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Granules in the improvement of oral heparin bioavailability

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ABSTRACT

Low molecular weight heparins (LMWHs), mainly used for the prevention of deep vein thrombosis, are so far only administered by parenteral route. Presumably, due to their large size and negative charge, LMWHs are not absorbed efficiently across the mucus of the gastrointestinal tract. Since parenteral administration requires medical assistance and is not the most convenient route of application, the development of an oral dosage form of heparin would improve patients' comfort and replace vitamin K antagonists. Thus, we developed granules of two LMWHs, enoxaparin and bemiparin, based on primary granules of microcrystalline cellulose and loaded with ethylcellulose (Aquacoat® ECD) or a polycationic polymer (Eudragit® RS30D (ERS)) or their mixtures. The highest maximal plasma anti-Xa activities and the highest bioavailabilities for enoxaparin granules (0.45 ± 0.12 IU/mL; 19.00 ± 0.30%, respectively) and for bemiparin granules (0.54 ± 0.08 IU/mL; 29.02 ± 4.12%, respectively) were found after oral administration of granules loaded with ERS alone at a dose of 600 IU anti-Xa/kg to rabbits. These results confirm the oral absorption of LMWH from granules loaded with polycationic polymer and open up this technology for peptides and proteins that are very sensitive to organic solvents and have poor drug absorption from the gastrointestinal tract.

1. Introduction

Discovered more than 90 years ago by Mclean (1916), unfractionated heparins (UFH) were used in clinic over 50 years and are still nowadays one of the most important anticoagulant drugs in current clinical use especially when a rapid effect is desired like in intensive healthcare or in surgery. Furthermore, thanks to their longer half-lives, less bleeding properties for a given antithrombotic effect and no laboratory monitoring requirement, low molecular weight heparins (LMWHs) replaced UFH step by step in the prevention and treatment of venous thrombo-embolism over the last few decades (Hirsh and Raschke, 2004). Despite their various advantages, LMWHs are molecules with large molecular weight, negatively charged structure and instability under acidic conditions obliging them to be administered by parenteral way and to be replaced by an orally active anticoagulant like warfarin for long-term outpatient therapy. However, warfarin presents a slow onset, a very low therapeutic index and a high possibility of drug/drug interactions limiting its practical utility. An oral dosage form of LMWH could be a very attractive alternative to usual oral anticoagulants and would result in shortened hospital stay, improvement of patient compliance and reduction in expenses (Arbit et al., 2006). From this observation, a lot of studies have been done during the last two decades to find a delivery system for the oral administration of heparin. These studies included strategies, e.g. cell-membrane permeabilization, tight junction modifications, increase of drug lipophilicity or protection against acidic pH of the stomach (Motlekar and Youan, 2006). Absorption enhancers were one of the chosen strategies to improve heparin absorption. Thanou et al. (2001a,b,c) used the Ca^{2+}-chelating ability of a poly(acrylate) derivative Carbopol 934P to interfere with the intercellular junctions and increase the paracellular permeability. Another strategy to circumvent the barrier function of the gastrointestinal tract is offered by thiol groups of polymeric adjuvants inhibiting protein tyrosine phosphatase involved in the closing process of tight junctions, via a GSH-mediated mechanism (Bernkop-Schnurch et al., 2003; Schmitz et al., 2005). Motlekar et al. investigated the absorption-enhancing effect of glycyrrhetinic acid (Motlekar et al., 2006a,b,c), L-arginine (Motlekar et al., 2006a,b,c), zonula occludens toxin synthetic peptide derivative (AT1002) (Motlekar et al., 2006a,b,c) or sodium caprate (Motlekar et al., 2005) on the intestinal permeability and bioavailability of ardeparin using rat model and Caco-2 cell culture model. Various other strategies are also under investigation such as dimethyl sulfoxide and deoxycholic acid conjugates (Kim et al., 2007) in monkeys, chitosan derivatives (Thanou et al., 2007) or Labrasol (Rama Prasad et al., 2004) in rats. Currently, the most effective formulation is the co-administration of heparin with N-[8-(2-hydroxybenzoyl)amino]caprylate, a carrier molecule that improves the oral absorption of heparin in humans (Berkowitz et al., 2003). Previous results obtained with various oral

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delivery systems of UFH or LMWH and based on their encapsulation into polymeric nano/microparticles (Hoffart et al., 2006; Jiao et al., 2002a,b; Lamprecht et al., 2007) have led to the gastrointestinal absorption of heparin in rabbits, with doses that were similar to those administered by intravenous infusion or subcutaneous injection in humans.

In this study, granules of LMWH were prepared by wet granulation and subsequently blended with two polymeric aqueous dispersions used alone or in different mixtures (Eudragit®RS30D and Aquacoat®ECD30). This new oral form of heparin, compared to nano/microparticles, may offer a more convenient, industrialisable and robust way of fabrication leading to an easier scale up process. Furthermore, the utilization of polymeric aqueous dispersions allows the toxicological issues usually related with the use of organic solvents to be avoided and a more safety product for the working staff as well as for patients to be obtained. Enoxaparin and bemiparin were selected as LMWH drugs and the optimized LMWH granules were administered orally to rabbits.

2. Materials and methods

2.1. Materials

Two low molecular weight heparins were used as active ingredients, sodium enoxaparin (MW 3500–5500 Da), Lovenox® (10,000 IU anti-Xa/1 mL) marketed by Sanofi-Aventis (Paris, France) and sodium bemiparin powder (MW 3000–4200 Da) kindly offered by Rovi Pharmaceutical Laboratories with respective anti-Xa/anti-IIa ratio of 3–4 and 8. Microcrystalline cellulose (MCC), Ceolus®KG-802 was a gift from Asahi Kasei Chemicals Corporation (Tokyo, Japan) and was used as a filler. An aqueous dispersion of ethylcellulose polymer, Aquacoat®ECD30 (AqC) from FMC BioPolymer (Newark, U.S.A.) or aqueous dispersion of ammonio methacrylate copolymer (MW 150,000 Da), Eudragit®RS30D (ERS), from Evonik Industries (Essen, Germany) were used as loading polymers. The reagents used for the measurement of the anti-Xa activity were supplied by Diagnostica Stago (Asnières-sur-Seine, France). Hydranal®-Coulomat AG and N,N-dimethylformamide used for the water content measurement were provided by Riedel-de Haën (Seelze, Germany) and Fluka (Steinheim, Germany), respectively. All other reagents were of analytical grade and used as supplied.

2.2. Methods

2.2.1. Preparation of LMWH granules

The preparation of granules of LMWH was carried out by the wet granulation technique divided into three usual steps: moistening, drying and granulation/sieving. Samples (containing different enoxaparin content, i.e. 500, 1000, 2000, 3000, 4000 and 5000 anti-Xa IU, respectively obtained by dilution of the commercial stock solution in a final volume of 2 mL; and 75,000 anti-Xa IU of bemiparin diluted in 2 mL of distilled water) of LMWH solution were first added to 1 g of MCC. The resulting wetted mass was pre-dried at 40 °C for 3 h. Then it was forced through a sieve with a mesh size of 600 μm. Final drying of granules at 40 °C lasted 24 h. The samples were prepared in triplicate and stored in a vacuum desiccator at 4 °C in the presence of a drying agent.

2.2.2. Preparation of polymers-loaded LMWH granules

One gram of LMWH granules was blended with either ERS or AqC or their mixtures (100:0; 75:25; 50:50; 25:75 and 0:100) corresponding to 400 mg of dry polymer powder. The resulting mass was pre-dried for 1.5 h at 40 °C in order to be forced through a sieve and the polymers-loaded LMWH granules were finally dried and stored as described above. Each formulation was made in triplicate.

2.2.3. Particle size distribution

The shape of granules was determined by transmission light microscopy performed by Olympus IX50 (Olympus France S.A.S., France) and the measurement of the size ($n = 180$) was performed by Kappa ImageBase software purchased from Kappa opto-electronics GmbH (Gleichen, Germany).

2.2.4. Water content measurement

The water content measurement of granules was determined by Karl Fisher titration realized by 756 KF Coulometer (Metrohm SA, Switzerland). This assay was performed by adding 10 mg of granules to 50 mL of Hydranal®-Coulomat AG and 50 mL of N,N-dimethylformamide into a titration cell without diaphragm. Each sample was measured in triplicate.

2.2.5. In vitro drug release

Granules (50 mg) were suspended in 20 mL of phosphate buffer saline (Na_2HPO_4 0.64%, KH_2PO_4 0.06%, NaCl 0.59%; pH 7.4). The suspension was incubated in a water bath at 37 °C under magnetic stirring at 200 rpm. At different times (5, 10, 15, 30, 45 min, 1, 2, 4, 6, 8 and 24 h) samples of 1.5 mL were withdrawn and replaced by 1.5 mL of fresh phosphate saline buffer. Each sample was filtered through Porafil® cellulose mixed ester membrane with a pore size of 0.20 μm (Macherey-Nagel, Düren, Germany). The amount of heparin released from the granules was determined by turbidimetric method (Hoffart et al., 2003). Aliquots of 0.5 mL of filtered samples were added to 0.5 mL of sodium acetate buffer (acetic acid 1.22%, sodium acetate 10.75%; pH 4.4) and mixed with 2 mL of 0.1% cetylpyridinium chloride in 0.94% NaCl aqueous solution. Samples were incubated at 37 °C for 1 h and then assayed at 500 nm by UV spectroscopy (Uvikon 922, Kontron, Eching, Germany). All experiments were performed in triplicate.

2.2.6. In vivo experiments

Experiments were carried out according to the French legislation on animal experiments. Granules were filled into hard gelatine capsules and orally administered to male New-Zealand rabbits (3478 ± 395 g) housed in separate cages, fasted overnight with water ad libitum. The administration of enoxaparin granules was performed by oral gavage at a dose of 600 anti-Xa IU/kg of body weight, that one of the bemiparin granules at doses of 600 and 1200 anti-Xa IU/kg, respectively. Blood samples (1.5 mL) were taken from the marginal ear vein at different times (2, 4, 6, 8, 10 and 24 h) and then, added to a constant volume of sodium citrate 0.129 M (0.17 mL) and centrifuged at $3000 \times g$ for 10 min.

The concentration of heparin was determined automatically (STA Compact Automate, Diagnostica Stago, France) with a factor Xa chromogenic assay (Stachrom Heparin, Diagnostica Stago) (Teien and Lie, 1977). Each of the heparin standards and plasma samples containing LMWH (25 μL) was mixed with 50 μL of antithrombin III solution. This solution was mixed with 100 μL of bovine factor Xa and incubated for 30 s at 37 °C. Factor Xa chromogen substrate (100 μL) was then added and incubated at 37 °C. The absorbance was determined at 405 nm every 2 s for 10–30 s of incubation. A linear relationship between Δabsorbance/min and the concentration of heparin in the range of 0–0.8 anti-Xa U/mL was obtained. This assay had a coefficient of variation of <7% at a limit of detection of 0.02 anti-Xa U/mL.

To calculate the relative bioavailability (F) of heparin granules, the solutions of enoxaparin and bemiparin were administered by subcutaneous injection to overnight fasted rabbits at respective dose of 300 and 150 anti-Xa IU/kg of body weight. The anti-Xa activity of heparin was measured as described above. After the determination of the area under the curve (AUC) of the concentration/time profile by the linear trapezoidal method, the relative bioavailabilities were calculated by the ratio of the respective AUC

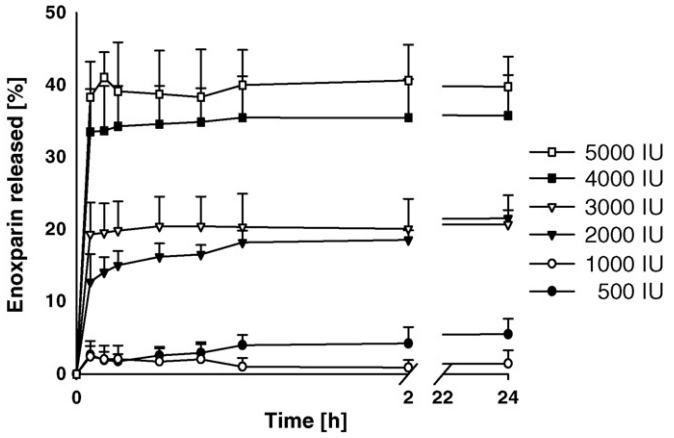

Fig. 1. Influence of enoxaparin content: Release profiles of enoxaparin from granules loaded with Eudragit®RS30D. Experiments were performed in phosphate buffer saline at 37 °C and pH 7.4 under 200 rpm stirring. Data are shown as mean ± S.D. (n = 3).

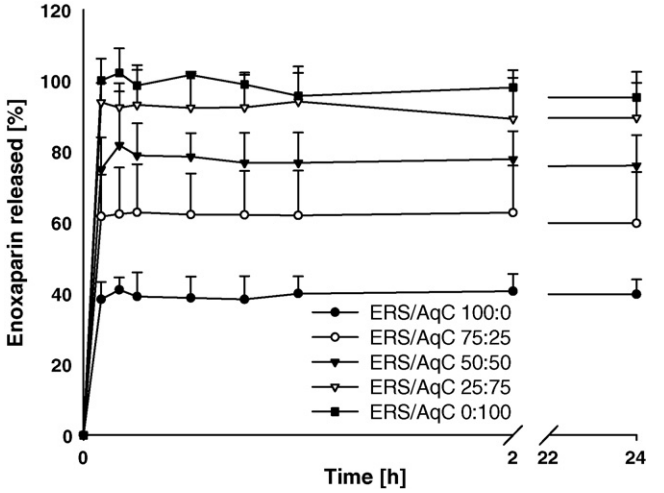

Fig. 2. Influence of the ratio of polymers: Release profile of enoxaparin (5000 anti-Xa IU) from granules loaded with polymers. Experiments were performed in phosphate buffer saline at 37 °C and pH 7.4 under 200 rpm stirring. Data are shown as mean ± S.D. (n = 3).

corrected by the administered doses. All experiments were performed in triplicate.

2.3. Statistical analysis

The results were expressed as mean values ± S.D. For the pairwise comparison the Mann–Whitney test was used to investigate differences statistically. In all cases, $P < 0.05$ was considered to be significant.

3. Results and discussion

The granules of enoxaparin, prepared with an average yield of 94.0 ± 2.9%, showed broad size distribution ranging from 5 to 335 μm. Independently on the heparin contents of the particles and polymers used, the water contents (2.8 ± 0.2%) measured after the final drying were in usual industrial range. Consequently to these parameters, low production costs and use of safe materials as an aqueous dispersion, the industrial production of enoxaparin granules can be easily considered.

Fig. 1 shows the effect of enoxaparin content (500, 1000, 2000, 3000, 4000 and 5000 anti-Xa IU) on release: the higher is the enoxaparin content the more the drug is released. The amount of drug released from the granules after 24 h of stirring ranged from 1.4 ± 1.8% to 40.1 ± 4.8%. For the lowest drug contents (500 resp. 1000 anti-Xa IU), only small amount of enoxaparin (4.0 ± 1.4% and 1.0 ± 1.2%, respectively after 1 h; 5.5 ± 2.1%, and 1.4 ± 1.8%, respectively after 24 h) was released from the granules probably due to the strong interaction of all enoxaparin with the positive groups of Eudragit®RS30D by electrostatic forces. The drug content however did not affect the shape of release profile that was composed of fast burst release followed by a plateau. It was supposed that on one hand, enoxaparin was partly adsorbed on the surface of granules and its easy desorption corresponded to a burst effect as noticed above and on the other hand enoxaparin partly and strongly interacted with Eudragit®RS30D leading to an incomplete release. Influence of enoxaparin content on its *in vitro* release presented for granules loaded with ERS only was also found for all other mixtures of polymers.

The effect of the ratio of polymers ERS/AqC (0:100; 25:75; 50:50; 75:25 and 100:0) on the release of enoxaparin is presented in Fig. 2. The decrease in the enoxaparin release has been observed with the increase in ERS content. Indeed, the percentage of enoxaparin released from the granules ranged from 38.3 ± 4.9% to 100.0 ± 6.1% when the ratio of ERS/AqC varied from 100:0 to 0:100. Thanks to its positively charged quaternary ammonium groups, ERS can form electrostatic bonds with negatively charged sulfate and carboxyl groups of enoxaparin. Thus, it might be possible to control the rate of release of enoxaparin from granules by modifying the Eudragit®RS30D/Aquacoat®ECD30 ratio. As an example, granules containing 5000 anti-Xa IU of enoxaparin were chosen, but influence of the ratio of polymers on *in vitro* release of active substance was also found for granules with different drug contents (2000, 3000 or 4000 anti-Xa IU).

The previous results are confirmed in Table 1, which presents the percentage of enoxaparin released from the granules after 24 h and summarizes the influence of enoxaparin content and polymers ratio. These values varied from 21.6 ± 1.1% for granules containing 2000 anti-Xa IU of enoxaparin and loaded with ERS alone to 95.2 ± 7.2% for granules with 5000 anti-Xa IU of enoxaparin and only loaded with AqC. However, the influence of polymers ratio seemed to be more important than those of drug content. Indeed, the percentage of released drug varied between 1.0 and 2.7 times when the drug content ranged between 2000 and 5000 anti-Xa IU whereas it varied between 2.4 and 4.6 times when the ratio of polymers ERS/AqC ranged between 100:0 and 0:100.

According to *in vitro* studies, studies *in vivo* were performed with granules loaded with 5000 anti-Xa IU of enoxaparin, which

Table 1
Percentage of enoxaparin released from the granules after 24 h of stirring (200 rpm) in phosphate buffer saline pH 7.4 at 37 °C (n = 3).

		Eudragit®RS30D/Aquacoat®ECD30 ratios				
		100:0	75:25	50:50	25:75	0:100
Incorporated enoxaparin (anti-Xa IU)	2000	21.6 ± 1.1	24.2 ± 0.6	45.2 ± 1.3	80.1 ± 5.8	93.9 ± 2.5
	3000	20.7 ± 4.1	39.6 ± 3.8	67.9 ± 2.8	89.7 ± 5.3	94.8 ± 4.8
	4000	35.8 ± 5.6	51.2 ± 3.2	71.7 ± 3.8	92.4 ± 8.2	93.7 ± 5.2
	5000	39.8 ± 4.2	59.7 ± 14.4	76.0 ± 8.6	89.5 ± 9.9	95.2 ± 7.2

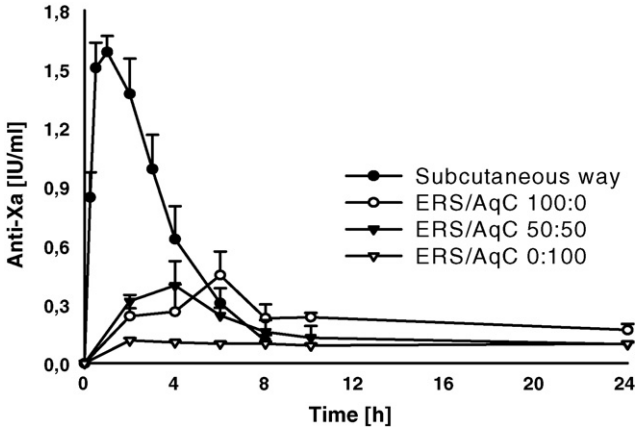

Fig. 3. *In vivo* release of enoxaparin: Anti-Xa activity over the time after a single oral administration of granules loaded with polymers to rabbits at a dose of 600 anti-Xa IU/kg. Data are mean ± S.D. ($n = 3$).

released the highest amount of the drug. Furthermore, they allowed a more comfortable oral administration to rabbits and decreased the volume of administered dose. The results of *in vivo* studies are presented in Fig. 3. After a single oral administration of fresh granules of enoxaparin (600 IU/kg) to rabbits, the highest anti-Xa response was observed for granules loaded with ERS alone (peak concentration of 0.45 ± 0.12 IU/mL 6 h after administration). Lower peak concentrations, 0.40 ± 0.12 and 0.12 ± 0.01 IU/mL, respectively, were observed 4 and 2 h after administration of granules loaded with ERS/AqC 50:50 and AqC alone. After subcutaneous injection of a single dose of 300 IU/kg of body weight to rabbits (peak concentration of 1.59 ± 0.08 IU/mL 1 h after administration) and determination of AUC, the bioavailability values (F) were calculated. The lowest bioavailability was observed for granules loaded with AqC alone (6.66 ± 0.12%) and the highest for granules loaded only with ERS (19.00 ± 0.30%); granules loaded with ERS/AqC 50:50 showed slightly lower bioavailability (15.53 ± 1.80%) than ERS granules (Table 2). These results demonstrated the improvement of enoxaparin absorption for granules loaded with ERS. This improvement may be due to an intimate contact between enoxaparin granules loaded with ERS and mucus resulting from electrostatic interactions between positive charges of ERS and negative charges of mucus especially related to sialic acid residues (Lamprecht et al., 2006). This interaction may also lead to a higher local concentration of heparin helping its diffusion and absorption through the mucocellular wall. As presented for polycationic chitosan (Thanou et al., 2007; Thanou et al., 2001a,b,c), ERS may also interact with the surface of cells and modify the structural organization of the tight junction associated proteins allowing an increased absorption of enoxaparin by paracellular way. Compared to previous studies made with microparticles or nanoparticles (Hoffart et al., 2006; Jiao et al., 2002a,b), granules of enoxaparin present a less intense but more sustained anti-Xa activity released profile over the curative dose of 0.2 IU anti-Xa IU/mL.

According to the results obtained with enoxaparin granules, we decided to use a second generation LMWH, bemiparin sodium with a lower molecular weight (3600 Da) and higher anti-Xa/anti-IIa ratio (8:1) than enoxaparin sodium.

The effect of ERS on the *in vitro* release of enoxaparin was also found with granules of bemiparin as shown in Table 3. Determined amounts of bemiparin released from unloaded and ERS loaded granules were 80.9 ± 2.7% and 70.3 ± 4.9% after 1 h, respectively and 80.3 ± 1.0% and 68.8 ± 5.4% after 24 h, respectively. Even if the presence of ERS in the granules decreased the drug release in dissolution medium, the difference of heparin release from ERS unloaded and ERS loaded granules of bemiparin was less significant than in the case of enoxaparin. This result can be explained by the use of a greater amount of heparin (75,000 IU) leading to the saturation of the ERS polymer quaternary ammonium groups by bemiparin and the release in dissolution medium of an important amount of heparin adsorbed on the surface of the granules. The increase in heparin contents in granules led to the decrease in the ratio between the amount of heparin strongly interacting with ERS polymer and the amount of heparin weakly adsorbed on the surface of the granules. Furthermore it is interesting to notice that bemiparin was not completely released from unloaded granules. This fact could be explained by the lower molecular weight and the lower chain length of bemiparin compared to enoxaparin which led to a better entrapment of bemiparin inside the matrix of cellulose.

After a single oral administration of fresh granules of bemiparin to rabbits at the dose of 600 or 1200 anti-Xa IU/kg, respectively, the highest responses were observed for ERS loaded granules with peak concentrations of 0.54 ± 0.08 and 0.38 ± 0.15 anti-Xa IU/mL 4 h after administration as expected (see Fig. 4). After a subcutaneous injection of bemiparin solution to rabbits at a dose of 150 anti-Xa IU/kg (peak concentration of 0.51 ± 0.08 IU/mL 1 h after administration), the AUC of ERS loaded granules administered at 600 or 1200 IU/kg of rabbit body weight were determined and their calculated bioavailabilities after 10 h were 29.02 ± 4.12% and 19.05 ± 4.47%, respectively (see Table 2). Lower pharmacokinetic parameters were observed after administration of unloaded granules (peak concentration of 0.10 ± 0.03 and 0.15 ± 0.04 IU/mL and bioavailabilities of 6.55 ± 1.84% and 7.76 ± 0.85% for a dose of 600 and 1200 IU/kg, respectively) showing the role of ERS polymer on the improvement of heparin absorption after its oral administration. The increase in the dose administered to rabbits did not show an increase in the biological activity of bemiparin. Indeed, no statistical difference was observed between the biological activities of bemiparin granules administered at a dose of 600 IU/kg (peak concentration of 0.54 ± 0.08 anti-Xa IU/mL at 4 h) and at a dose of

Table 2
Pharmacokinetic parameters of granules after a single oral administration to rabbits compared with subcutaneous administration of enoxaparin ($n = 3$).

Formulations	Dose (anti-Xa IU/kg)	T_{max} (h)	C_{max} (anti-Xa IU/mL)	$AUC_{0\ to\ 10\,h}$ (IU h/mL/kg)	$F_{10\,h}$ (%)
Enoxaparin					
Enoxaparin solution	300	1	1.59 ± 0.08	2.19 ± 0.14	100
Aquacoat®ECD30	600	2	0.12 ± 0.01	0.29 ± 0.01	6.66 ± 0.12
Aquacoat®ECD30 Eudragit®RS30D 50:50	600	4	0.40 ± 0.12	0.68 ± 0.08	15.53 ± 1.80
Eudragit®RS30D	600	6	0.45 ± 0.12	0.83 ± 0.02	19.00 ± 0.30
Bemiparin					
Bemiparin solution	150	1	0.51 ± 0.08	0.91 ± 0.16	100
Unloaded granules	1200	2	0.15 ± 0.04	0.32 ± 0.03	7.76 ± 0.85
Loaded granules	1200	4	0.38 ± 0.15	0.77 ± 0.18	19.05 ± 4.47
Unloaded granules	600	2	0.10 ± 0.03	0.24 ± 0.07	6.55 ± 1.84
Loaded granules	600	4	0.54 ± 0.08	1.05 ± 0.15	29.02 ± 4.12

Table 3
Release profile of bemiparin (75,000 anti-Xa IU) from granules loaded with polymers. Experiments were performed in phosphate buffer saline at 37 °C and pH 7.4 under 200 rpm stirring. Data are shown as mean ± S.D. ($n = 3$).

	Bemiparin released (%)						
Time	5 min	15 min	30 min	1 h	2 h	4 h	24 h
Unloaded granules	87.8 ± 6.6	80.4 ± 5.6	85.3 ± 6.6	80.9 ± 2.7	82.3 ± 1.7	83.7 ± 1.5	80.3 ± 1.0
Loaded granules	74.3 ± 3.8	70.5 ± 7.4	71.2 ± 9.3	70.3 ± 4.9	73.3 ± 6.0	71.5 ± 5.3	68.8 ± 5.4

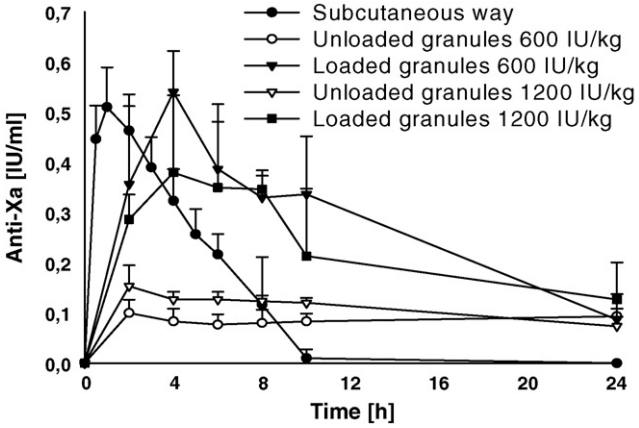

Fig. 4. *In vivo* release of bemiparin: Anti-Xa activity over the time after a single oral administration of loaded and unloaded granules to rabbits at a dose of 600 and 1200 anti-Xa IU/kg. Data are mean ± S.D. ($n = 3$).

1200 IU/kg (peak concentration was only 0.38 ± 0.15 anti-Xa IU/mL at 4 h). Since there is no statistical difference, it can be hypothesized that absorption of bemiparin through the intestinal wall may be limited by a saturable mechanism.

4. Conclusion

In this study, we tried to modify the permeability factor of heparin by using different ratios of polymers. The use of AqC seemed to increase enoxaparin release on the one hand but on the other hand decrease its permeability. Contrary to AqC, ERS decreased the release of enoxaparin or bemiparin and increased their *in vivo* permeabilities probably thanks to its mucoadhesivity and its ability to open the tight junctions. Furthermore, thanks to granules, we developed a very interesting new solid form of heparin because of its very easy, non-toxic, and industrialisable way of manufacture. The aim of our next studies would be to quantify the modification of heparin permeability created by polycationic polymers or by other intestinal absorption enhancers and to determine if this modification of permeability is controlled over the time.

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