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Pharmacokinetic and pharmacodynamic studies following oral administration of erythropoietin mucoadhesive tablets to beagle dogs

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Abstract

Oral administration of mucoadhesive tablets containing erythropoietin (EPO) and an absorption enhancer Labrasol was studied in rats and dogs. Mucoadhesive tablets were prepared using Sylysia 550 holding the absorption enhancer and Carbopol 974P as a mucoadhesive agent. Mucoadhesive tablets were covered with a water-insoluble backing layer made of cellulose acetate and a pH-sensitive covering layer made of Eudragit L/Eudragit S. Tablet was administered into the rat jejunum at EPO dose of 100 IU/kg and serum samples were collected for 6 h. Serum EPO level was analysed with a standard ELISA procedure. After administration, rats showed a maximum serum EPO level of C_{max} 70.6 ± 8.9 mIU/ml. Oral administration of a single tablet containing 100 IU/kg EPO to beagle dogs showed a C_{max} of 24.6 ± 4.1. When EPO dose was increased to 500 IU/kg and the number of tablets was also increased to 5, the C_{max} was 54.8 ± 9.0 mIU/ml. However, when EPO, 100 IU/kg dose was divided into five tablets, the C_{max} was 15.5 ± 1.8 mIU/ml. In the absence of absorption enhancer, the C_{max} was 35.8 ± 3.8 with 500 IU/kg dose distributed among five tablets. Pharmacodynamic studies were carried out following oral administration of mucoadhesive tablets for 6 consecutive days at an EPO dose of 500 IU/kg. Whole blood samples were collected and percent circulating reticulocytes were counted using Miller technique. The increase in percent circulating reticulocytes was found to be 1.7% on day 8 following oral administration. As a control study, EPO was administered by i.v. route at a dose of 300 IU/kg for 3 consecutive days and the percent circulating reticulocytes were counted. Mucoadhesive tablets showed promising results as an oral drug delivery system for protein therapeutics.

Keywords: Erythropoietin; Labrasol; Mucoadhesive tablets; Oral; Reticulocytes

1. Introduction

Erythropoietin (EPO) is a hormone primarily produced by the kidney cells, which is responsible for the regulation of erythropoiesis in mammals (Goldwasser and Kung, 1968). EPO is used in a number of clinical circumstances to treat anemia due to renal failure, cancer, bone marrow transplantation, AIDS, etc. (Winerals et al., 1986; Eschbach et al., 1987; Urabe et al., 1988; Spivak, 1994; Markham and Bryson, 1995). Recombinant human EPO available as a commercial product is obtained using recombinant DNA technology. The molecular weight of the hormone is about 30 kDa (Davis et al., 1987). It has been well established and proved for the safe use in humans. Presently, EPO is administered either as an intravenous or subcutaneous injection two to three 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 (Maitani et al., 1996; Mizuno et al., 1992; Shimoda et al., 1995).

Oral delivery of protein and peptide drugs poses a challenge to pharmaceutical scientists. Present study was aimed at developing an oral dosage form, which is the preferred route of administration. Earlier studies involving oral delivery of EPO using liposomes have been reported by Maitani et al. (1996, 1999). However, our present study involves the use of mucoadhesive tablet systems. Mucoadhesive tablets have been reported in the literature mostly for local delivery of the drug to the oral mucosa than for the delivery of large molecular weight protein drugs. However, a few reports are available for the delivery of salmon calcitonin and insulin (Guggi et al., 2003; Krauland et al., 2004). In our present study, an attempt was made to improve

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the absorption of EPO following oral administration to beagle dogs and to study their subsequent pharmacological activity.

2. Materials and methods

2.1. Materials

Cellulose acetate (CA) (CA-398-10NF) (Eastman Chemical, Kingsport, USA), Eudragit® L 100 and Eudragit® S 100 (Röhm GmbH, Germany), Labrasol® (Gattefosse, Lyon, France), Carbopol® 974P (BF Goodrich Co., Cleveland, USA), porpus silicon dioxide (Sylysia® 550, Fuji Silysia Co., Japan) and triethyl citrate (TEC) (Wako Pure Chemical Industries, Osaka, Japan) were obtained. All other materials used were of reagent grade and were used as received. The membrane spreading apparatus (Bakerapplicator®) was developed in technical collaboration with Imoto Seisakusho (Kyoto, Japan). A commercially available erythropoietin injection (ESPO® 24,000 IU/0.5 ml) manufactured by Kirin Breweries (Japan) and 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). Male beagle dogs used in the present study and standard solid meal of commercial food (Lab D stock®) were obtained from Nippon Nousan Co. Ltd.

2.3. Preparation of water-insoluble and pH-sensitive membranes

A water-insoluble backing layer made of CA was prepared using a solvent evaporation technique. CA solution (20%, w/v) containing TEC (20%, w/w, of the polymer) was prepared in acetone. A thin membrane of CA was prepared by spreading the CA solution on a Teflon coated plate (30 cm × 40 cm) using Bakerapplicator having a clearance of 200 μm. The CA membrane was allowed to dry at room temperature. The thickness of the dried membrane was measured using a Sony μ-mate (Sony, Japan). The thickness of the membrane obtained was 40.0 ± 0.3 μm. The enteric polymer membrane was also prepared by the above technique using Eudragit L 100/Eudragit S 100 with a 50 μm clearance applicator. The enteric polymer solution was prepared by dissolving 20% (w/v) of Eudragit L 100/Eudragit S 100 along with TEC (70%, w/w, of the polymer) in a mixture of methanol:methylene chloride (1:2). The thickness of the dried enteric membrane was 23.4 ± 1.1 μm.

2.4. Preparation of mucoadhesive tablet

Sylysia 550 and Labrasol (500 mg each) were mixed and to this mixture was added Carbopol 974P (10 mg/tablet). On mixing well, lyophilized EPO was added and mixed to obtain a uniform distribution of the drug. This mixture was then weighed into the tablet dye (0.5 in.) and a further 10 mg/tablet of Carbopol 974P was added on to the surface. Tablets were prepared by direct compression of the above mixture at a compression force of 3 tonnes using a hand press machine (Shimadzu, Japan). The tablets were 0.5 in. in diameter and 0.75 mm in thickness.

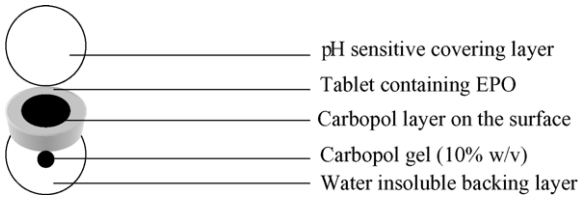

Fig. 1. Schematic presentation of the EPO mucoadhesive tablet depicting different layers in the tablet.

The prepared tablets were placed with Carbopol side facing upwards on a water-insoluble membrane containing Carbopol gel (10%, w/v), which helps hold the tablet to the water-insoluble membrane (Fig. 1). The water-insoluble membrane was placed around the tablet except for the upper surface (Carbopol side). The membrane was fixed using small amount of CA solution (to act as glue). The surface was covered with enteric polymer film and was sealed using the respective enteric polymer solution.

2.5. Pharmacokinetic studies of EPO after oral/i.v. administration of EPO test formulations to rats

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). After blank blood samples (0.35 ml) were obtained, the hairs on the abdominal region were shaved and a 3 cm midline incision was made through the skin and muscle without damaging other internal organs. The small intestine was exposed and a small incision was made in the jejunum, where the blood supply was sparse. Mucoadhesive tablet containing EPO equivalent to an EPO dose of 100 IU/kg was placed 5 cm away from the point of incision using a smooth end fine forceps with proper care not to damage the intestinal wall. The tablet was placed in such a way that the enteric side was facing the intestinal wall while the water-insoluble backing layer was away from the wall. The incision was sutured and then sealed carefully, not to close the intestinal flow using a synthetic adhesive, Aron alpha® (Sankyo Corporation). 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 intra-jejunum administration of the mucoadhesive tablets. In order to determine the bioavailability, EPO solution was injected into the left jugular vein of another group of rats, at a dose of 50 IU/kg. A blank blood sample was collected prior to the injection. Blood samples were collected from the right jugular vein at 2, 20, 40, 60, 120, 180, 360 and 480 min after administration. 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.

2.6. Pharmacokinetic studies of EPO after oral/i.v. administration of EPO test formulations to dogs

Adult male beagle dogs (11–12 kg body weight) were fasted overnight for at least 12 h with access to water ad libitum. Test formulations were administered at 10:00 a.m. with 30 ml of luke-warm water in a cross-over manner with 1 week washout period. The test tablets were orally administered to the dogs. The animals were deprived of any food after oral administration of the tablets, until the last sampling time. However, they had free access to water. Group 1 received one tablet (100 IU/kg), group 2 received one tablet (500 IU/kg), group 3 received five tablets (100 IU/kg), group 4 received five tablets (500 IU/kg) and group 5 received five tablets (500 IU/kg) without any absorption enhancer. When five tablets were administered to the dog, the tablets were covered with pH-sensitive membrane of different composition (two tablets with Eudragit L 100, one tablet with Eudragit L 100:Eudragit S 100, 90:10; two tablets with Eudragit L 100:Eudragit S 100, 80:20). These tablets were wrapped into an Oblate film (PIP Health, Osaka, Japan) with little talc between each tablet so that they do not stick with each other.

A blank blood sample (0.5 ml) was taken prior to the administration of the test formulations. All the experiments were carried out at the same time of the day to exclude the influence of circadian rhythm. After oral administration of test formulations, blood samples were collected from the jugular vein at 1, 2, 3, 4, 5 and 6 h intervals. In order to determine the bioavailability, 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. The collected blood samples were allowed to clot by standing at room temperature and serum samples were obtained by centrifugation at 12,000 rpm for 15 min at 8 °C using Kubota 1700 centrifuge (Kubota). The serum samples were collected and stored at −80 °C until analysis.

2.7. Pharmacodynamic studies of EPO after oral/i.v. administration of EPO test preparations to dogs

Adult male beagle dogs (11–12 kg body weight) were fasted overnight for at least 12 h with access to water ad libitum. Test formulations were administered at 10:00 a.m. with 30 ml of luke-warm water in a cross-over manner with 1 week washout period. The animals were served with a solid commercial meal (450 g), at 4 h after oral administration of the test preparations. The animals had free access to water throughout the experiment. Four dogs were used as a group. Mucoadhesive tablets containing EPO were administered to dogs (five tablets of 500 IU/kg) everyday at 10:00 a.m. for 6 consecutive days. Blood samples were collected in EDTA-2K coated sample tubes on every administration day at 4 h after oral administration of the tablet and everyday at the same time for a further period of 4 days (total 10 days). A blank blood sample was taken prior to the administration of the test formulations. As a control, pharmacodynamic studies following i.v. injection of EPO solution (300 IU/kg) for 3 consecutive days were also carried out. Blood samples were collected in EDTA-2K coated sample tubes for 8 days. All the experiments were carried out at the same time of the day to exclude the influence of circadian rhythm. The percent circulating reticulocytes were counted on the same day after collection of the blood sample. All the animal experiments were carried out in accordance with the Guidelines for Animal Experimentation in Kyoto Pharmaceutical University.

2.8. Analytical methods

2.8.1. Serum EPO analysis by ELISA

The serum EPO level was determined by an ELISA method. 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 (marketed EPOS used in the present study) 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, 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 microplate reader (MTP-300 microplate reader, Corona Electric, Japan).

2.8.2. Measurement of circulating reticulocytes

Circulating reticulocytes were measured using a previously reported Miller technique (Brecher and Schneiderman, 1950). Briefly, 20 μl of the blood sample was stained with new methylene blue (Brecher, 1949). The mixture was allowed to stand for 10 min and was then placed on a glass slide and thin smears were made and air-dried. The slide was placed under an optical microscope (Shimadzu, Kyoto, Japan) and reticulocytes were counted under oil immersion objective and a 10× eyepiece into which a Miller disc was inserted. Twenty squares were counted and the reticulocytes percentage was calculated as follows:

$$\frac{100 \times \text{reticulocytes in large squares}}{\text{red cells in small squares} \times 9}. \quad (1)$$

2.9. Pharmacokinetic analysis

Pharmacokinetic parameters were determined from the serum EPO concentrations versus time data by a non-compartmental pharmacokinetic analysis method using WinHARMONY software developed by us (Yoshikawa et al., 1998). The maximum drug concentration (C_{max}) and the time to reach maximum concentration (T_{max}) were determined from the

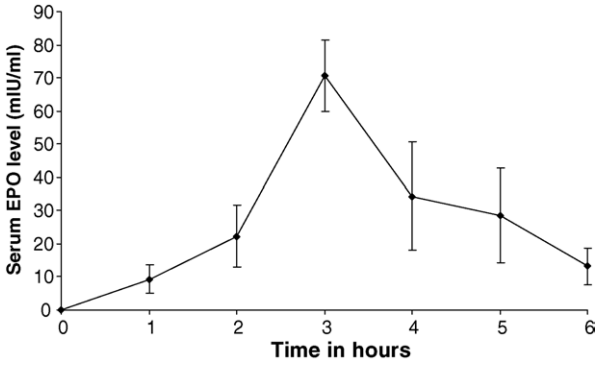

Fig. 2. Serum EPO level vs. time profiles following intra-jejunum administration of mucoadhesive tablets containing EPO (100 IU/kg) with Labrasol as an absorption enhancer (50 mg/tablet) to rats. Each point represents the mean ± S.E. ($n = 4$).

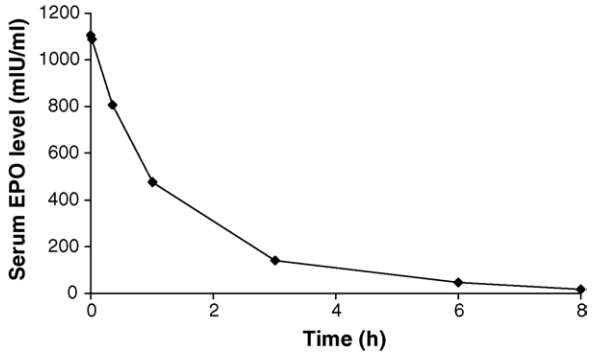

Fig. 3. Serum EPO level vs. time profiles following i.v. injection of EPO solution (50 IU/kg) to dogs. Each point represents the mean ± S.E. ($n = 4$).

authentic serum concentration–time data. The area under the plasma drug concentration versus time curve (AUC) after oral administration was calculated using the linear trapezoidal rule up to the last measured drug concentration and the percent BA was calculated by using the following equation:

$$\%\text{BA} = \frac{\text{AUC}_{\text{oral}}}{\text{AUC}_{\text{i.v.}}} \times \frac{\text{dose}_{\text{i.v.}}}{\text{dose}_{\text{oral}}} \times 100. \quad (2)$$

2.10. Statistical analysis

All values are expressed as their mean ± S.E. Levels of significance were evaluated using Tukey's test.

3. Results

Absorption of EPO from the rat small intestine was studied with a mucoadhesive tablet containing Labrasol as an absorption enhancer, Sylysia 550 as an adsorbent and Carbopol 974P as a mucoadhesive agent. In the preliminary experiment, mucoadhesive tablets containing EPO was administered to the jejunum of the rat small intestine at a dose level of 100 IU/kg, the maximum serum EPO level was 70.6 ± 9.0 mIU/ml. The $T_{\max}$ was found to be 3.3 ± 0.3 h (Fig. 2). Table 1 shows the pharmacokinetic parameters of EPO where the mean AUC was 161.7 ± 21.4 mIU h/ml. On the other hand, EPO solution was injected i.v. at 50 IU/kg, to another group of rats showed an AUC of 1127.2 ± 28.7 mIU h/ml (Fig. 3). By comparing the two AUC values, the mean BA of EPO was calculated to be 7.2% following intra-jejunum administration to the rat. Based on this preliminary experiment, we carried forward the study on beagle dogs.

Oral administration of single mucoadhesive tablet containing EPO at a dose level of 100 IU/kg to beagle dogs showed a $C_{\max}$ of 24.6 ± 4.1 mIU/ml with a $T_{\max}$ of 4.0 ± 0.0 h (Fig. 4). When a single mucoadhesive tablet containing 500 IU/kg dose was administered to the dogs, the $C_{\max}$ was lowered to 17.1 ± 3.7 mIU/ml as shown in Fig. 4. Increasing the number of tablets to five per dog, while keeping the dose constant, showed a $C_{\max}$ of 15.5 ± 1.8 and 54.8 ± 9.0 mIU/ml with 100 and 500 IU/kg dose, respectively (Fig. 4). Next, absorption of EPO was also studied when an absorption enhancer was not formulated. The $C_{\max}$ in this case was 35.8 ± 3.8 mIU/ml (Fig. 5). Administration of five tablets per dog with 500 IU/kg showed a significantly higher $\text{AUC}_{0-6\,\text{h}}$ of 94.2 ± 17.5 mIU h/ml as compared to single mucoadhesive tablet of the same dose which showed an $\text{AUC}_{0-6\,\text{h}}$ of 36.4 ± 12.8 mIU h/ml. Table 2 shows the various pharmacokinetic parameters of EPO in dogs, calculated by non-compartmental pharmacokinetic analysis. Based on these results, further pharmacodynamic studies were carried out using a dose of 500 IU/kg divided into five tablets.

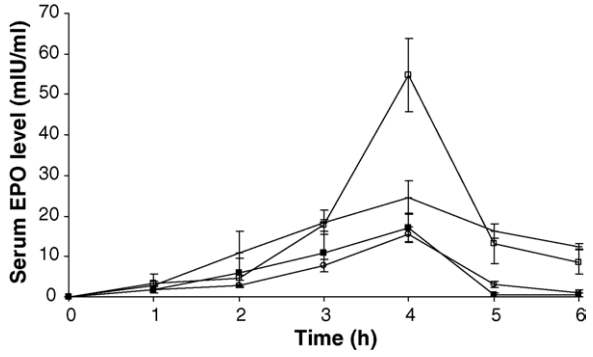

Fig. 4. Serum EPO level vs. time profiles following oral administration of mucoadhesive tablets containing EPO with Labrasol as an absorption enhancer (50 mg/tablet) to each dog. (+) 100 IU/kg (single tablet), (■) 500 IU/kg (single tablet), (□) 100 IU/kg (five tablets) and (◯) 500 IU/kg (five tablets). Each point represents the mean ± S.E. ($n = 3$).

Table 1
Pharmacokinetic parameters of EPO following oral/i.v. administration of test formulations to rats

Dosage form	Route	Absorption enhancer	Dose (IU/kg)	$C_{\max}$ (mIU/ml)	$T_{\max}$ (h)	$\text{AUC}_{0-6\,\text{h}}$ (mIU h/ml)	BA (%)
Tablet	Intra-jejunum	Labrasol	100	70.6 ± 9.0	3.3 ± 0.3	161.7 ± 21.4	7.2
Solution	i.v.	–	50	–	–	1127.2 ± 28.7	100

Table 2
Pharmacokinetic parameters of EPO following oral/i.v. administration of test formulations to beagle dogs

Dosage form	Route	No. of tablets	Absorption enhancer	Dose (IU/kg)	C_{max} (mIU/ml)	T_{max} (h)	$AUC_{0-6\,h}$ (mIU h/ml)	BA (%)
Tablet	Oral	1	Labrasol	100	24.6 ± 4.1	4.0 ± 0.0	72.7 ± 14.9	2.1
Tablet	Oral	1	Labrasol	500	17.1 ± 3.7	4.0 ± 0.0	36.4 ± 12.8	0.2
Tablet	Oral	5	Labrasol	100	15.5 ± 1.8	4.0 ± 0.0	31.5 ± 4.5	0.9
Tablet	Oral	5	Labrasol	500	54.8 ± 9.0*	4.0 ± 0.0	94.2 ± 17.5	0.6
Tablet	Oral	5	–	500	35.8 ± 3.8	3.7 ± 0.3	101.9 ± 23.1	0.6
Solution	i.v.	–	–	50	–	–	1712.9 ± 159.9	100

* $p < 0.05$, significantly different from 100 IU/kg/5 tablets and 500 IU/kg/tablet.

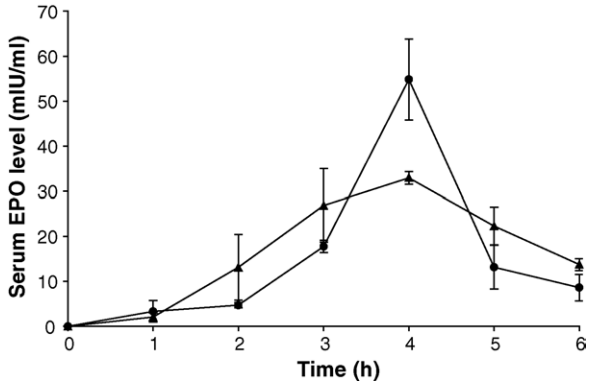

Fig. 5. Serum EPO level vs. time profiles following oral administration of five mucoadhesive tablets containing EPO (500 IU/kg) to each dog. (●) With Labrasol as absorption enhancer (50 mg/tablet) and (▲) without absorption enhancer. Each point represents the mean ± S.E. ($n = 3$).

Percent circulating reticulocytes studies was first carried out in a control group of animals, which did not receive any EPO dose or formulation, where no change in the circulating reticulocytes was observed (Fig. 6). An increase in the circulating reticulocytes was observed after mucoadhesive tablets were administered to the dogs at a dose of 500 IU/kg. Percent circulating reticulocytes increased from 0.9 to 1.6 on day 3, on day 5 the increase was 1.9 which was found to move upwards on day 8 towards 2.7 after which a decrease in circulating reticulocytes was observed (Fig. 7). A positive control study carried out following i.v. injection of 300 IU/kg for 3 consecutive days to beagle dogs showed higher circulating reticulocytes on day

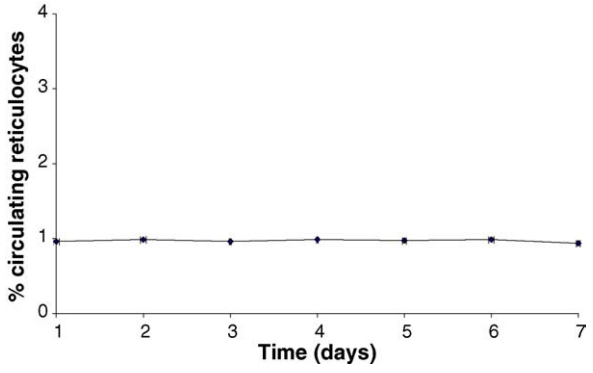

Fig. 6. Percent circulating reticulocytes in dog blood (control group). Each point represents the mean ± S.E. ($n = 4$).

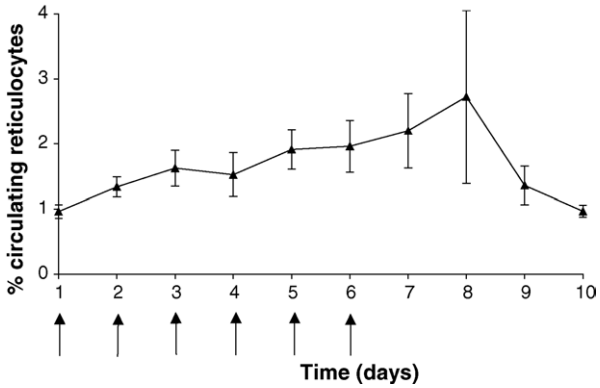

Fig. 7. Percent circulating reticulocytes in dog blood following oral administration of five mucoadhesive tablets containing EPO (500 IU/kg) with Labrasol as an absorption enhancer (50 mg/tablet) for 6 consecutive days. Administration days are indicated by arrow. Each point represents the mean ± S.E. ($n = 4$).

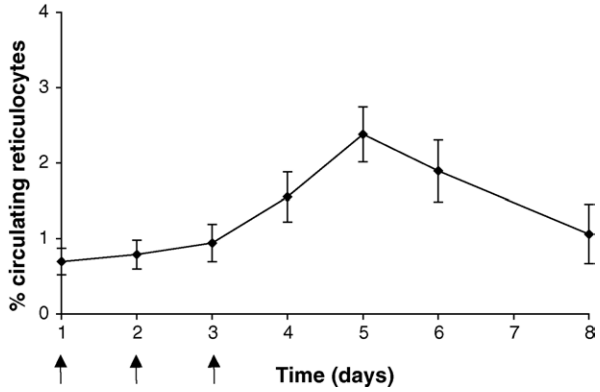

Fig. 8. Percent circulating reticulocytes in dog blood following i.v. administration of EPO solution (300 IU/kg) for 3 consecutive days. Administration days are indicated by arrow. Each point represents the mean ± S.E. ($n = 4$).

4 (1.6), which was sustained until day 5 following which a decrease was observed (Fig. 8).

4. Discussion

Oral delivery of drugs is the most attractive route of administration. However, oral administration of protein and peptide drugs has not been an easy task. In our present study, we have made an attempt to use a mucoadhesive tablet system with a unidirectional release that is capable of delivering the drug intact

to the small intestine. The use of Sylysia as an adsorbent for the absorption enhancer, Labrasol, has been recently reported from our laboratory (Ito et al., 2005, in press). Porous silicon dioxide particles are capable of holding Labrasol, which is a liquid. However, the final formulation remains to be a powder. Based on our past experience, we chose Labrasol as an absorption enhancer in the present study (Eaimtrakarn et al., 2002; Rama Prasad et al., 2004; Venkatesan et al., 2005). The mucoadhesive tablet has been designed in such a way that the tablet is protected from harsh GI conditions on the one side by a water-insoluble backing membrane and the other side by a pH-sensitive covering membrane (Fig. 1). The backing membrane is used to cover all sides of the tablet except for the surfacial layer, which is covered by the pH-sensitive layer. When the tablet is administered orally, the tablet passes through various stages and reaches the small intestine intact. On reaching the small intestine, the pH-sensitive layer made of Eudragit L/Eudragit S starts dissolving, where the threshold pH of these enteric polymers are 6.0 and 6.8. Once the pH-sensitive layer is dissolved, the surfacial layer of the tablet containing Carbopol 974P is exposed, which being a mucoadhesive agent will help attach the tablet to the mucosal wall of the small intestine. Once, the tablet is attached to the intestinal wall, EPO is expected to be absorbed from the tablet. The presence of absorption enhancer along with EPO is expected to be released simultaneously from the porous silicon dioxide particles which will help in the absorption of the protein drug. The liquid filled microporous particles start to release the absorption enhancer when in contact with the intestinal fluids. The released absorption enhancer is expected to increase the absorption of EPO either by membrane perturbation or by formation of a microemulsion (Ito et al., in press), the exact mechanism by which absorption takes place is still not known.

Administration of a single tablet at a dose of 100 IU/kg to beagle dogs showed an increase in the serum EPO levels as compared to 500 IU/kg. This could be attributed to the reason that when the tablet is attached to the intestinal wall, Carbopol which is present on the surface of the tablet, swells and absorb the intestinal fluid and may retard the release of the drug and absorption enhancer from the tablet as shown in our previous results in gel-type gastro-intestinal patch systems (Venkatesan et al., 2005). Secondly, the increase in dose leads to higher concentration of the drug in a single tablet and this leads to intermolecular aggregation when a large molecule like EPO is released from the tablet, which might also lead to decreased serum EPO level with higher dose. As 100 IU/kg showed good serum EPO levels, we increased the dose to 500 IU/kg but distributed among five different tablets (100 IU/kg/tablet). This lead to an increased serum EPO levels. However, the C_{max} was not as high as expected with the increase in dose. Therefore, we decided to decrease the dose back to 100 IU/kg though distributed among five tablets (20 IU/kg/tablet). However, this showed a decrease in serum EPO levels as compared to single tablet of 100 IU/kg. This was in contrary to the results obtained with 500 IU/kg. In the case of five tablets study, tablets were covered with different pH-sensitive layers. In this way, attachment of tablets among themselves was avoided when the pH-sensitive layer was dissolved. The next day after administration of the tablets, the feces of the dogs were observed. The water-insoluble membrane along with some undissolved part of tablets was found in the feces and all the five tablets were separate. Higher ratio of Eudragit S 100 could also be used however; it would lead to dissolving of the pH-sensitive layer down the small intestine, say nearer to ileum, which was not desired. This was after our finding that jejunum is a better absorption site than ileum for EPO (Venkatesan et al., 2005). The higher serum EPO level with single tablet administration of 100 IU/kg dose could be attributed to better absorption from the tablet in the presence of an absorption enhancer. The same dose, when split between five tablets, the tablets after attaching to the intestinal wall release the drug and absorption enhancer at different site in the intestine because of different pH-sensitive layers. However, in the case of five tablets, the dose is less between each tablet (ca. 20 IU/kg/tablet). As not all the drug is released and or absorbed from the tablet, the serum EPO level within the study period was found to be less. This is the reason that higher C_{max} was obtained with 500 IU/kg/5 tablets system than to 100 IU/kg/tablet system, though precise dose proportionality was not obtained.

The presence of an absorption enhancer did not increase the serum EPO levels to a significant level. In spite of the absence of Labrasol, serum EPO levels were achieved using the mucoadhesive tablets. This clearly indicates that when a drug is delivered very close to the absorption site in a unidirectional way and if the concentration of the drug is maintained without any dilution or degradation at the absorption site due to intestinal fluids, absorption of the drug can be increased. Also Carbopol has an absorption promoter action and has been reported for the oral delivery of protein and peptide drugs (Lehr et al., 1992; Lueben et al., 1995, 1996; Bernkop-Schnurch and Gilge, 2000). However, with the use of an absorption enhancer, a synergistic effect could be obtained. Whitehead et al. (2004) reported a similar finding recently.

Pharmacodynamic studies revealed that the orally administered mucoadhesive tablets showed a pharmacological effect. The absorbed EPO was found to exert its pharmacological action in comparison to a control study where no EPO was administered to the dogs ($p < 0.001$). However, the pharmacological results obtained with dogs treated with i.v. injection and those with oral mucoadhesive tablets were similar ($p < 0.05$). This result is surprising as the relative bioavailability with an oral mucoadhesive tablet was only 0.6%. The answer for this is presently not known to us. The mucoadhesive tablets after loosing their mucoadhesiveness might get detached from the intestinal wall and the tablet with the remaining unabsorbed drug passes through the ileum and the large intestine where there is possibility of the drug to be taken up. However, this needs to be verified scientifically. The water-insoluble film along with very little unabsorbed portion of the tablet was seen during the fecal examination of the dogs on the subsequent day. Maitani et al. (1996) reported the oral administration of EPO containing liposomes where only two of the five rats used showed an increase in circulating reticulocytes. In the case of liposomal formulations the dose was very high (8000–25,000 IU/kg) and a single oral administration was carried out. Oral administration of mucoadhesive tablets for 6 consecutive days showed pharmacological activity. The phar-

macological activity of EPO following i.v. injection was short lived in spite of three consecutive administrations. This clearly indicates the need for a repeated administration to bring in an effective pharmacological activity. With the oral administration of mucoadhesive tablets it has been proved that oral route itself has an advantage against i.v. therapy though repeated administration may be required to bring in a pharmacological response. However, the system needs drastic up gradation to bring into use. Our system shows that with lower dose and consecutive administration, promising results can be obtained. Further modification and development of the present dosage form may lead to newer type of drug delivery system with the potential to deliver therapeutic protein and peptide drugs orally.

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References

Bernkop-Schnurch, A., Gilge, B., 2000. Anionic mucoadhesive polymers as auxiliary agents for the peroral administration of (poly) peptide drugs: influence of the gastric juice. Drug Dev. Ind. Pharm. 26, 107–113.

Brecher, G., Schneiderman, M., 1950. A time-saving device for the counting of reticulocytes. Am. J. Clin. Pathol. 20, 1079–1083.

Brecher, G., 1949. New methylene blue as a reticulocytes stain. Am. J. Clin. Pathol. 19, 895–896.

Davis, J.M., Aralkawas, T., Strickland, T.W., Yphantis, D.A., 1987. Characterization of recombinant human erythropoietin produced in Chinese hamster ovary cells. Biochemistry 26, 2633–2638.

Eaimtrakarn, S., Rama Prasad, Y.V., Ohno, T., Konishi, T., Yoshikawa, Y., Shibata, N., Takada, K., 2002. Absorption enhancing effect of Labrasol on the intestinal absorption of insulin in rats. J. Drug Target 10, 255–260.

Eschbach, J.W., Egrie, J.C., Downing, M.R., Browne, J.K., Adamson, J.W., 1987. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. N. Engl. J. Med. 316, 73–78.

Goldwasser, E., Kung, C.K.H., 1968. Progress in the purification of erythropoietin. Ann. N. Y. Acad. Sci. 149, 49–53.

Guggi, D., Kast, C.E., Bernkop-Schnurch, A., 2003. In vivo evaluation of an oral salmon calcitonin-delivery system based on a thiolated chitosan carrier matrix. Pharm. Res. 20, 1989–1994.

Ito, Y., Arai, H., Uchino, K., Iwasaki, K., Shibata, N., Takada, K., 2005. Effect of adsorbents on the absorption of lansoprazole with surfactant. Int. J. Pharm. 289, 69–77.

Ito, Y., Kusawake, T., Ishida, M., Tawa, R., Shibata, N., Takada, K. Oral solid gentamicin preparation using emulsifier and adsorbent. J. Control. Release, in press.

Krauland, A.H., Guggi, D., Bernkop-Schnurch, A., 2004. Oral insulin delivery: the potential of thiolated chitosan-insulin tablets on non-diabetic rats. J. Control. Release 95, 547–555.

Lehr, C.M., Bouwstra, J.A., Kok, W., de Boer, A.G., Tukker, J.J., Verhoef, J.C., Breimer, D.D., Junginger, H.E., 1992. Effects of the mucoadhesive polymer polycarbophil on the intestinal absorption of a peptide drug in the rat. J. Pharm. Pharmacol. 44, 402–407.

Lueben, H.L., de Leeuw, B.J., Langemeyer, M.W.E., de Boer, A.G., Verhoef, J.C., Junginger, H.E., 1996. Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin in vivo. Pharm. Res. 13, 1668–1672.

Lueben, H.L., Verhoef, J.C., Borchard, G., Lehr, C.M., de Boer, A.G., Junginger, H.E., 1995. Mucoadhesive polymers in peroral peptide drug delivery. II. Carbomer and polycarbophil are potent inhibitors of the intestinal proteolytic enzyme trypsin. Pharm. Res. 12, 1293–1298.

Maitani, Y., Hazama, M., Tojo, Y., Simoda, N., Nagai, T., 1996. Oral administration of recombinant human erythropoietin in liposomes in rats: influence of lipid composition and size of liposomes on bioavailability. J. Pharm. Sci. 85, 440–445.

Maitani, Y., Moriya, H., Shimoda, N., Takayama, K., Nagai, T., 1999. Distribution characteristics of entrapped recombinant human erythropoietin in liposomes and its intestinal absorption in rats. Int. J. Pharm. 185, 13–22.

Markham, A., Bryson, H., 1995. Epoetin alfa, a review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in non-renal applications. Drugs 49, 232–254.

Mizuno, A., Ueda, M., Kawanishi, G., 1992. Effects of salicylate and other enhancers on rectal absorption of erythropoietin in rats. J. Pharm. Pharmacol. 44, 570–573.

Rama Prasad, Y.V., Minamimoto, T., Yoshikawa, Y., Shibata, N., Mori, S., Matsuura, A., Takada, K., 2004. In situ intestinal absorption studies on low molecular weight heparin in rats using Labrasol as absorption enhancer. Int. J. Pharm. 271, 225–232.

Shimoda, N., Maitani, Y., Machida, Y., Nagai, T., 1995. Effects of dose, pH and osmolarity on intranasal absorption of recombinant human erythropoietin in rats. Biol. Pharm. Bull. 18, 734–739.

Spivak, J., 1994. Recombinant human erythropoietin and the anaemia of cancer. Blood 84, 997–1004.

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., 1988. Effect of recombinant human erythropoietin on the anemia of chronic renal failure. Int. J. Cell Cloning 6, 179–191.

Venkatesan, N., Yoshimitsu, J., Ito, Y., Shibata, N., Takada, K., 2005. Liquid filled nanoparticles as a drug delivery tool for protein therapeutics. Biomaterials 26, 7154–7163.

Whitehead, K., Shen, Z., Mitragotri, S., 2004. Oral delivery of macromolecules using intestinal patches: application for insulin delivery. J. Control. Release 98, 37–45.

Winerals, C.G., Olivar, D.O., Pippard, M.J., Reid, C., Downing, M.R., Cotes, P.R., 1986. Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis. Lancet ii, 1175–1177.

Yoshikawa, Y., Kato, K., Sone, H., Takada, K., 1998. Development and evaluation of non-compartmental pharmacokinetic analysis program "Win-HARMONY" 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. 29, 475–487.