

Liquid filled nanoparticles as a drug delivery tool for protein therapeutics

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Abstract

In the present study, an attempt was made to study the feasibility of nanoparticulate adsorbents in the presence of an absorption enhancer, as a drug delivery tool for the administration of erythropoietin (EPO) to the small intestine. Liquid filled nano- and micro-particles (LFNPS/LFMPS) were prepared using solid adsorbents such as porous silicon dioxide (Sylysia 550), carbon nanotubes (CNTs), carbon nanohorns, fullerene, charcoal and bamboo charcoal. Surfactants such as a saturated polyglycolysed C8–C18 glyceride (Gelucire 44/14), PEG-8 capryl/caprylic acid glycerides (Labrasol) and polyoxyethylene hydrogenated castor oil derivative (HCO-60) were used as an absorption enhancer at 50 mg/kg along with casein/lactoferrin as enzyme inhibitors. The absorption of EPO was studied by measuring serum EPO levels by an ELISA method after small intestinal administration of EPO-LFNPS preparation to rats at the EPO dose level of 100 IU/kg. Among the adsorbents studied, CNTs showed the highest serum EPO level of 62.7 ± 11.6 mIU/ml. In addition, with the use of casein, EPO absorption was improved, C_{\max} 143.1 ± 15.2 mIU/ml. Labrasol showed the highest absorption enhancing effect after intra-jejunum administration than Gelucire 44/14 and HCO-60, 25.6 ± 3.2 and 22.2 ± 3.6 mIU/ml, respectively. Jejunum was found to be the best absorption site for the absorption of EPO from LFNPS. The use of CNTs as LFNPS, improved the bioavailability of EPO to 11.5% following intra-small intestinal administration.

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1. Introduction

Erythropoietin (EPO) is a sialoglycoprotein hormone produced primarily by the kidney [1,2]. EPO regulates red blood cell production through stimulation of erythropoiesis [3]. Since the innovation of recombinant DNA technology, the pharmaceutical industry is capable of producing highly purified therapeutic protein and peptide drugs. However, their major route of administration still remains to be the parenteral route.

With the use of recombinant DNA technology, recombinant human EPO could be manufactured in large quantities for therapeutic purposes [4,5]. Recombinant human EPO has a molecular weight of 30 kDa, and is similar in structure, composition and biological activity to those of natural EPO isolated from human urine [6,7]. EPO is used in a number of clinical circumstances to treat anemia due to renal failure, cancer, bone marrow transplantation, AIDS, etc. [8–12]. Presently, EPO is administered either as an intravenous or subcutaneous injection 2–3 times a week, depending on the patient requirement. Except for these parenteral routes of administration, no other patient friendly routes are presently available, although studies on various other routes have been carried out such as the oral, rectal and intranasal [13–15].

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Oral route is considered to be the most attractive and convenient route of drug delivery including high patient compliance. However, a series of barriers have to be overcome in achieving a successful oral delivery. The barrier includes, low oral bioavailability (BA), i.e. poor membrane permeability and enzymatic degradation in the GIT [16–18]. Several drug delivery systems have been studied in the recent past years to achieve a successful BA following their oral administration. However, little success has been achieved in terms of commercialization. Drug carrier systems studied includes liposomes [19], nanoparticles [20], micro-spheres [21], hydrogels [22], mucoadhesive systems [23,24] and micro-emulsions [25,26].

In our present study, we have made an attempt to improve the absorption of EPO using porous adsorbents such as carbon nanotubes (CNTs), Sylysia-550[®], charcoal, bamboo charcoal, fullerene and carbon nanohorns as a drug carrier and Labrasol[®], Gelucire[®] 44/14 and HCO-60[®] as absorption enhancers. Recently, we have reported the usefulness of porous adsorbents for the oral delivery of lansoprazole [27]. CNTs have gained attention since its discovery by Iijima [28] for its various application [29]. However, no specific report to our knowledge exists on the use of CNTs for the purpose of oral drug delivery. CNTs are finite carbon structure consisting of needle-like tubes. These are produced using an arc-discharge evaporation method similar to that used for fullerene synthesis. The needles grow at the negative end of the electrode used for the arc discharge [28]. CNTs can be broadly classified into two categories, i.e. multi-walled nanotubes (MWNTs) and single-walled nanotubes (SWNTs). MWNTs comprises of carbon sheets co-axially arranged in a cylindrical shape. The number of co-axial tubes range from 2 to 50 and their diameter ranges between 1.4 and 100 nm [28,30]. Unlike MWNTs, SWNTs are formed in the gas phase [31]. SWNTs show a diameter range from 0.4 to 3 nm [30]. Invariably, both CNTs are capped at the ends. However, opening of CNTs has been reported and has been shown to be capable of obtaining capillarity-induced filling [32–34] (Fig. 1a). In our present study, we have used SWNTs that are open-ended.

Carbon nanohorns are composed of graphite carbon atom structure similar to CNTs. The only difference is, they have an irregular horn-like shape (graphitic cones) (Fig. 1b). Each tube has a diameter of 2–3 nm. The advantage of carbon nanohorns is that upon aggregation, they form a secondary particle (100–120 nm in size), which provides an extremely large surface area and is easy for the gas/liquid to penetrate inside [35–37]. The use of CNTs conjugated to protein has been reported by Kam et al. [38]. They have studied the uptake of CNT–protein conjugate by HL-60 and human T cells. Pantarotto et al. [39] have reported the usefulness of

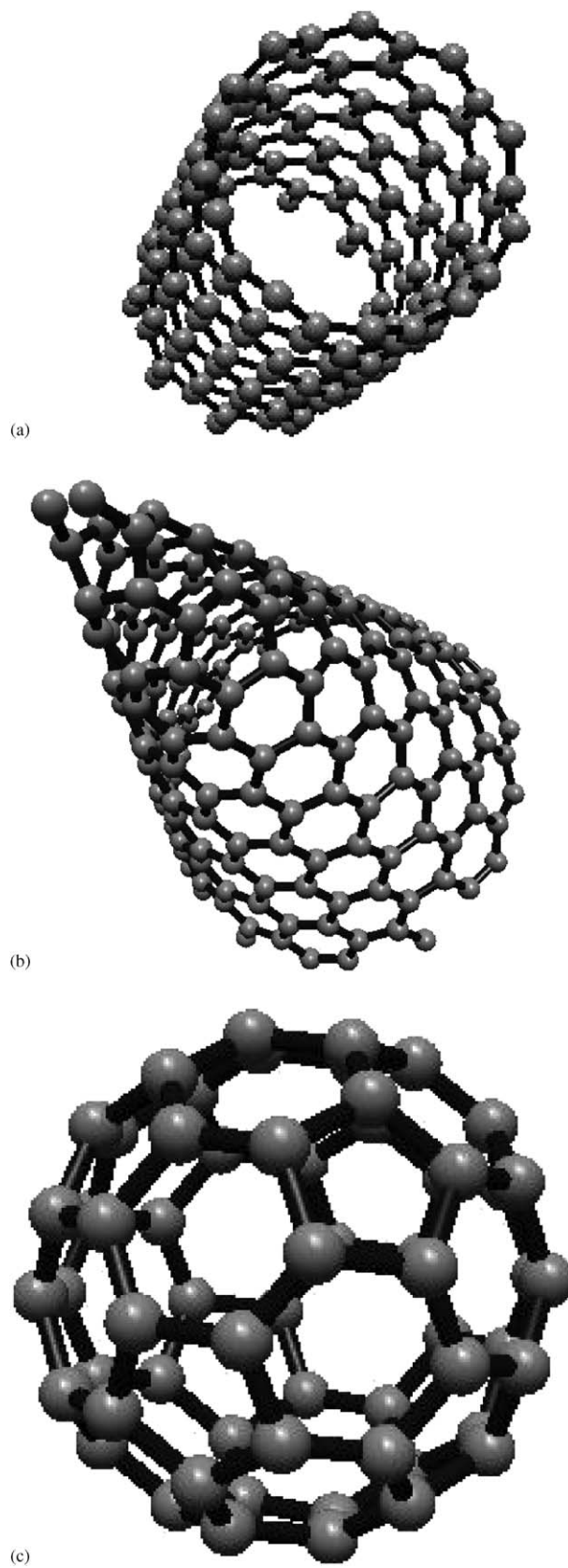


Fig. 1. Computer generated images of (a) carbon nanotube, (b) carbon nanohorn structure and (c) fullerene (Bucky ball structure).

functionalized CNTs (f-CNTs) for the delivery of plasmid DNA. The biomedical application of f-CNTs has been well reviewed by Bianco et al. [40]. Recently, Murakami et al. [41] have reported the usefulness of carbon nanohorns for the purpose of sustained drug delivery. In our present study, liquid filled nanoparticle system (LFNPS) was studied as an oral protein delivery system alongside with liquid filled micro-particle system (LFMPS).

2. Experimental

2.1. Materials

CNTs, fullerene (SES Research, Houston, USA), carbon nanohorns (ARV, Aichi, Japan), charcoal (30–60 mesh, Nacalai Tesque, Kyoto, Japan), bamboo charcoal (30–60 mesh, in-house preparation), porous silicon dioxide (Sylsisa 550, Fuji Sylsisa, Japan), Labrasol[®] and Gelucire[®] 44/14 (Gattefosse, Lyon, France), polyoxyethylene hydrogenated castor oil derivative (HCO-60[®]) (Nikko Chemical, Tokyo, Japan), sodium starch glycolate (Explotab[®], Edward Mendell Co. Inc., New York, USA) was obtained through Kimura Sangyo Corporation Ltd. (Tokyo, Japan), lactoferrin (from bovine milk) and casein (Wako Pure Chemical Industries, Osaka, Japan) were also obtained. All other materials used were of reagent grade and were used as received. A commercially available erythropoietin injection (ESPO[®] 24,000 IU/0.5 ml) marketed by Sankyo Corporation (Tokyo, Japan) was used.

2.2. Animals

Male Wistar rats used in the present study were obtained from Nippon SLC (Hamamatsu, Japan) and standard solid meal of commercial food (LabDiet[®]) was purchased from Nippon Nousan (Yokohama, Japan).

2.3. Preparation of EPO-LFNPS

Adsorbent and absorption enhancer were weighed into a bottle, and mixed well (in the case of Gelucire 44/14 and HCO-60, after they were melted by warming them in a water bath at 45 °C). To this mixture, Explotab was added and mixed well using a micro-spatula so as to bring uniform distribution of the contents. Finally, EPO solution was added to this mixture and mixed. Formulation details are as described in Table 1.

2.4. Effect of enzyme inhibitor

To study the effect of enzyme inhibitor on the absorption of EPO from the small intestine, casein and lactoferrin were compared. Formulation was prepared as described under Section 2.3 to which casein or lactoferrin were added (Table 1).

Table 1
Formulation of the test preparations containing EPO used in this study

Formulation code	Adsorbent	Adsorbent (mg/kg)	Surfactant (50 mg/kg)	Enzyme inhibitor (25 mg/kg)	Additive (2.5 mg/kg)	Dosage form	Type of delivery system	EPO dose (IU/kg)	Administration site/route
A	Sylsisa 550	30	Labrasol	—	—	Solid	LFMPS	100	Jejunum
B	Carbon nanotube	5	Labrasol	—	—	Solid	LFNPS	100	Jejunum
C	Carbon nanohorn	18	Labrasol	—	—	Solid	LFNPS	100	Jejunum
D	Fullerene	150	Labrasol	—	—	Solid	LFNPS	100	Jejunum
E	Charcoal	58	Labrasol	—	—	Solid	LFMPS	100	Jejunum
F	Bamboo charcoal	65	Labrasol	—	—	Solid	LFMPS	100	Jejunum
G	Carbon nanotube	5	Labrasol	Casein	Explotab	Solid	LFNPS	100	Jejunum
H	Carbon nanotube	5	Labrasol	Lactoferrin	Explotab	Solid	LFNPS	100	Jejunum
I	Carbon nanotube	5	Gelucire 44/14	Casein	Explotab	Solid	LFNPS	100	Jejunum
J	Carbon nanotube	5	HCO-60	Casein	Explotab	Solid	LFNPS	100	Jejunum
K	—	—	Labrasol	—	—	Solution	—	100	Jejunum
L	—	—	—	—	—	Solution	—	100	Jejunum
M	—	—	—	—	—	Solution	—	50	Intravenous

2.5. Absorption studies of EPO following administration of LFNPS to rat small intestine

Absorption studies were carried out with male Wistar rats (380–400 g body weight). Rats were fasted 12–16 h with access to water *ad libitum*. The rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The hairs on the abdominal region were shaved and a 3 cm midline incision was made without damaging other internal organs. The small intestine was exposed and based on the site of administration; a small incision was made in the duodenum/jejunum/ileum, where the blood supply was sparse. Each rat was administered with LFNPS containing EPO equivalent to an EPO dose of 100 IU/kg. The incision was sutured and then sealed carefully, not to close the intestinal flow using a synthetic adhesive, Aron alpha[®] (Sankyo Corporation, Tokyo, Japan). The sealed area was checked for any bleeding and then placed back into the abdominal cavity of the rat. The abdominal cavity was then sutured. The body temperature of the rats was maintained at 37 °C by heating with a lamp over the animals. Blood samples (0.35 ml) were collected from the right jugular vein at 1, 2, 3, 4, 5 and 6 h after administration in the case of small intestinal administration with LFNPS. In order to determine the BA, EPO solution was injected into the left jugular vein, at a dose of 50 IU/kg. Blood samples were collected from the right jugular vein at 2, 20, 40, 60, 120, 180, 360 and 480 min after administration. A control study was carried out by direct administration of EPO solution with and without absorption enhancer to the jejunum. A blank blood sample was collected prior to the administration of the formulation/*i.v.* injection. The animals were kept anaesthetized throughout the experiment. The collected blood samples were allowed to clot by standing at room temperature for 30 min and serum samples were obtained by centrifugation at 12,000 rpm for 15 min at 8 °C using Kubota 1700 centrifuge (Kubota, Tokyo, Japan). The serum samples were collected and stored at –80 °C until analysis. Experiment on animals was carried out in accordance with the Guidelines for Animal Experimentation in Kyoto Pharmaceutical University.

2.6. Serum EPO analysis by ELISA

The serum EPO level was determined by an ELISA method. Twenty microlitre of the serum sample was used for analysis. The method involved the use of a standard EPO ELISA kit (Roche Diagnostics GmbH, Germany). The kit was slightly modified in the case of calibration standard samples wherein the use of ESPO at the concentration range as mentioned in the standard assay kit was used. This was done in order to overcome any difference between the supplied standard and the EPO used in the absorption studies. Accuracy of the standard concentrations was compared with the standard concentration supplied along with the kit. Accuracy level was found to be greater than 95%. All other reagents and procedure were used/carried out as mentioned in the supply manual. The ELISA plate was placed on a plate-shaker at 300 rpm/3 h/25 °C (Titramax 101, Heidolph Instruments, Germany), and the ELISA plate was washed using a plate washer, Dia-washer II (Dia-Iatron Co., Ltd., USA). Finally,

absorbance was measured at 450 nm using a micro-plate reader (MTP-300 micro-plate reader, Corona Electric, Japan).

2.7. Pharmacokinetic analysis

Pharmacokinetic parameters were determined from the serum EPO concentrations vs. time data by a non-compartmental pharmacokinetic analysis method using WinHARMONY software developed by us [42]. The maximum drug concentration (C_{\max}) and the time to reach maximum concentration (T_{\max}) were determined from the authentic serum concentration–time data. The area under the plasma drug concentration vs. time curve (AUC) after oral administration was calculated using the linear trapezoidal rule up to the last measured drug concentration and the per cent BA was calculated by using the following equation:

$$\% \text{ BA} = \left(\frac{\text{AUC}_{\text{oral}}}{\text{AUC}_{\text{i.v.}}} \right) \left(\frac{\text{Dose}_{\text{i.v.}}}{\text{Dose}_{\text{oral}}} \right) \times 100. \quad (1)$$

2.8. Statistics

All values are expressed as their mean \pm standard error (SE). Levels of significance were evaluated using ANOVA with Fisher's PLSD multiple range test.

3. Results

3.1. Effect of adsorbents on serum EPO levels

Porous adsorbents were used in the present study as a drug carrier system for oral administration of EPO. The efficiency of the drug carrier system along with absorption enhancer was evaluated by determining the serum EPO level vs. time curve. At first, different adsorbents were studied in the presence of Labrasol as absorption enhancer. The absorption enhancer and absorption site were chosen based on a pilot study. The amount of adsorbent varied in each formulation as their efficiency to hold (adsorb) the absorption enhancer and drug solution varied. The final formulation was solid mass, which could be easily distributed with minimum amount of fluid present in the small intestine. The formulation containing EPO was administered to the jejunum part of the small intestine and their serum EPO levels were measured (Fig. 2). CNTs (formulation B), when used as adsorbent, showed the maximum serum EPO level, mean C_{\max} 62.7 ± 11.6 mIU/ml followed by fullerene (formulation D), charcoal (formulation E), bamboo charcoal (formulation F), Sylsysis 550 (formulation A) and carbon nanohorns (formulation C) which showed mean C_{\max} of 50.0 ± 11.4 , 40.4 ± 13.1 , 34.1 ± 7.5 , 25.9 ± 3.7 and 18.75 ± 5.3 mIU/ml, respectively. Table 2 shows the pharmacokinetic parameters of EPO calculated by non-compartmental pharmacokinetic analysis. The BA of EPO from various adsorbent system studied were found to be 6.7%, 5.7%, 3.9%, 4.8%,

5.3% and 4.0% for CNTs, fullerene, charcoal, bamboo charcoal, Sylysia 550 and carbon nanohorns, respectively. Since the highest BA was obtained with CNTs, all further studies were carried out using CNTs as an adsorbent.

3.2. Effect of enzyme inhibitors

To help the increase of absorption of EPO from the rat small intestine and to keep them protected from the intestinal enzymes, we used two different enzyme inhibitors: casein and lactoferrin. The mean serum EPO C_{max} obtained with the use of casein (formulation G) as enzyme inhibitor was 143.1 ± 15.2 mIU/ml while with lactoferrin (formulation H) C_{max} was 71.8 ± 14.9 mIU/ml (Fig. 3). However, when CNTs was

used without any enzyme inhibitor (formulation B), the C_{max} was 62.7 ± 11.6 . The BA was 7.4% and 11.5% for lactoferrin and casein, respectively, while it was 6.7% in the absence of an enzyme inhibitor. Henceforth, all further studies were carried out using casein as enzyme inhibitor in the formulation, irrespective of the absorption enhancer used.

3.3. Effect of absorption enhancer

The effect of absorption enhancer on the absorption of EPO was studied using CNTs as the adsorbent, where Labrasol, Gelucire 44/14 and HCO-60 were used as absorption enhancer. Following the intra-jejunal administration of CNTs containing various absorption enhancers, the mean C_{max} of EPO was 143.1 ± 15.2 , 25.6 ± 3.2 and 22.2 ± 3.6 mIU/ml with Labrasol (formulation G), Gelucire 44/14 (formulation I) and HCO-60 (formulation J), respectively (Fig. 4). Labrasol as an absorption enhancer showed the highest BA of 11.5% while Gelucire 44/14 and HCO-60 showed 3.5% and 3.6% of BA, respectively (Table 2).

3.4. Effect of administration site

Duodenum, jejunum and ileum were chosen as the administration site. The mean C_{max} obtained following administration of CNTs containing Labrasol as absorption enhancer in the case of duodenum was 28.5 ± 2.2 mIU/ml, while jejunum and ileum showed a C_{max} of 143.1 ± 15.2 and 25.6 ± 3.2 mIU/ml, respectively (Fig. 5). The mean $AUC_{0-\infty}$ in the case of jejunum administration was 256.3 ± 9.7 while it was 123.7 ± 4.2

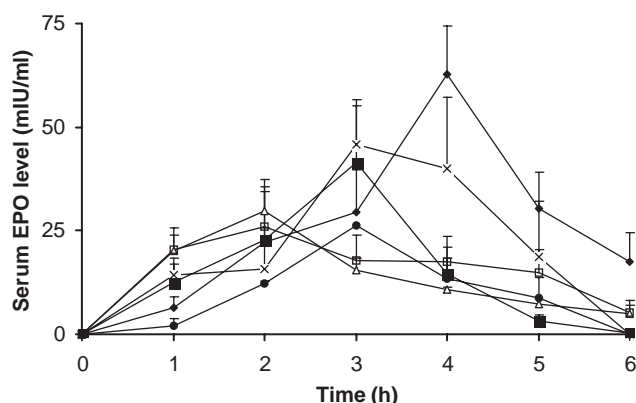


Fig. 2. Serum EPO level vs. time profiles after intra-jejunal administration of EPO (100IU/kg) solid preparations containing different adsorbents with Labrasol as an absorption enhancer (50mg/kg) to rats: (◆) formulation A; (□) formulation B; (●) formulation C; (×) formulation D; (■) formulation E; and (△) formulation F. Each point represents the mean \pm SE ($n = 4$).

Table 2

Pharmacokinetic parameters of EPO following administration of test preparations to rat small intestine

Formulation code	C_{max} (mIU/ml)	T_{max} (h)	$AUC_{0-\infty}$ (mIU h/ml)	BA (%)
A	25.9 ± 3.7	2.3 ± 0.3	119.5 ± 40.6	5.3
B	62.7 ± 11.6	4.0 ± 0.0	$160.1 \pm 6.7^{a,b}$	6.7
C	18.7 ± 5.3	2.5 ± 0.4	91.1 ± 11.6	4.0
D	50.0 ± 11.4	3.3 ± 0.3	128.3 ± 40.5	5.7
E	40.4 ± 13.1	3.2 ± 0.5	80.0 ± 11.7	3.9
F	34.1 ± 7.5	1.6 ± 0.3	111.3 ± 16.1	4.8
G	143.1 ± 15.2	3.6 ± 0.3	$256.3 \pm 9.7^{a,b,c}$	11.5
H	71.8 ± 14.9	4.3 ± 0.3	175.3 ± 26.6	7.4
I	25.6 ± 3.2	3.8 ± 0.3	74.4 ± 7.5	3.5
J	22.2 ± 2.6	4.0 ± 0.5	75.7 ± 11.1	3.6
K	22.6 ± 2.2	0.5 ± 0.06	43.8 ± 15.7	1.9
L	3.1 ± 2.0	1.04 ± 0.1	13.9 ± 9.1	0.6
M	—	—	1127.2 ± 28.7	100

^a $p < 0.05$, formulation B is significantly different from formulations E and F; formulation G is significantly different from formulations B and H.

^b $p < 0.01$, formulation B significantly different from formulation A.

^c $p < 0.0001$, formulation G is significantly different from formulations I and J.

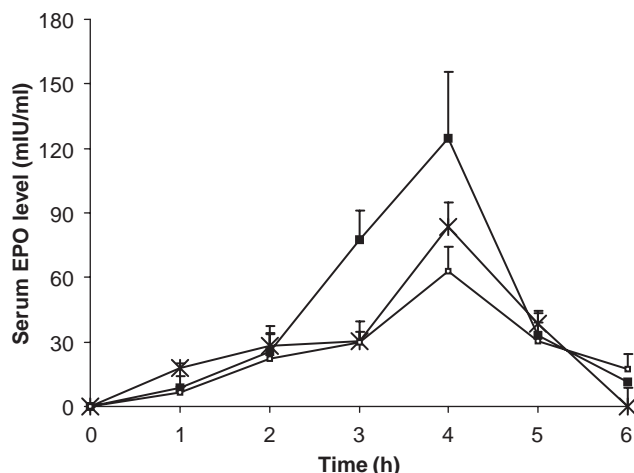


Fig. 3. Effect of enzyme inhibitor on serum EPO level vs. time profiles after intra-jejunal administration of EPO (100 IU/kg) solid preparations containing carbon nanotubes as an adsorbent and Labrasol as an absorption enhancer (50 mg/kg) to rats: (□) formulation B; (■) formulation G; and (×) formulation H. Each point represents the mean \pm SE ($n = 4$).

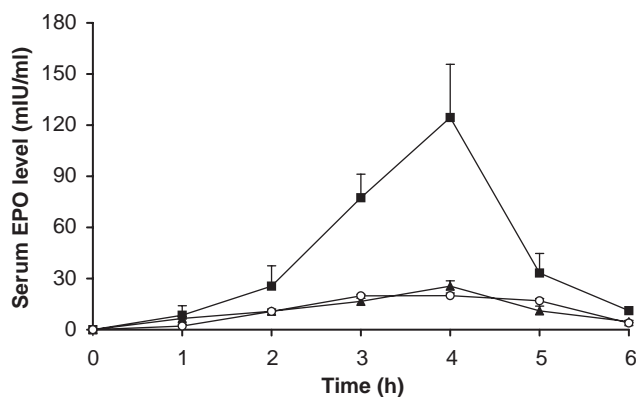


Fig. 4. Effect of absorption enhancer on serum EPO level vs. time profiles after intra-jejunal administration of EPO (100 IU/kg) solid preparations containing carbon nanotubes as an adsorbent and three different absorption enhancers (50 mg/kg) to rats: (■) formulation G; (○) formulation I; and (▲) formulation J. Each point represents the mean \pm SE ($n = 4$).

and 108.6 ± 5.8 mIU h/ml with duodenum and ileum, respectively (Table 3).

Administration of EPO–Labrasol solution increased the absorption of EPO from the small intestine, mean C_{\max} 16.2 ± 3.6 mIU/ml as compared to EPO solution alone, mean C_{\max} 3.1 ± 2.0 mIU/ml (Fig. 6). However, the mean C_{\max} was 143.1 ± 15.2 mIU/ml in the presence of an adsorbent (CNTs). This clearly indicates the need for an adsorbent based delivery system.

4. Discussion

Successful delivery of a protein/peptide drug by the oral route has several barriers such as degradation by

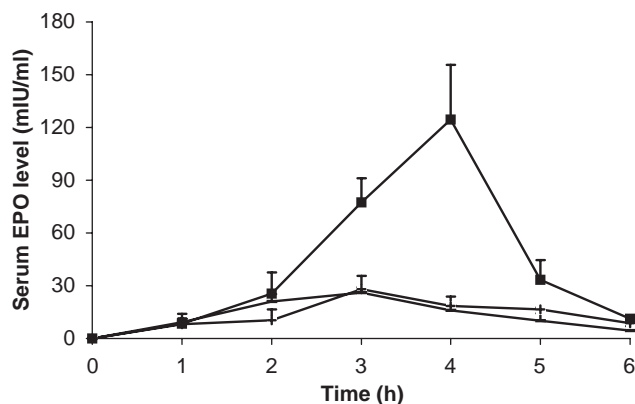


Fig. 5. Effect of administration site on serum EPO level vs. time profiles after intra-jejunal administration of EPO (100 IU/kg) solid preparation G to rats: (+) duodenum; (■) jejunum; and (–) ileum. Each point represents the mean \pm SE ($n = 4$).

hydrolytic and proteolytic enzymes and low membrane permeability. Like with many other protein and peptide drugs, the drawback with EPO is the non-availability of the oral route for effective administration of the protein. EPO, like other protein drugs, on oral administration is denatured by the harsh gastric conditions and enzymes present there in. This makes it difficult for the oral administration of EPO.

In our present study, we have made an attempt to administer EPO to the small intestine of rats and their serum EPO level was determined. The proposed system, a liquid filled nanoparticulate system can be described as a hollow nanoparticulate system capable of holding the drug and absorption enhancer in the liquid form and delivering them intact to the site of absorption (small intestine in this case). For this purpose, we studied the use of Sulyisia 550, CNTs, carbon nanohorns, fullerene, charcoal and bamboo charcoal as adsorbents.

Present study, was also aimed at identifying the suitable absorption enhancer for oral EPO administration with the above-mentioned adsorbents. We studied three different absorption enhancers Labrasol, Gelucire 44/14 and HCO-60. Labrasol and Gelucire 44/14 are saturated polyglycolysed glycerides. Labrasol is predominantly made of C8 and C10 alkyl chain lengths, while Gelucire 44/14, is C8–C18 glyceride, with a predominant C12 fatty acid chain. HCO-60 is 60 mol hydrogenated castor oil, a non-ionic surfactant. The selected absorption enhancers had an HLB value of 14. In our earlier studies, Labrasol was used to improve the absorption of a protein drug–insulin, a glycopeptide–vancomycin and a poorly absorbable drug gentamicin [23,43–46]. Hence, we first kept the absorption enhancer constant and tried the use of various adsorbents. It was found that CNTs gave the maximum serum EPO level as compared to other adsorbents. In this system, the protein drug and absorption enhancer are expected to be entrapped/

Table 3
Pharmacokinetic parameters of EPO following administration of the test preparation G to different sites of the rat small intestine

Administration site	C_{\max} (mIU/ml)	T_{\max} (h)	$AUC_{0-\infty}$ (mIU h/ml)	BA (%)
Duodenum	28.5 ± 2.2	3.0 ± 0.5	123.7 ± 4.2	5.7
Jejunum	143.1 ± 15.2	3.6 ± 0.3	256.3 ± 9.7^a	11.5
Ileum	25.6 ± 3.2	3.3 ± 0.8	108.6 ± 5.8	4.4

^a $p < 0.001$, significantly different from duodenum and ileum.

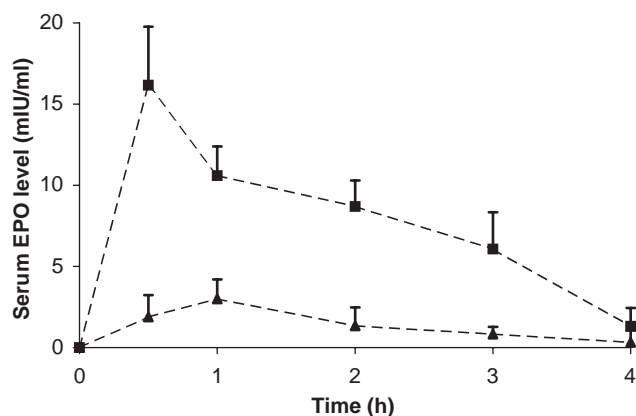


Fig. 6. Serum EPO levels vs. time profiles after the administration of EPO solution (-■-) with and (-▲-) without absorption enhancer, Labrasol (94 mg/kg), into the rat jejunum, EPO 100 IU/kg. Each point represents the mean \pm SE ($n = 4$).

bound within the porous adsorbent. The higher serum EPO level obtained in the case of CNTs could be attributed to their size and structure. One reason may be due to the number of adsorption site available for the drug and absorption enhancers to get themselves adsorbed. As it could be see from Fig. 7, there is a pore in individual tube, channels, formed at the contact of three tubes in the bundle, the groove formed at the contact between adjacent tubes on the outside of the bundle, and the surface, which are available as adsorption site. A solution of EPO, which was added to this system, makes the EPO to be filled along with the absorption enhancer or to get adsorbed onto the surface. In the case of CNTs, it is presumed that, when the liquid filled nanoparticulate system comes in contact with the intestinal fluid, there is a possibility for the formation of micro-emulsion between Labrasol–EPO–intestinal fluids, which subsequently helps in the absorption of EPO from the small intestine. Though this was the expected mechanism even with other adsorbents, we got the maximum serum EPO level with CNTs as an adsorbent. Fullerene are graphite sheets rolled into a ball (Bucky balls) and they have an average diameter of 0.7 nm. Fullerene, which was found to be the second best adsorbent in the present study, next to CNTs have a closed structure as compared CNTs. In the case of

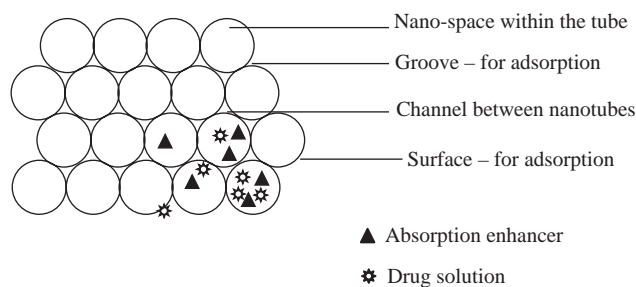


Fig. 7. Computer generated images of a bundle of carbon nanotubes and schematic presentation of the spaces available for adsorption.

charcoal and bamboo charcoal, the particle size was 280–500 μm and hence their effectiveness as a delivery system was far less than CNTs which was having a much smaller size, giving more surface area and more pores which might have acted as carrier space for the drug and the absorption enhancer. On the other hand, carbon nanohorns, which are said to possess more surface area available for adsorption than CNTs [36,37] did not elicit higher serum EPO level, this shows that not only the adsorption space which was thought to be of importance to act as an effective drug delivery tool, but also the structure of the adsorbent is of importance. Sylysia 550 is comparatively having a larger particle size similar to those of charcoal and bamboo charcoal, it was found to act as a better drug delivery carrier in our earlier studies where lansoprazole was the drug [27]. However, with a macro-molecular drug like EPO, they did show a comparable efficiency to that of CNTs. The structural difference between adsorbents and their surface properties might be attributed to the difference in their action. However, a detailed study in this direction is necessary for confirmation.

The effect of enzyme inhibitor on the oral absorption of EPO from jejunum was carried out with two different enzyme inhibitors: casein and lactoferrin. Both casein and lactoferrin are dietary constituent and hence they are safe for use. The use of casein and lactoferrin as enzyme inhibitors in gastrointestinal absorption studies have been reported [47–49]. The increased serum EPO level with casein may be attributed to their effective inhibition activity of trypsin and chymotrypsin in the

intestinal lumen and cathepsin B activity in lysosome [45]. Since the mechanism of inhibition is said to be competitive, the effect of casein must be reversible.

Gelucire 44/14, the two numbers in their names corresponds to their melting point and HLB value, respectively [50]. Gelucire 44/14 has been shown to increase the BA of orally administered drugs [51–54]. Gelucire 44/14 is capable of forming sub-micron emulsions when they come in contact with the physiological fluids in the small intestine. The possible mechanism by which both Labrasol and Gelucire 44/14 could act is by changing the fluidity of the membrane structure and thereby increasing the permeability of the drug through passive diffusion. However, so far there is no concrete evidence discussing the mechanism of action of Labrasol or Gelucire 44/14.

In the present study, Labrasol containing formulation was found to be more effective as an absorption enhancer as compared to Gelucire 44/14 or HCO-60 containing formulations. Gelucire 44/14 and HCO-60 both being solid at room temperature, were added to the nanoparticulate system after bringing them to a liquid state by melting them. However, when the drug was added to the nanoparticulate system containing the adsorbent at room temperature, the absorption enhancers had by then solidified, blocking the pores, giving little space for the drug to enter into the nanospace available within the nanoparticulate system. Thus, most of the drug might have got adsorbed onto the surface of the particulate system, leading to their quick release when administered and their subsequent degradation by the intestinal enzymes as they have little protection against intestinal enzymes, when adsorbed onto the surface, leading to poor absorption and less serum EPO level.

Among the chosen site of administration within the small intestine, jejunum was found to be better as compared to duodenum and ileum. The decreased absorption from duodenum may be attributed to the increased flow of digestive fluid which might have lead to the poor absorption from the site of administration. The decreased absorption from the ileum may be attributed to the less surface area available for diffusion by the protein [55]. Another possible reason could be the type of cells present in the ileum. In the case of jejunum, the surface area available for the absorption of the protein by diffusion into the intestinal layer is more [55].

EPO when administered as solution into the jejunum, without any absorption enhancer, showed lower serum EPO level. However, co-administration of EPO solution along with Labrasol solution, showed higher serum EPO level as compared to EPO solution itself. This clearly suggests the need for an absorption enhancer. However, the use of nanoparticulate system as adsorbent for Labrasol was found to be effective than Labrasol alone. This is evident from the maximum serum EPO level

achieved with EPO-CNTs system. EPO–Labrasol solution when administered to the jejunum is set to flow and leads to dilution of both the drug and the absorption enhancer. This also gives less time for the absorption enhancer to act effectively and help the protein molecule to diffuse into the systemic circulation. Dilution, in this case, is brought about by the intestinal fluid, which may also inactivate EPO and thereby decrease the serum EPO level. There is also no protection for the drug from the harsh intestinal enzymes, which might degrade the drug on oral administration. Therefore, the usefulness of nanoparticulate system for the oral delivery of protein drug, EPO, has been confirmed through these studies.

5. Conclusion

The use of nanoparticulate system for the effective administration of EPO could be achieved in combination with suitable absorption enhancer to appropriate absorption site. It has been proved in this study that by using the correct choice of absorption enhancer and adsorbent, high BA, 11.5%, of EPO was obtained following intra-jejunum administration to rats. It is also possible to use a low dose of the drug effectively and there by reducing the cost of medication. Studies related to uptake, distribution and toxicity are presently being carried out. Based on these results further pharmacodynamic studies will be carried out.

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References

- [1] Jacobson LO, Goldwasser E, Fried W, Plzak K. Role of the kidney in erythropoiesis. *Nature* 1957;179:633–4.
- [2] Schuster SJ, Wilson JH, Erslev AJ, Caro J. Physiologic regulation and tissue localization of renal erythropoietin messenger RNA. *Blood* 1987;70:316–8.
- [3] Filmanowicz E, Gurney CW. Studies on erythropoiesis XVI. Response to a single dose of erythropoietin in polycythemic mouse. *J Lab Clin Med* 1961;57:65–72.
- [4] Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, Chen KK, Fox GM, Martin F, Stabinsky Z, Badrawi SM, Lai PH, Goldwasser E. Cloning and expression of the human erythropoietin gene. *Proc Natl Acad Sci USA* 1985;82:7580–4.

- [5] Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, Seehra J, Jones SS, Hewick R, Fritsch EF, Kawakita M, Shimizu T, Miyake T. Isolation and characterization of genomic and DNA clones of human erythropoietin. *Nature* 1985;313:806–10.
- [6] Egrie JC, Strickland TW, Lane J, Aoki K, Cohen AM, Smalling R, Trail G, Lin FK, Browne JK, Hines DK. Characterization and biological effects of recombinant human erythropoietin. *Immunobiology* 1986;173:213–24.
- [7] Davis JM, Aralkawa T, Stricklan TW, Yphantis DA. Characterization of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Biochemistry* 1987;26:2633–8.
- [8] Winerals CG, Olivar DO, Pippard MJ, Reid C, Downing MR, Cotes PR. Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis. *Lancet* 1986;ii:1175–7.
- [9] Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. *N Engl J Med* 1987;316:73–8.
- [10] Urabe A, Takaku T, Mizoguchi H, Kubo K, Ota K, Shimizu N, Tanaka K, Miura N, Nihei H, Koshikawa S, Akizawa T, Akiyama N, Otsubo O, Kawaguchi Y, Maeda T. Effect of recombinant human erythropoietin on the anaemia of chronic renal failure. *Int J Cell Cloning* 1988;6:179–91.
- [11] Spivak J. Recombinant human erythropoietin and the anaemia of cancer. *Blood* 1994;84:997–1004.
- [12] Markham A, Bryson H. Epoetin alfa, a review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in non-renal applications. *Drugs* 1995;49:232–54.
- [13] Maitani Y, Moriya H, Shimoda N, Takayama K, Nagai T. Distribution characteristics of entrapped recombinant human erythropoietin in liposomes and its intestinal absorption in rats. *Int J Pharm* 1999;185:13–22.
- [14] Mizuno A, Ueda M, Kawanishi G. Effects of salicylate and other enhancers on rectal absorption of erythropoietin in rats. *J Pharm Pharmacol* 1992;44:570–3.
- [15] Shimoda N, Maitani Y, Machida Y, Nagai T. Effects of dose, pH and osmolarity on intranasal absorption of recombinant human erythropoietin in rats. *Biol Pharm Bull* 1995;18:734–9.
- [16] Saffran M, Kumar GS, Savariar C, Burnham JC, Williams F, Neckers DC. A new approach to the oral administration of insulin and other peptide drugs. *Science* 1986;233:1081–4.
- [17] Saffran M, Pansky B, Budd GC, Williams FE. Insulin and the gastrointestinal tract. *J Control Rel* 1997;46:89–98.
- [18] Fix JA. Oral controlled release technology for peptides: status and future prospects. *Pharm Res* 1996;13:1760–4.
- [19] Patel H, Ryman BE. Systemic and oral administration of liposomes. In: Knight CG, editor. *Liposomes: from physical structure to therapeutic applications*. Amsterdam: Elsevier; 1981. p. 409–41.
- [20] Dange C, Michael C, Aprahamian M, Couvreur P, Devis S. Nanoparticles as carriers for oral peptide delivery. *J Control Rel* 1990;13:233–9.
- [21] Paolo C, Francesco VM, Silavano L. Polyphosphazene microspheres for insulin delivery. *Int J Pharm* 2000;211:57–65.
- [22] Lowman A, Morishita M, Kajita M, Nagai T, Peppas N. Oral delivery of insulin using pH-responsive complexation gels. *J Pharm Sci* 1999;88:933–7.
- [23] Eaimtrakarn S, Itoh Y, Kishimoto J, Yoshikawa Y, Shibata N, Murakami M, Takada K. Gastrointestinal mucoadhesive patch system (GI-MAPS) for oral administration of G-CSF, a model protein. *Biomaterials* 2002;23:145–52.
- [24] Shen Z, Mitragotri S. Intestinal patches for oral drug delivery. *Pharm Res* 2002;19:391–5.
- [25] Patel DG, Ritchel WA, Chalasani P. Biological activity of insulin in microemulsion in mice. *J Pharm Sci* 1991;80:613–4.
- [26] Morishita M, Matuzawa A, Takayama K, Isowa K, Nagai T. Improving insulin enteral absorption using water in oil in water emulsion. *Int J Pharm* 1998;172:189–98.
- [27] Ito Y, Arai H, Uchino K, Iwasaki K, Shibata N, Takada K. Effect of adsorbents on the absorption of lansoprazole with surfactant. *Int J Pharm* 2005;289:69–77.
- [28] Iijima S. Helical microtubules of graphitic carbon. *Nature* 1991;354:56–8.
- [29] Baughman RH, Zakhido AA, deHeer WA. Carbon nanotubes—the route toward applications. *Science* 2002;297:787–92.
- [30] Ding RG, Lu GQ, Yan ZF, Wilson MA. Recent advances in the preparation and utilization of carbon nanotubes for hydrogen storage. *J Nanosci Nanotechnol* 2001;1:7–29.
- [31] Iijima S, Ichihashi T. Single-shell carbon nanotubes of 1 nm diameter. *Nature* 1993;363:603–5.
- [32] Ajayan PM, Iijima S. Capillarity-induced filling of carbon nanotubes. *Nature* 1993;361:333–4.
- [33] Tsang SC, Harris PJF, Green MLH. Thinning and opening of carbon nanotubes by oxidation using carbon dioxide. *Nature* 1993;362:520–2.
- [34] Ajayan PM, Ebbesen TW, Ichihashi T, Iijima S, Tanigaki K, Hiura H. Opening carbon nanotubes with oxygen and implications for filling. *Nature* 1993;362:522–5.
- [35] Iijima S, Yudasaka M, Yamada R, Bandow S, Suenaga K, Kokai F, Takahashi K. Nano-aggregates of single-walled graphitic carbon nano-horns. *Chem Phys Lett* 1999;309:165–70.
- [36] Berber S, Kwon YK, Tomanek D. Electronic and structural properties of carbon nanohorns. *Phys Rev B* 2000;62:R2291–2294.
- [37] Kasuya D, Yudasaka M, Takahashi K, Kokai F, Iijima S. Selective production of single-wall carbon nanohorn aggregates and their formation mechanism. *J Phys Chem B* 2002;106:4947–51.
- [38] Kam NWS, Jessop TC, Wender PA, Dai H. Nanotube molecular transporters: internalization of carbon nanotube-protein conjugates into mammalian cells. *J Am Chem Soc* 2004;126:6850–1.
- [39] Pantarotto D, Singh R, McCarthy D, Erhardt M, Briand JP, Prato M, Kostarelos K, Bianco A. Functionalized carbon nanotubes for plasmid DNA gene delivery. *Angew Chem Int Ed* 2004;43:5242–6.
- [40] Bianco A, Kostarelos K, Partidos CD, Prato M. Biomedical applications of functionalised carbon nanotubes. *Chem Commun* 2005:571–7.
- [41] Murakami T, Ajima K, Miyawaki J, Yudasaka M, Iijima S, Shiba K. Drug-loaded carbon nanohorns: adsorption and release of dexamethasone in vitro. *Mol Pharm* 2004;1(6):399–405.
- [42] Yoshikawa Y, Kato K, Sone H, Takada K. Development and evaluation of noncompartmental pharmacokinetic analysis program “WinHARMONY” using Visual BASIC language having a function of an automatic recognition of terminal elimination phase of plasma drug concentration vs time profile. *Jpn J Clin Pharmacol* 1998;29:475–8.
- [43] Eaimtrakarn S, Rama Prasad YV, Ohno T, Konishi T, Yoshikawa Y, Shibata N, Takada K. Absorption enhancing effect of Labrasol on the intestinal absorption of insulin in rats. *J Drug Target* 2002;10:255–60.
- [44] Rama Prasad YV, Puthli SP, Eaimtrakarn S, Ishida I, Yoshikawa Y, Shibata N, Takada K. Enhanced intestinal absorption of vancomycin with Labrasol and *D-α*-tocopheryl PEF 1000 succinate in rats. *Int J Pharm* 2003;250:181–90.
- [45] Hu Z, Tawa R, Konishi T, Shibata N, Takada K. A novel emulsifier, Labrasol, enhances gastrointestinal absorption of gentamicin. *Life Sci* 2001;69:2899–910.

- [46] Hu Z, Rama Prasad YV, Tawa R, Konishi T, Ishida M, Shibata N, Takada K. Diethyl ether fraction of Labrasol having a stronger absorption enhancing effect on gentamicin than Labrasol itself. *Int J Pharm* 2002;234:223–35.
- [47] Yan X, Wang X, Zhang X, Zhang Q. Gastrointestinal absorption of recombinant hirudin-2 in rats. *J Pharmacol Exp Ther* 2004;208:774–9.
- [48] Ohtani S, Shirasu K, Ogawara KI, Higaki K, Kimura T. Evaluation of inhibitory activity of casein on proteases in rat intestine. *Pharm Res* 2003;20:611–7.
- [49] Dial EJ, Dohrman AJ, Romero JJ, Lichtenberger LM. Recombinant human lactoferrin prevents NSAID-induced intestinal bleeding in rodents. *J Pharm Pharmacol* 2005;57:93–9.
- [50] Craig DQM. The use of glycerides as controlled release matrices. In: Karsa DR, Stephenson RA, editors. *Excipients and delivery systems for pharmaceutical formulations*. London: Royal Society of Chemistry; 1995. p. 148–73.
- [51] Sheen PC, Kin SI, Petillo JJ, Serajuddin ATM. Bioavailability of a poorly water-soluble drug from tablet and solid dispersions in humans. *J Pharm Sci* 1991;80:712–4.
- [52] Pozzi F, Longo A, Lazzarini C, Carenzi A. Formulations of ubidecarenon with improved bioavailability. *Eur J Pharm Biopharm* 1991;37:243–6.
- [53] Aungst BJ, Nguyen NH, Rogers NJ, Rowe SM, Hussain MA, White SJ, Shum L. Amphiphilic vehicles improve the oral bioavailability of a poorly soluble HIV protease inhibitor at high doses. *Int J Pharm* 1997;156:79–88.
- [54] Barker SA, Yap SP, Yuen KH, McCoy CP, Murphy JR, Craig DQM. An investigation into the structure and bioavailability of α -tocopherol dispersions in Gelucire 44/14. *J Control Rel* 2003;91:477–88.
- [55] Kararli TT. Comparison of the gastrointestinal anatomy, physiology and biochemistry of humans and commonly used laboratory animals. *Biopharm Drug Dispos* 1995;16:351–80.