final volume of the solution in each compartment was 2.5 ml. The concentrations of enalaprilat and fluorescein in the donor compartment were 1 mM and 5 μM, respectively.

Samples (250 μl) were withdrawn from the acceptor compartment at 25-min intervals up to 175 min and were replaced with fresh Ringer buffer containing 10 mM glucose. The viability of the tissue was monitored by recording the potential difference (PD) and transepithelial electrical resistance (TEER) (the average values were in the range 20–35 Ω cm^2) every 25 min. TEER and PD were measured by a multichannel voltage–current clamp (VCC MC6, Physiologic Instruments, USA).

Apparent permeability coefficients (P_{app} in cm/s $\times 10^{-6}$) of investigated substances were calculated according to Eq. (3):

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot c_0} \quad (3)$$

where dQ/dt is the steady-state appearance rate on the acceptor side of the tissue, A is the exposed area of the tissue (1 cm^2) and c_0 is the initial concentration of the drug in the donor compartment.

3. Results and discussion

3.1. Solubility of enalaprilat in different solvents

The choice of a particular method of encapsulation of substance in a colloidal carrier is most commonly determined by the solubility characteristics of the drug and polymer (Kristl et al., 1996). We found that the solubility of enalaprilat is very low in many organic solvents such as acetone, chloroform, tetrahydrofuran, but better in benzyl alcohol (22.4 g/l), which was therefore used in nanoparticle preparation. Additionally, benzyl alcohol is widely used because of its acceptability in parenteral formulations (as an antimicrobial substance) and its solubilizing properties. It is miscible with water in the ratio of 1:25 (m/v) which allows polymeric nanoparticles to be prepared by the solvent-diffusion method.

3.2. The influence of PVA concentration on PMMA nanoparticle size

Polyvinyl alcohol (PVA) concentrations of 10, 15 and 20% (w/w) in the external phase of the

Table 1
Influence of the percentage of PVA in the external phase of the primary emulsion on the average diameter (*d*), polydispersity index (*PI*) and zeta potential (*ZP*) of PMMA nanoparticles with entrapped enalaprilat

PVA (%)	*d* (nm)	*PI*	*ZP* (mV)
10	612 ± 45	1.00	-43.3 ± 0.4
15	488 ± 18	0.71 ± 0.28	-46.7 ± 1.1
20	297 ± 2	0.43 ± 0.03	-58.7 ± 2.1

primary emulsion were used to evaluate the effect of stabilizer concentration on the PMMA nanoparticle size. The mean nanoparticle size was found to decrease with increasing PVA concentration (Table 1). The polydispersity index also decreased with increasing PVA concentration indicating a more homogeneous size distribution. It can thus be concluded that with increasing PVA concentration more PVA molecules overlay the surface of the droplets, providing increased protection of the latter against coalescence and resulting in smaller emulsion droplets. Since nanoparticles were formed from the emulsion droplets after solvent diffusion, their size is dependent on the size and the stability of the emulsion droplets. Nanoparticles have a negative surface charge. Commonly, zeta potential is an index of the stability of the nanoparticles. Under most conditions, the higher the absolute value of the zeta potential of the nanoparticles, the larger the charge on their surface, leading to stronger repulsive interactions between the dispersed nanoparticles, and higher stability and more uniform size (Feng and Huang, 2001). Zeta potential and physical stability of PMMA nanoparticles were observed to increase with increasing PVA concentration. The smallest PMMA nanoparticles were obtained using 20% PVA in the external phase of the dispersion, and the same concentration was used for preparing PLGA nanoparticles.

3.3. Physical stability of PMMA and PLGA nanoparticles

In further studies the physical stability of PMMA and PLGA nanoparticle dispersions was studied with 1.1% PVA as stabiliser (20% PVA in the external phase of the primary emulsion). The mean diameter of drug-free PMMA and PLGA nanoparticles was 214 and 183 nm, respectively, and that of drug loaded PMMA and PLGA nanoparticles was 297 and 204 nm. Nanoparticle size and polydispersity were both affected by presence of the drug (Table 2). The mean diameter and the polydispersity index were higher for drug loaded nanoparticles, because both drug and polymer are present in the inner phase of the primary emulsion, influencing the efficiency of the emulsification process.

The physical stability of drug free and drug loaded PMMA nanoparticles and drug free PLGA nanoparticles was very good, although the absolute value of zeta potential decreased. The mean diameter and polydispersity index of these dispersions did not increase after 15 days, while

Table 2
Influence of incorporated drug and storage time on the average diameter (*d*), polydispersity index (*PI*) and zeta potential (*ZP*) of PMMA and PLGA nanoparticles with 20% PVA in the external phase of the primary emulsion

		t (day)	*d* (nm)	*PI*	*ZP* (mV)
	Drug free	1	214 ± 4	0.06 ± 0.04	-65.6 ± 1.4
		15	209 ± 4	0.03 ± 0.04	-47.1 ± 2.1
PMMA NP	Drug loaded	1	297 ± 2	0.43 ± 0.03	-58.7 ± 2.1
		15	298 ± 15	0.05 ± 0.07	-44.6 ± 0.7
	Drug free	1	183 ± 5	0.22 ± 0.04	-51.1 ± 3.5
		15	181 ± 6	0.03 ± 0.01	-17.3 ± 0.6
PLGA NP	Drug loaded	1	204 ± 6	0.13 ± 0.1	-33.4 ± 1.5
		15	730 ± 200	1	-29.0 ± 2.8

Table 3
Efficiency of enalaprilat incorporation expressed as drug content and drug entrapment for PMMA and PLGA nanoparticles with different PVA concentrations in the external phase of the primary emulsion

	PVA (%)	Drug content (% w/w)	Drug entrapment (%)
PMMA NP	10	7.0 ± 0.6	24.5 ± 2.3
	20	6.9 ± 0.4	24.2 ± 1.2
PLGA NP	20	13.2 ± 0.5	46.4 ± 1.7

the diameter of PLGA nanoparticles with incorporated drug increased significantly with time.

3.4. Efficiency of enalaprilat encapsulation

The efficiency of drug incorporation into nanoparticles is generally limited by the large surface area of the latter, as well as by the solubility of the drug in water. These two factors accelerate drug loss into the aqueous phase during nanoparticle preparation (Govender et al., 2000). The theoretical enalaprilat content of PMMA and PLGA nanoparticles was 28.6% (w/w). The results (Table 3) show that more enalaprilat is incorporated into PLGA nanoparticles (130 mg enalaprilat/g nanoparticles) than into PMMA nanoparticles (70 mg enalaprilat/g nanoparticles). The concentration of PVA in the dispersion influences particle size but not drug entrapment. PMMA nanoparticles with 10 and 20% PVA have the same drug content.

3.5. Lyophilization of nanoparticle dispersions

Nanoparticle dispersions were freeze-dried in order to obtain dry formulations for oral administration. The influence of freeze-drying on re-dispersibility was investigated. When PMMA nanoparticles were freeze-dried without a lyoprotectant, full re-dispersion of the particles could not be achieved on rehydration. The mean size of PMMA nanoparticles increased after lyophilization from 297 to 600 nm.

Trehalose, a non-reducing disaccharide of glucose, was chosen as lyoprotectant in this study. The re-dispersibility of PLGA nanoparticles was markedly improved on the addition of trehalose prior to freeze-drying (Table 4). After lyophilization, the mean particle size increased from 204 to 210 or 283 nm depending on trehalose concentration. The best results were obtained when trehalose was used at a weight ratio of 2 g trehalose/1 g nanoparticles.

3.6. In vitro release studies

The in vitro release of enalaprilat from nanoparticle dispersions is illustrated in Fig. 2. The release rate from PMMA and PLGA nanoparticles was monitored in water, gastric fluid pH 1.2 and intestinal fluid pH 7.5 and found to be similar in all media. After 1 min, 60–70% of the drug was released with small differences between different acceptor media. It can be concluded that the released amount of enalaprilat is located in the water phase of the dispersion and on the surface of nanoparticles. These results

Table 4
The mean diameter (d), polydispersity index (PI) and zeta potential (ZP) of PLGA nanoparticles with enalaprilat, before and after lyophilization

	NP:T (w/w)	d (nm)	PI	ZP (mV)
Before lyophilization	0	204 ± 6	0.13 ± 0.1	-33.4 ± 1.5
After lyophilization	0	283 ± 65	0.90 ± 0.09	-43.2 ± 3.5
	1:1	255 ± 30	0.91 ± 0.15	-40.3 ± 2.1
	1:2	210 ± 12	0.59 ± 0.11	-54.5 ± 1.3

The lyoprotectant trehalose (T) was added at a weight ratio of 1:1 or 1:2; nanoparticles:trehalose, (NP:T).

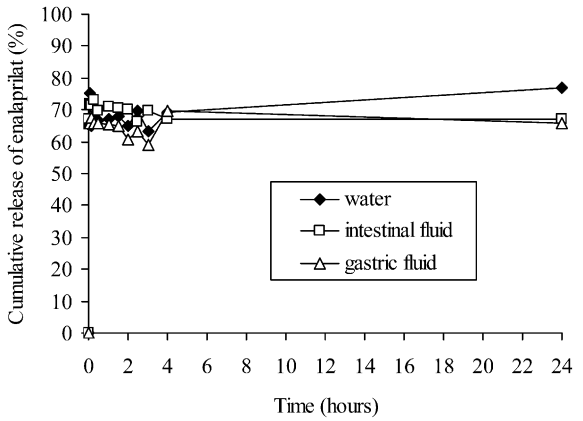

Fig. 2. Cumulative in vitro release of enalaprilat from PLGA nanoparticle dispersions to different media.

agree with the results of enalaprilat entrapment (Table 3). The remaining fraction of the drug was incorporated in nanoparticles and, after 1 day, was still not totally released to the media. The release study was performed over 15 days, after which around 75% of drug was released in water and intestinal fluid and 100% in gastric fluid.

3.7. Ex vivo permeability of enalaprilat across rat jejunum

A variety of techniques may be applied to estimate drug diffusion through the gastrointestinal tract. While in vivo data in humans are most relevant, many different in vitro, ex vivo and in vivo approaches have been used to evaluate nanoparticle uptake in GIT—different animals, cell cultures, isolated tissues, side-by-side diffusion chambers, etc. (Delie, 1998). These methods allow the implicit assumptions to be examined as to whether they are likely to be valid in any particular case. To study enalaprilat permeability we chose side-by-side diffusion chambers. The viable rat jejunum constitutes a suitable model membrane for studying the permeation of free enalaprilat or enalaprilat entrapped in nanoparticles. Viability of the tissue was and maintained and monitored during the experiments. The potential difference and transepithelial electrical resistance of jejunum remained unchanged over 3 h. The measured steady-state appearance rate and the calculated apparent permeability coefficients for paracellular transport of marker fluorescein provided evidence for the integrity of the tissue. In our ex vivo permeability studies PLGA nanoparticles with enalaprilat were tested because they contain higher amounts of entrapped enalaprilat.

Table 5 shows the comparison of the ex vivo permeability of enalaprilat in solution and enalaprilat in nanoparticle dispersions. The results show relatively high intra-individual variability. Although the average P_{app} value for enalaprilat in PLGA nanoparticle dispersion is higher than that for enalaprilat in solution, the differences are not significant as indicated by independent samples t-test ($P = 0.15$). Both enalaprilat and fluorescein exhibit similar values for P_{app}, suggesting that they may have similar transport mechanisms.

There are three possible routes of absorption for nanoparticles: paracellular passage of particles, particle absorption by endocytosis and absorption of particles at the intestinal lymphatic tissues, i.e. Peyer's patches. The absorption of nanoparticles at intestinal lymphatic tissues was not investigated since, during the isolation of the intestinal tissue, care was taken to avoid visible Peyer's patches in segments used for the permeability experiments. It may be concluded that in this study the contribution of nanoparticles to enalaprilat permeability (i.e. via paracellular passage of particles or particle permeation through

Table 5
Ex vivo apparent permeability coefficient (P_{app}) of enalaprilat through rat jejunum when applied in solution (A) or in PLGA nanoparticle dispersion (B)

A		B	
P_{app} (enalaprilat)	P_{app} (fluorescein)	P_{app} (enalaprilat)	P_{app} (fluorescein)
2.88	4.35	4.07	4.14
2.52	3.74	4.25	4.03
4.46	5.26	5.50	6.83
3.29 ± 1.03	4.45 ± 0.76	4.61 ± 0.78*	5.00 ± 1.59

P_{app} ($cm/s \times 10^{-6}$) values for permeability of transport marker fluorescein are also given. The concentrations of enalaprilat and fluorescein in the donor compartment were 1 mM and 5 μM, respectively.

* Non-significant, $P = 0.15$ as indicated by independent samples t-test.

endocytosis) was negligible, since the permeability of enalaprilat in the presence of nanoparticles was not significantly improved compared with enalaprilat alone.

4. Conclusions

Enalaprilat loaded PMMA and PLGA nanoparticles were prepared by the emulsification-diffusion method. More enalaprilat is incorporated in PLGA nanoparticles (130 mg enalaprilat/g nanoparticles) than in PMMA nanoparticles (70 mg enalaprilat/g nanoparticles). The concentration of stabilizer PVA in the dispersion has an influence on particle size but not on drug entrapment. The mean nanoparticle size and the polydispersity index were found to decrease with increasing PVA concentration. By comparing the ex vivo transport through isolated rat jejunum of enalaprilat in solution with that of enalaprilat in nanoparticle dispersions, it can be concluded that the gastrointestinal permeation of enalaprilat cannot be significantly improved by the administration of enalaprilat-loaded PLGA nanoparticles.

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