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In situ intestinal absorption studies on low molecular weight heparin in rats using Labrasol as absorption enhancer

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Abstract

Oral absorption of low molecular weight heparin (LMWH) is limited by its molecular size and negative charge. Development of its oral formulations would allow outpatient treatment with LMWH and decrease the hospital expenses. Studies were aimed at evaluating Labrasol for improving intestinal absorption of LMWH. Formulations containing LMWH and Labrasol were administered to duodenum, jejunum, and ileum of the fasted rats. The doses of LMWH and Labrasol were 200 IU/kg and 50 mg/kg, respectively. Reversibility of absorption enhancing effect of Labrasol was assessed by administering LMWH to jejunum after 0.5 and 1 h of administration of Labrasol. The effect of different doses of Labrasol on LMWH absorption was studied by administering Labrasol at 50, 100, and 200 mg/kg doses. Administration of LMWH formulation to jejunum resulted in the highest plasma anti-Xa activity (0.50±0.03 IU/ml) compared to duodenum (0.19±0.03 IU/ml), and ileum (0.29±0.06 IU/ml) and the anti-Xa levels were maintained above the therapeutic level for about 160 min. The absorption of LMWH was negligible when LMWH was administered at 0.5 and 1 h post-Labrasol administration. Increasing the dose of Labrasol has decreased the absorption of LMWH from jejunum. Labrasol increased the intestinal absorption of LMWH, and jejunum was found to be the best site of absorption. Intestinal membrane permeability changes induced by Labrasol were transient and reversible. Maintaining high drug concentration gradient across intestinal wall is important to obtain increased intestinal LMWH absorption.

Keywords: Low molecular weight heparin; Labrasol; Deep vein thrombosis; Anticoagulant; Oral heparin

1. Introduction

Heparin is the anticoagulant of choice in the treatment of deep vein thrombosis and pulmonary embolism (Agnelli and Sonaglia, 2000; Ageno, 2000). Unfractionated heparin (UH) is a naturally occurring glycosaminoglycan that exists as a heterogenous mixture of oligosaccharides composed of alternating chains of D-glucosamine and uronic acid (Hirsh et al., 1992). Heparin is sulfated, highly acidic and has a negative ionic charge. In many countries, low molecular weight heparins (LMWHs) have replaced UH for the prevention and treatment of venous thrombo-embolism (Boneu, 2000) due mainly to a longer half-life and less bleeding for a given antithrombotic effect compared to UH. Furthermore, the frequency of heparin-induced thrombocytopenia is lower with LMWHs and almost non-existent when

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used in the short-term because of less immunogenic nature (Lane et al., 1984). LMWHs have a molecular weight of approximately 4500 Da and, compared to UH (12,000 Da), show better distribution and less binding to non-anticoagulant-related plasma proteins and platelets (Heit, 1998). The gastrointestinal (GI) absorption of LMWH by itself has not been reliable because of the relatively large size and the negative charge of this molecule. Hence, LMWHs are administered parenterally in clinical practice, which is not only expensive but also painful. The availability of oral formulations of LMWHs would result in decreased hospital stay, better patient compliance and reduced expenses associated with parenteral formulations. Different strategies are under investigation to improve oral absorption of LMWHs. A carrier compound, sodium *N*-[10(2-hydroxybenzoyl)amino]decanoate (SNAD), was found to increase the enteral absorption of LMWH in pigs (Salartash et al., 2000). SNAD is claimed to neutralize LMWH making it more lipophilic through non-covalent bonding and consequently favoring its permeation through the intestinal wall. Intestinal absorption of LMWH in rats and human subjects was reported (Nissan et al., 2000) using sodium cholate as absorption promoter through rectal administration. Enhanced intestinal absorption of LMWH following intraduodenal administration in rats and pigs was reported with the use of Carbopol (Thanou et al., 2001a). There was a report of increased intestinal absorption of LMWH with a chitosan derivative, mono-*N*-carboxymethyl chitosan, in rats (Thanou et al., 2001b). Besides oral route, other non-parenteral routes such as nasal (Arnold et al., 2002) and transdermal (Mitragotri and Kost, 2001) routes are also under investigation.

In our earlier studies, Labrasol was found to enhance the intestinal absorption of hydrophilic drug gentamicin (Hu et al., 2001), and high molecular weight drugs such as insulin (Eaimtrakarn et al., 2002) and vancomycin (Rama Prasad et al., 2003). Labrasol is a surfactant that contains saturated polyglycolyzed C_6–C_{14} glycerides and its NMR characterization indicated that it is a mixture consisting of 30% mono-, di- and triglycerides of C_8 and C_{10} fatty acids, 50% of mono- and di-esters of poly(ethylene glycol) (PEG) and 20% of free PEG 400 (Kreilgaard et al., 2000). It shows high tolerance and low toxicity, and is having a LD_{50} of 22 g/kg in rats. Caprylocaproyl macrogolglyceride (Labrasol) is included as a pharmaceutical excipient in European Pharmacopoeia in 1998. In the present study, Labrasol was evaluated for its ability to improve the intestinal absorption of LMWH in rats by in situ administration to the small intestine.

2. Materials and methods

2.1. Materials

LMWH (Parnaparin sodium, anti-Xa factor activity (aXa): 85.4 IU/mg) was a gift sample from Shimizu Pharmaceutical Co., Ltd. (Shizuoka, Japan). Labrasol (Gattefösse, France) was obtained from CBC Co., Ltd. (Tokyo, Japan). Male Wistar rats used in the study were obtained from Nippon SLC Company (Hamamatsu, Japan) and standard solid meal of commercial food (LabDiet®) was purchased from Nippon Nousan Co., Ltd. (Yokohama, Japan). All other materials used were of reagent grade and were used as received.

2.2. LMWH preparations

The composition of formulations used in the present study is shown in Table 1. LMWH was initially dissolved in deionised water and Labrasol was added and upon mixing, transparent formulations were obtained. The ratio of Labrasol and water was maintained at 1:1 in all the formulations. LMWH formulations were equilibrated at ambient temperature overnight and then used in animal experiments.

2.3. Absorption studies

Male Wistar rats (300–350 g) fasted overnight for at least 12 h were used in the study. The rats were anaesthetized by intraperitoneal administration of sodium pentobarbital solution (50 mg/kg). The abdominal cavity was cut opened and upper small intestine was isolated. A small pore was made in the duodenum with a 23 G needle and the LMWH formulation (formulation B) was administered at a dose of 200 IU/kg of LMWH and 50 mg/kg of Labrasol. The pore was sealed with synthetic glue. The same formulation at the same dose of LMWH and Labrasol was also administered to the jejunum (30 cm from ileo–caecal junction) and ileum (15 cm from ileo–caecal junction) of the rats to find

Table 1
LMWH formulations used in absorption studies

Formulation	Volume of solution (μl/kg)	Dose of Labrasol (mg/kg)	Site of administration
A	100.0	0.0	Duodenum, jejunum, and ileum
B	100.0	50.0	Duodenum, jejunum, and ileum
C	200.0	100.0	Jejunum
D	400.0	200.0	Jejunum

The dose of LMWH was 200 IU/kg in all the experiments.
The ratio of water and Labrasol was 1:1 in all the experiments except in formulation A.

out the best site of LMWH absorption in the small intestine. Blood samples of 0.5 ml each were collected from the right jugular vein at 0.25, 0.5, 1, 2, 3, and 4 h intervals. The blank blood sample was taken at 5 min prior to the administration of test preparations. The blood samples were collected into syringes containing 0.05 ml of 3.2% (w/v) trisodium citrate dihydrate solution as anticoagulant. The samples were mixed well and immediately cooled on an ice bath. Plasma was obtained from whole blood by centrifugation at 5000 rpm for 20 min at 4 °C using Kubota 1720 centrifuge (Tokyo, Japan), and then stored at −80 °C until analysis.

Studies were also carried out with increased doses of Labrasol, i.e. 100 and 200 mg/kg to find out the effect of the dose of Labrasol on LMWH absorption from jejunum. Factor Xa inhibition activity (IU/ml) was measured in plasma samples by the Testzym® Heparin S Kit (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). All the animal experiments were carried out in accordance with the Guidelines for Animal Experimentation in Kyoto Pharmaceutical University.

2.4. Reversibility studies

An absorption enhancer should elicit its effect without causing any permanent damage to the intestinal membrane. The enhancer induced membrane structure or fluidity changes should return to normal to allow for the normal physiological function of the intestinal wall. The reversibility of the absorption enhancing effect of Labrasol was studied by administering Labrasol alone to the jejunum followed by the administration of LMWH solution to the same site at 0.5 and 1 h after Labrasol administration. The blood samples were collected from that time onwards for 4 h.

2.5. Pharmacokinetic analysis

The time to reach maximum plasma anti-Xa activity (T_{max}) and the maximum plasma anti-Xa activity (C_{max}) were determined from the authentic plasma anti-Xa activity versus time data. The area under the plasma anti-Xa activity versus time curve up to 4 h ($AUC_{0–4\,h}$) and the area under the first-moment curve up to 4 h ($AUMC_{0–4\,h}$) after administration of the test preparations were calculated using the linear trapezoidal rule up to the last measured anti-Xa activity. The mean residence time (MRT) was calculated by $AUMC_{0-\infty}/AUC_{0-\infty}$.

2.6. Statistical analysis

All values are expressed as their mean ± S.E. Statistical tests of significance were performed with GraphPad Prism 4 (Graphpad Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA). Post hoc statistical comparison of the means from individual groups was performed using Tukey's multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

3. Results

The plasma anti-Xa activity versus time profiles following administration of LMWH formulation containing Labrasol to rat duodenum, jejunum, and ileum are shown in Fig. 1. The pharmacokinetic parameters such as T_{max}, C_{max}, MRT, and $AUC_{0–4\,h}$ obtained after administration of different LMWH formulations are given in Table 2. The T_{max} and C_{max} values given in Table 2 were the mean of T_{max} and C_{max} values of individual rats ($n = 3$). The plasma anti-Xa activity

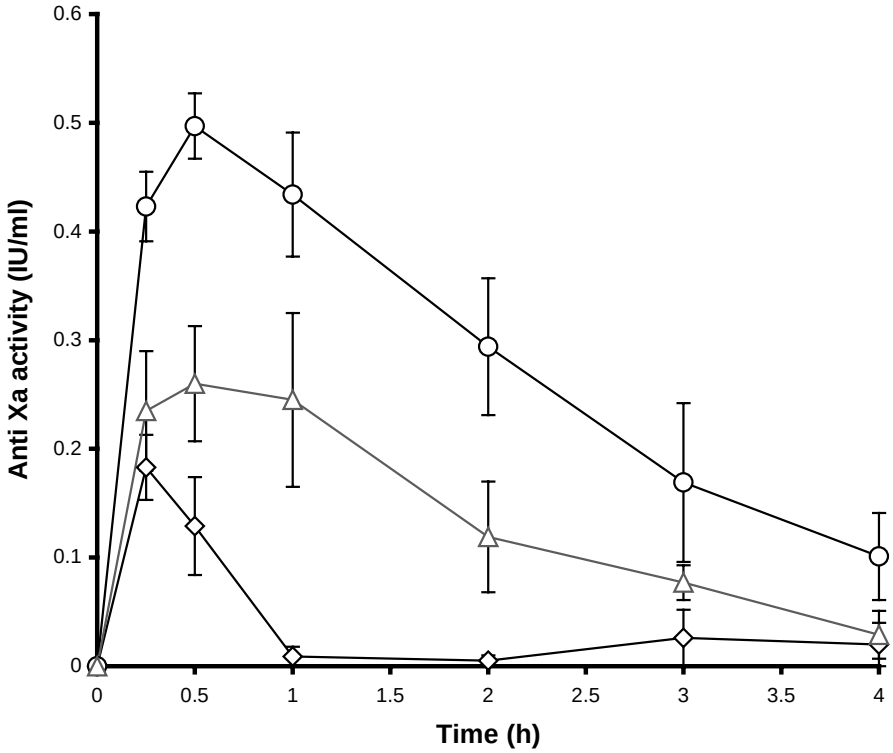

Fig. 1. Plasma anti-Xa activity vs. time profiles following administration of LMWH formulation to rat duodenum (◇), jejunum (○), and ileum (△). LMWH was dissolved in water and Labrasol was added to it. The ratio of water and Labrasol was 1:1. The dose of LMWH was 200 IU/kg and that of Labrasol was 50 mg/kg. Values are the mean ± S.E. of three animals.

was negligible following the administration of LMWH solution alone to duodenum, jejunum, and ileum (formulation A). Except for jejunal administration, the anti-Xa activity was not detected in the plasma samples obtained following duodenal and ileal administration. A transient rise in plasma anti-Xa activity was observed and returned to base level within 1 h following duodenal administration of 50 mg/kg of Labrasol along with 200 IU/kg of LMWH (formulation B). The C_{max} and T_{max} values obtained were 0.19±0.03 IU/ml and 0.33 ± 0.08 h, respectively. The C_{max} value obtained was just below the therapeutic plasma anti-Xa activity of 0.2 IU/ml. Administration of formulation B to jejunum resulted in an increased plasma anti-Xa levels than those observed with duodenal administration. The C_{max} value obtained (0.50±0.03 IU/ml) was

Table 2
Pharmacokinetic parameters of LMWH after administration of test preparations

Formulation	Site of administration	C_{max} (IU/ml)	T_{max} (h)	MRT[a] (h)	$AUC_{0-4\,h}$ (IU h/ml)
B	Duodenum	0.19 ± 0.03	0.33 ± 0.08	0.79 ± 0.43	0.14 ± 0.07
	Jejunum	0.50 ± 0.03*	0.50 ± 0.00	2.12 ± 0.35	1.13 ± 0.20**
	Ileum	0.29 ± 0.06	0.67 ± 0.17	1.51 ± 0.40	0.55 ± 0.15
C	Jejunum	0.35 ± 0.04	0.67 ± 0.17	1.83 ± 0.18	0.63 ± 0.09
D	Jejunum	0.19 ± 0.07	0.75 ± 0.25	1.12 ± 0.31	0.36 ± 0.23

[a] $MRT = AUMC_{0-\infty}/AUC_{0-\infty}$.
* Significantly different from formulations B (duodenum) and D ($P < 0.01$).
** Significantly different from formulations B (duodenum, $P < 0.01$) and D ($P < 0.05$).

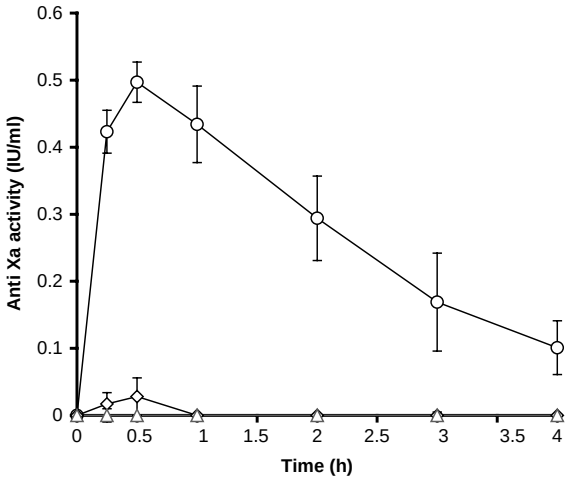

Fig. 2. Plasma anti-Xa activity vs. time profiles following administration of LMWH solution after 0 h (○), 0.5 h (◇), and 1 h (△) of administration of Labrasol to rat jejunum. LMWH was dissolved in water. The dose of LMWH was 200 IU/kg and that of Labrasol was 50 mg/kg. Values are the mean ± S.E. of three animals.

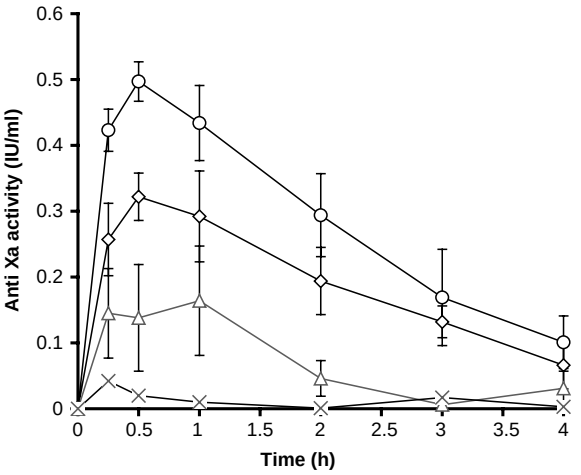

Fig. 3. Plasma anti-Xa activity vs. time profiles following administration of LMWH formulations at Labrasol doses of 0 mg/kg (×), 50 mg/kg (○), 100 mg/kg (◇), and 200 mg/kg (△). LMWH was dissolved in water and Labrasol was added to it. The ratio of water and Labrasol was 1:1. The dose of LMWH was 200 IU/kg. Values are the mean ± S.E. of three animals.

about 2.6 times more than that observed with duodenal administration. The T_{max} was observed at 0.5 ± 0.00 h and the anti-Xa activity was maintained over 0.2 IU/ml for about 160 min.

The plasma anti-Xa activity was decreased following administration of formulation B to rat ileum. The C_{max} value was 0.29 ± 0.06 IU/ml and the therapeutic level was maintained for about 70 min. The T_{max} was high (0.67 ± 0.17 h) compared to the other two sites of administration. $AUC_{0-4\,h}$ values of 0.14 ± 0.07, 1.13 ± 0.20 and 0.55 ± 0.15 IU h/ml were obtained following the duodenal, jejunal, and ileal administrations, respectively. The $AUC_{0-4\,h}$ value obtained following jejunal administration was significantly ($P < 0.05$) higher than that obtained following duodenal administration. However, there was no significant difference in the MRT values observed following the administration of formulation B to duodenum, jejunum, and ileum.

In the above studies jejunum was found to be the best site of absorption for LMWH with Labrasol. Further studies were carried out on jejunum. The results of the study on the reversibility of absorption enhancing effect of Labrasol are shown in Fig. 2. When Labrasol and LMWH solutions were administered simultaneously to rat jejunum, a C_{max} value of 0.50 IU/ml was obtained. Administration of LMWH solution at 0.5 h and 1 h after administration of Labrasol resulted in the absence of absorption of LMWH. The plasma anti-Xa levels observed were negligible and were similar to those observed in the absence of Labrasol (formulation A). The results indicate that the absorption enhancing effect of Labrasol on rat jejunum was transient and the membrane quickly regained its barrier function.

The results of the study with increased doses of Labrasol are shown in Fig. 3. When 100 mg/kg of Labrasol was administered (formulation C), the C_{max} value was decreased to 0.35 ± 0.04 IU/ml compared to 50 mg/kg dose (0.50 ± 0.03 IU/ml) and the T_{max} was observed at 0.67 ± 0.17 h. Further increase in Labrasol dose to 200 mg/kg (formulation D) resulted in significant decrease in C_{max} value (0.19 ± 0.07 IU/ml) compared to 50 mg/kg and the T_{max} value observed (0.75 ± 0.25 h) was highly variable among the individual rats. For the purpose of comparison, the plasma anti-Xa activity versus time profile in the absence of Labrasol is also given in Fig. 3. The absorption of LMWH was very low in the absence of Labrasol and a C_{max} value of 0.04 ± 0.02 IU/ml was obtained at 0.17 ± 0.08 h. The results indicate that increasing the dose of Labrasol decreased the plasma anti-Xa activity and the absorption of LMWH was negligible in the

absence of Labrasol even at the best site of absorption, i.e. jejunum. Increased dose of Labrasol has resulted in decreased $AUC_{0-4\,h}$ values (Table 2), and the value obtained with 200 mg/kg dose (0.36 ± 0.23) was significantly lower than that obtained with 50 mg/kg dose (1.13 ± 0.20). There was not much difference in the MRT values obtained with 50, 100, and 200 mg/kg doses of Labrasol.

4. Discussion

There are many physicochemical properties that are associated with poor intestinal wall permeability of highly polar and macromolecular drugs. These include low octanol/aqueous partitioning, the presence of strongly charged functional groups, high molecular weight, a substantial number of hydrogen-bonding functional groups and high polar surface area (Aungst and Saitoh, 1996). Absorption of large and more hydrophilic drugs is mostly limited to the paracellular pathway. Entry of molecules through the paracellular pathway is primarily restricted through the tight junction (Madara, 1989). Hydrophilic and low molecular weight drugs can cross the bimolecular lipid membrane of GI tract covered with mucus layer by a filtration process through membrane. However, LMWH has a relatively large molecular weight and it cannot pass through the membrane. One approach to overcome the membrane resistance to the permeation of drugs is to co-administer drugs with absorption enhancing agents. Therefore, the present work was designed to study whether the membrane permeability of a hydrophilic and high molecular weight drug such as LMWH can be modified through the use of a surfactant (Labrasol).

Surfactants that are too hydrophobic are poor enhancers and surfactants that are very hydrophilic cannot partition into the hydrophobic environment of the lipid bi-layer (Swenson and Curatolo, 1992). A medium length alkyl chain surfactant may penetrate the lipid bi-layer easily. Labrasol is a surfactant that contains predominantly alkyl chain lengths of C_8 and C_{10}. Hence, in the present study Labrasol was used as an absorption enhancer for hydrophilic drug, LMWH. Administration of LMWH at a dose of 200 IU/kg alone to duodenum, jejunum or ileum resulted in very low or negligible plasma anti-Xa levels. The highest anti-Xa level obtained was 0.04 IU/ml following jejunal administration. This indicates that intestinal LMWH absorption was restricted by the barrier function of the intestinal epithelium. Co-administration of Labrasol at a dose of 50 mg/kg with LMWH increased the plasma anti-Xa activity from duodenum, jejunum, and ileum. Labrasol-induced LMWH absorption was higher from jejunum than from duodenum and ileum. The C_{max} value obtained from jejunal administration was 2.6 and 1.7 times more than those obtained with duodenal and ileal administrations. It has been reported that plasma anti-Xa concentration required to induce a 50% antithrombotic effect is 0.12 IU/ml, whereas concentrations exceeding 0.2 IU/ml always result in an evident antithrombotic effect (Bianchini et al., 1995). Plasma anti-Xa level of 0.2 IU/ml was maintained for about 160 min with jejunal administration compared to 0 and 70 min observed with duodenal and ileal administrations, respectively. The results of the study clearly indicate that jejunum was the best site of absorption for LMWH with Labrasol.

The absorption of many drugs is intestinal site-dependent and it may the reason for a transient absorption of LMWH (0.04 IU/ml) observed in jejunum following the administration of formulation A compared to no absorption in duodenum and ileum. Similarly, the effects of the absorption enhancing agents are also often intestinal site-dependent. The precise reason(s) for increased absorption of LMWH with the addition of Labrasol is not known. As a class it has been speculated that surfactants increase the permeability of drugs via disruption or fluidization of the cell membrane and subsequently increase transcellular transport (Liu et al., 1999). But this surfactant-induced intestinal permeation enhancement is correlated with acute epithelial damage. There were reports of intestinal damage caused by surfactants resulting in the release of cellular components. The extent of damage depends upon the type of surfactant, the quantity of surfactant and the time of exposure. However, there were many reports of rapid reversibility in the acute damage caused by different surfactants immediately after their removal from the intestinal site (Swenson and Curatolo, 1992; Erickson, 1988; Nakanishi et al., 1983). The results of the reversibility study also indicate that the Labrasol-induced increase in intestinal absorption of LMWH was transient and reversible. The plasma anti-Xa levels observed following ad-

ministration of LMWH at 0.5 and 1 h after Labrasol administration were almost close to base level and were similar to those observed with formulation A. The results indicate that Labrasol-induced changes in the intestinal membrane, if any at 50 mg/kg, were lasted for less than 0.5 h. However, further studies involving the estimation of cellular components (e.g. LDH and proteins) released after the administration of formulations are to be carried out to establish the safety of Labrasol.

The results of the recent studies (Thanou et al., 2001a) indicated the involvement of paracellular route in the intestinal absorption of LMWH. However, other studies have indicated that Labrasol rapidly decreased the short circuit current (I_{sc}) across jejunal membrane mounted on Ussing-type diffusion chamber (Koga et al., 2002). I_{sc} is identical with the total flow of electrogenic ions through the membrane. The studies concluded that the effect of Labrasol on active transport via ion flux was strong. The results of the study also indicate that Labrasol has no significant effect on the membrane resistance (R_m), and the membrane barrier function was maintained normally. So, the lowering of I_{sc} by Labrasol might have contributed to the increased active transport of LMWH. However, mechanistic studies are needed to establish the role of paracellular route and active transport in the intestinal absorption of LMWH with Labrasol. Non-ionic surfactants with low polydispersity (the ratio of weight-weighed average diameter (d_w) and number-weighed average diameter (d_n) is approximately 1) change the membrane lipid fluidity thereby influencing the membrane permeability of drugs (Koga et al., 1999). The d_w/d_n value of Labrasol is 1.2. The low polydispersity of Labrasol might also have resulted in increased membrane permeability of LMWH following jejunal administration.

Administration of LMWH formulations at higher doses of Labrasol has resulted in decreased plasma anti-Xa levels. The C_{max} values obtained with 100 and 200 mg/kg dose of Labrasol were lower than that obtained with 50 mg/kg dose. The C_{max} value obtained with 200 mg/kg dose of Labrasol was even lower than the therapeutic anti-Xa level of 0.2 IU/ml. In this study the dose of LMWH was maintained at 200 IU/kg and the ratio of water and Labrasol was maintained at 1:1. As a result the volume of solution administered was 200 μl/kg for 100 mg/kg dose and 400 μl/kg for 200 mg/kg dose. Hence the entire dose of LMWH was diluted in either 200 or 400 μl of solution administered per kilogram body weight. This might have resulted in decreased concentration gradient across the intestinal membrane, which acts as driving force for passive diffusion of hydrophilic and high molecular weight drugs. Labrasol was reported to have no significant effect on the membrane resistance, an indicator of paracellular route (Koga et al., 2002). Labrasol was found to increase the active transport of drug molecules. The active transport carriers might have saturated with 50 mg/kg dose of Labrasol itself resulting in no further enhancing effect on LMWH absorption with increased Labrasol dose. But the decrease in the concentration gradient due to increased formulation volume resulted in decreased absorption of LMWH with increased doses of Labrasol.

Labrasol increased the intestinal absorption of hydrophilic macromolecular drug, LMWH. Jejunum was found to be the best site for Labrasol-induced absorption of LMWH. The plasma anti-Xa activity obtained with 50 mg/kg dose of Labrasol was above therapeutic level and sustained for about 160 min. Labrasol-induced changes in the permeability of intestinal epithelium were transient and reversible. Increasing the dose of Labrasol resulted in decreased plasma anti-Xa levels indicating the importance of maintaining a high drug concentration gradient across the intestinal wall.

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