MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY
Vol. 28, No. 9, pp. 1091–1099, 2002

RESEARCH PAPER

Low Molecular Weight Heparin-Loaded Polymeric Nanoparticles: Formulation, Characterization, and Release Characteristics

V. Hoffart,[1] N. Ubrich,[1,*] C. Simonin,[1]
V. Babak,[1] C. Vigneron,[2] M. Hoffman,[1]
T. Lecompte,[3] and P. Maincent[1]

[1]*Laboratoire de Pharmacie Galénique, EA 3452, Faculté de Pharmacie, 5 rue Albert Lebrun, B.P. 403, 54001 Nancy cedex, France*
[2]*Laboratoire d'Hématologie, EA 3452, Faculté de Pharmacie, 5 rue Albert Lebrun, B.P. 403, 54001 Nancy cedex, France*
[3]*Laboratoire d'Hématologie Biologique, EA 3452, CHU Brabois, 54500 Vandoeuvre-lès-Nancy, France*

ABSTRACT

The aim of the present work was to investigate the preparation of low molecular weight heparin (LMWH) nanoparticles (NP) as potential oral heparin carriers. The NP were formulated using an ultrasound probe by water-in-oil-in-water (w/o/w) emulsification and solvent evaporation with two biodegradable polymers [poly-ε-caprolactone, PCL and poly(D,L-lactic-co-glycolic acid) 50/50, PLGA] and two non-biodegradable positively charged polymers (Eudragit RS and RL) used alone or in combination. The mean diameter of LMWH-loaded NP ranged from 240 to 490 nm and was dependent on the reduced viscosity of the polymeric organic solution. The surface potential of LMWH NP prepared with Eudragit polymers used alone or blended with PCL and PLGA was changed dramatically from strong positive values obtained with unloaded NP to negative values. The highest encapsulation efficiencies were observed when Eudragit polymers took

*Corresponding author. Fax: 00 33 (0)3 83 17 88 79; E-mail: ubrich@pharma.uhp-nancy.fr

DOI: 10.1081/DDC-120014576
0363-9045 (Print); 1520-5762 (Online)

part in the composition of the polymeric matrix, compared with PCL and PLGA NP exhibiting low LMWH entrapment. The in vitro LMWH release in phosphate buffer from all formulations ranged from 10 to 25% and was more important (two- to threefold) when esterase was added into the dissolution medium. The in vitro biological activity of released LMWH, determined by the anti-factor Xa activity with a chromogenic substrate, was preserved after the encapsulation process, making these NP good candidates for oral administration.

Key Words: *Drug delivery; Low molecular weight heparin; Nanoparticle; W/O/W emulsion; Eudragit; Biodegradable polymer*

INTRODUCTION

Heparin is the anticoagulant of choice for the prevention of thromboembolism and the treatment of venous thrombosis and pulmonary embolism.[1] However, unfractionated heparin (UFH) has pharmacokinetic and biological limitations that led to the development of low molecular weight heparins (LMWH). These are derived from standard heparin by either chemical or enzymatic depolymerization to yield fragments that are approximately one-third the size of unfractionated heparin. Compared with UFH, LMWH have the main advantages of (i) to canceling the well-known bleeding complication of all anticoagulants, owing to their ability to mainly inactivate factor Xa,[2] (ii) reducing non-specific binding to plasma proteins[3] and consequently conferring excellent availability,[4] (iii) reducing binding to macrophages and endothelial cells with an associated increase in plasma half-life,[5] (iv) possibly reducing binding to osteoclasts resulting in less activation of osteoclats and an associated reduction in bone loss.[6] However, owing to their lack of oral absorption as for UFH, LMWH have to be administered parenterally, which does not represent the most physiological and desirable route for patients. An oral heparin formulation could consequently have tremendous clinical success. Several attempts to develop effective oral heparin formulations have been reported. For example, heparin complexes with spermine and lysine salts,[7] hydrophobic organic bases[8] and ethylenediaminetetraacetic acid (EDTA),[9] or oil-in-water emulsions[10] and liposomes[11] have been developed without exhibiting significant oral bioavailability in animals. Moreover, problems such as the toxicity of adjuvants and the lack of stability of the formulations have hampered their clinical development. Other dosage forms including proteinoid microspheres obtained from thermally condensed amino acid mixtures[12,13] have shown significant plasma heparin concentrations after oral administration in rats, as evidenced by an increase in activated partial thromboplastic time (APTT). More recently, the novel absorption enhancers sodium *N*-[8-(2-hydroxybenzoyl)amino] caprylate (SNAC)[14,15] and sodium *N*-[10-(2-hydroxybenzoyl)amino] decanoate[16] co-administered orally with unfractionated heparin have been shown to improve the heparin absorption. We also reported that UFH-loaded polymeric nanoparticles administered orally in rabbits could be used to facilitate oral heparin delivery.[17,18] Consequently, owing to the clinical advantages of LMWH compared to UFH, in this preliminary study we investigated the design and in vitro characterization of LMWH-loaded nanoparticles (NP) prepared with blends of biodegradable polymers [poly-ε-caprolactone, PCL and poly(D,L-lactic-*co*-glycolic acid) 50/50, PLGA] and non-biodegradable positively charged polymers (Eudragit RS and RL). The resulting NP prepared by the double emulsion and solvent evaporation method were first compared in terms of size, surface potential, encapsulation efficiency, and drug release in a saline phosphate buffer with or without esterase. Second, the biological activity of heparin released from the formulations suspended in the dissolution medium was checked by evaluating the anti-factor Xa activity with a chromogenic substrate.

MATERIALS AND METHODS

Eudragit® RS PO and RL PO (MW 150,000) were kindly supplied by Röhm GmbH (Darmstadt, Germany). Poly(D,L-lactic-*co*-glycolic acid) 50/50

(PLGA, MW 40,000) and poly-ε-caprolactone (PCL, MW 42,000) were respectively purchased from Medisorb Technologies International (Welmington, DE) and Aldrich Chemicals Company (Steinheim, Germany). Esterase from porcine liver (20 units/mg) and polyvinylalcohol (PVA) (MW 30,000, 88% hydrolyzed), chosen as the surface active agent for the second emulsion, were supplied by Sigma. Marketed low molecular weight heparin (HBPM, Innohep®, 20,000 IU anti-Xa/2 mL injectable solution) was a gift from Leo (Saint-Quentin-en-Yvelines, France). The kit used for the measurement of the anti-Xa activity (Stachrom® Heparin) was purchased from Diagnostica Stago (Asnières-sur-Seine, France). All other chemical reagents were of analytical grade and used as supplied.

Preparation of Nanoparticles

The LMWH-loaded NP were prepared by the water-in-oil-in-water (w/o/w) emulsion and evaporation method.[19] Briefly, 1 mL of aqueous LMWH solution (5000 IU) was first emulsified in methylene chloride (10 mL) containing the polymer(s) (0.25 g) with an ultrasound probe for 30 sec at 60 W. The resulting water-in-oil (w/o) emulsion was then poured into 40 mL of a PVA aqueous solution (0.1%) and emulsified by sonication for 1 min at 60 W, involving the formation of the w/o/w emulsion. After evaporation of methylene chloride under reduced pressure for 15 min, NP were isolated by centrifugation (Biofuge Stratos, Heraeus Instruments, Germany) at 45,000**g** for 30 min. The supernatant was removed and NP were resuspended in deionized water (3 mL) and stored at 4°C.

Blank NP were prepared in the same way, as well as nanoparticles formulated with blends of biodegradable and non-biodegradable polymers (ratio 1/1).

Drug Entrapment Efficiency

The amount of LMWH entrapped within NP was determined by nephelometry[20] by measuring the amount of non-entrapped drug into the external solution recovered after centrifugation of the colloidal suspension. Aliquots (1 mL) of each aqueous sample were reacted at 37°C for 1 hr with 1 mL of acetate buffer (1 M, pH 5) followed by 4 mL of a cetylpyridinium solution (0.1%) in NaCl 0.94% and assayed in triplicate at 500 nm by spectrophotometry. The drug entrapment efficiency was expressed as the percentage of LMWH entrapped with respect to the theoretical value, whereas the drug loading was presented as the amount of LMWH entrapped per gram of polymer. Since the indirect method that evaluated the amount of non-entrapped LMWH into the aqueous phase has been validated by the direct method after the dissolution of NP in methylene chloride, the drug content was determined indirectly within the aqueous phase, which is an easier and more rapid method.

Particle Mean Diameter and Surface Potential

The mean diameter of NP and their surface potential were evaluated with a Malvern Zetamaster (Malvern Instruments, U.K.) using respectively photon correlation spectroscopy and electrophoretic mobility. The results were all normalized with respect to a polystyrene standard suspension (Malvern Instruments). Each sample was measured in triplicate.

Determination of the Reduced Viscosity of the Organic Polymeric Solutions

The reduced viscosity of the various polymeric solutions was determined with a Ubbelohde viscosimeter (Bioblock Scientific, France) equipped with a glass capillary (∅ 0.56 mm), after dissolution of each polymer (250 mg) in methylene chloride (10 mL).

In Vitro Drug Release

Fifty milligrams of unloaded and LMWH-loaded NP were suspended in 20 mL of saline phosphate buffer (PBS, 0.011 M, NaCl, 0.15 M, pH 7.4) in a flask containing Tween® 80 (0.1%). The suspensions were gently stirred (150 rpm) at 37°C in a water bath. One milliliter of suspension was withdrawn at appropriate intervals and centrifuged at 45,000**g** for 30 min. The supernatant was removed and assayed for LMWH release. The amount of LMWH recovered within the release medium was determined by the nephelometric method described previously. All experiments were performed in triplicate.

A second series of experiments was done including esterase (25 mg, 50 units/mL) in the release medium in order to evaluate the influence of the enzyme on heparin released in vitro from NP. To

compensate for the loss of activity of the enzyme at 37°C, 25 mg of esterase was added to the release medium every 6 hr.

In Vitro Biological Activity of LMWH

The biological activity of LMWH released from NP after 24 hr in PBS at 37°C was evaluated by measuring the anti-factor Xa activity with a chromogenic substrate by use of a standard kit from Diagnostica Stago, according to the method described by the supplier.[21] The assay had a coefficient of variation <5% at a limit of detection of 0.05 IU/mL. Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

Size and Surface Potential

As reported in Table 1, the size of LMWH-loaded nanoparticles prepared with each single polymer or blends of polymers ranged from 240 to 490 nm. The smallest NP were obtained using Eudragit. Furthermore, the highest charged Eudragit, i.e., RL, which carries more quaternary ammonium groups than RS, displayed the smallest mean diameter (blank NP: 187 nm, LMWH-NP: 240 nm). In addition, when Eudragit polymers were used in combination with PCL or PLGA, the mean size of both unloaded and LMWH-loaded NP was smaller than when the two biodegradable polymers were used alone. This is probably a consequence of the inner organic phase viscosity. As shown in Table 2, the lowest reduced viscosity (measured in methylene chloride at the same concentration as for each formulation, 2.5%, w/v) was observed with both Eudragit RS and RL: 0.2 dL/g compared with 0.37 dL/g (PLGA) and 1.18 dL/g (PCL). It is well known that during the emulsification process, the lower the viscosity of the dispersed phase, the smaller the mean diameter. This is confirmed by our results, since the diameter of NP was correlated with the observed data of reduced viscosity. Although the mean diameter was larger after

Table 1

Mean Diameter, Surface Potential, Encapsulation Efficiency, and Drug Loading of Unloaded and Loaded LMWH Nanoparticles Prepared by the w/o/w Emulsion and Solvent Evaporation Method with Biodegradable (PCL and PLGA) and Non-biodegradable (Eudragit RS and RL) Polymers Used Alone or in Combination (Ratio 1/1). Drug-Loaded Nanoparticles Were Formulated with 5000 IU of LMWH. Data Are Expressed as Mean±SD (n = 3)

	Blank Nanoparticles		LMWH-Loaded Nanoparticles			
Polymer	Mean Diameter (nm)	Zeta Potential (mV)	Mean Diameter (nm)	Zeta Potential (mV)	Encapsulation Efficiency (%)	Drug Loading (IU/g Polymer)
PCL	379±68	−3.3±1.9	489±68	−5.8±0.7	16.0±2.6	3207±528
PLGA	339±14	−5.5±0.7	390±53	−9.1±0.3	10.6±3.3	2121±651
RS	225±21	35.7±2.9	301±38	−26.7±0.8	37.9±3.1	7587±620
RL	187±11	52.8±3.8	240±23	−45.7±1.7	56.0±2.3	11207±463
RS/PCL	304±42	32.7±1.0	408±16	−26.8±1.2	31.0±2.3	6203±462
RS/PLGA	346±23	35.5±1.3	294±51	−26.5±0.2	23.3±4.0	4653±803
RS/RL/PLGA	359±17	37.1±0.9	294±45	−44.7±0.4	24.9±2.5	4987±496

Table 2

Reduced Viscosity of Various Polymeric Solutions Obtained from Biodegradable Polymers (PCL and PLGA) and Non-biodegradable Polymers (Eudragit RS and RL) Dissolved in Methylene Chloride (2.5%, w/v) and Measured with a Ubbelohde Viscosimeter Equipped with a Glass Capillary (∅ 0.56 mm)

Polymer	PCL	PLGA	Eudragit RS	Eudragit RL
Reduced viscosity (dL/g)	1.18	0.37	0.23	0.20

LMWH encapsulation (except for two formulations), it did not change the size rank order. The zeta potential data of unloaded NP (Table 1) reflect the charges of the raw polymers. Indeed, the polycationic Eudragits bearing positive charges, conferred by the quaternary ammonium groups, presented the highest potential surface: +55 mV for Eudragit RL which carries 8.8 to 12% of ammonium groups vs. +30 mV for Eudragit RS characterized by 4.5 to 6.8% of positively charged groups. As reported many times, PCL and PLGA, being mostly uncharged polymers, displayed negative zeta potential values, close to neutrality. On the contrary, blends of PCL and PLGA with Eudragit RS and/or RL (ratio 1/1) showed a strong positive potential surface but lower than that obtained with Eudragits used alone, as expected. A dramatic change occurred when LMWH was encapsulated within NP. Indeed, LMWH PCL and PLGA NP exhibited a higher negative zeta potential. However, it was somewhat limited since the absolute difference represented only a few millivolts (2.5 to 4.5 mV). At the opposite end, the change in zeta potential with Eudragit used alone or in combination with PCL and PLGA was dramatically and significantly modified, going from strong positive to strong negative values. This is probably the consequence of a higher incorporation of LMWH, a negatively charged drug bearing sulfate and carboxyle groups bound through ionic interactions onto the positively charged groups of Eudragit. This hypothesis is corroborated by the encapsulation data of LMWH presented in Table 1.

Encapsulation Efficiency

The highest encapsulation ratio, in terms of drug loading and entrapment efficiency, was observed with Eudragit RL and RS (56 and 37.9% respectively), and the lowest with PCL and PLGA NP. Based on preliminary results which showed a very limited release of unfractionated heparin from NP prepared with Eudragit RL,[22] it was decided not to prepare NP of LMWH with this polymer used alone or in combination with PCL and PLGA. The critical influence of the polycationic polymers has been demonstrated, since the drug loading inside NP formulated with blends of Eudragit (RS and RL) with PCL and PLGA was twofold higher with regard to PCL and PLGA used alone. The previous encapsulation results can be explained by interactions between the anionic drug and the uncharged and charged polymers. Indeed, the higher the charge of NP, the higher the entrapment efficiency. At the opposite end, negatively charged NP such as PCL and PLGA NP exhibited a lower entrapment of the anionic drug. Increasing the positive charges of the particles led to a higher encapsulation ratio. At the molecular level, electrostatic interactions occurred between sulfate and carboxyle groups of LMWH and quaternary ammonium groups of the polycationic polymers. This is strengthened by the fact that Eudragit RL NP (i.e., the highest charged polymer) exhibited the highest drug loading owing to a higher surface potential. Moreover, compared with our previous results obtained with unfractionated heparin,[17,22] the encapsulation efficiencies were much lower with LMWH. The latter is obtained from enzymatic degradation or chemical modification of standard heparin and is thereby much smaller and less charged. Although the charge density of LMWH is similar to that of UFH, the overall charge is much lower, probably leading to fewer electrostatic bindings between the drug and the polycationic polymers. In addition, the smaller size and molecular weight of LMWH, as well as its very hydrophilic nature, can lead to an increased diffusion of the drug into the external aqueous phase before the precipitation of the polymer(s) and consequently to a decrease in encapsulation. The low encapsulation efficiencies with PCL (16%) and PLGA (10.6%) may be related to the viscosity of the organic phase (Table 2). Indeed, it can be assumed that an increase in reduced viscosity of the polymeric organic phase slowed down the organic solvent diffusion into the aqueous phase. Consequently, the polymer precipitation occurred more slowly, involving a significant leakage of the drug into the external aqueous phase. Therefore, there are probably two mechanisms that may be useful to describe the incorporation process. The first one corresponding to a low encapsulation efficiency is related to the incorporation of the inner aqueous droplets within the hydrophobic polymeric matrix and may reflect a significant tendency of LMWH to escape to the external aqueous phase. The second mechanism corresponds to ionic interactions between the anionic drug and the positively charged groups of Eudragit polymers. It can therefore be postulated that LMWH was mainly encapsulated inside the core of PCL and PLGA NP, whereas it was adsorbed by electrostatic binding within NP prepared with Eudragit. Isothiocyanate fluorescein (FITC)-labeled LMWH-loaded microparticles pre-

pared according to a similar double emulsion technique (except for the volume of the external aqueous phase and the mixing process) and observed by confocal microscopy (data not shown) confirmed this hypothesis. Indeed, FITC-LMWH was mainly distributed on the outside surface of particles prepared with Eudragit polymers, specifically bound onto the hydrophilic quaternary ammonium groups directed toward the external aqueous phase. On the contrary, FITC-LMWH was mainly located in the core of PCL and PLGA microparticles. Due to their very small size, it was impossible to carry out the same observations with NP.

In Vitro LMWH Release

Figure 1 illustrates the release profiles of LMWH from nanoparticles prepared with (A) PCL used alone or in combination with Eudragit RS and (B) PLGA with or without Eudragits, vs. time up to 24 hr. It can be seen that the highest release percentage was obtained with a single biodegradable polymer, i.e., PCL or PLGA. Whatever the formulation, it is very important to note a low and biphasic release pattern characterized by a burst release of LMWH observed as early as the first sampling time (30 min) and followed by a plateau up to 24 hr. This plateau means that no more subsequent release of LMWH occurred during the following hours. As soon as Eudragit, the polycationic polymer, was added to the formulations, the release (expressed as a percentage) was decreased, as expected and probably owing to strong interactions between the polymers and LMWH which are difficult to disrupt at the pH of the experimental conditions. However, when the amount of LMWH released from NP

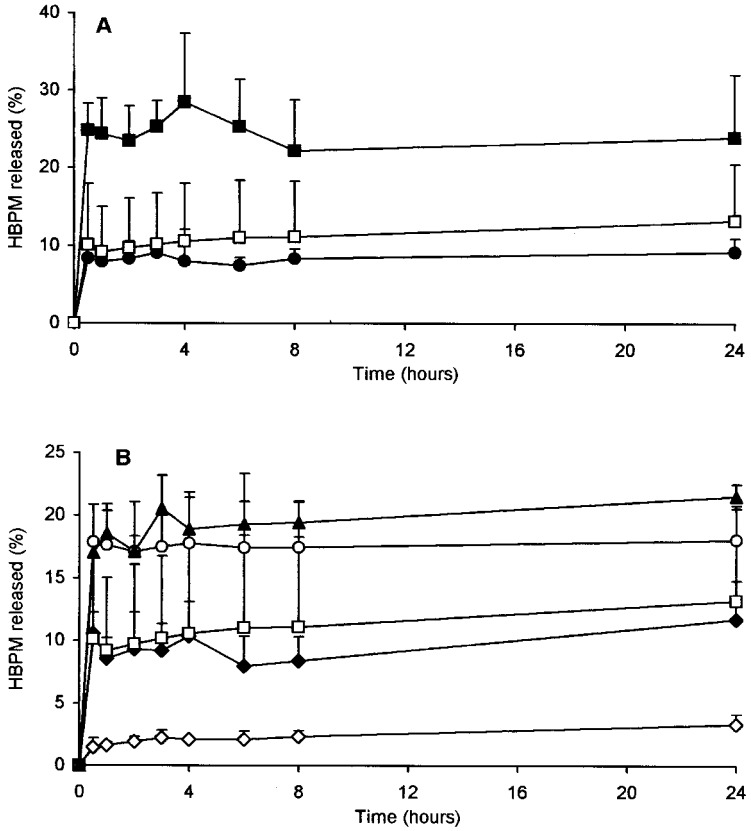

Figure 1. Release profiles of LMWH from polymeric nanoparticles prepared by the double emulsion technique with 5000 IU of LMWH in the internal aqueous phase (1 mL) and biodegradable or non-biodegradable polymers used alone or in combination (250 mg, ratio 1/1). (A) PCL (■), Eudragit RS (□), RS/PCL (●). (B) Eudragit RS (□), Eudragit RL (◇), PLGA (▲), RS/PLGA (◆), RS/RL/PLGA (○). Experiments were performed in phosphate buffer at 37°C and pH 7.4. Data shown as mean ± SD ($n = 3$).

was expressed in IU per gram of polymer, the differences were not significant. This is mainly due to the differences in drug loading, which was higher when Eudragit polymers were used for the preparation of NP. Basically, there were no major differences in the release patterns obtained with biodegradable polymers used alone or in combination with Eudragit RS and RL. The low encapsulation efficiency in PCL (16%) or PLGA (10.6%) NP can explain the partial release of LMWH with a significant burst effect. Indeed, as previously reported by Polakovic et al. with lidocaine nanospheres,[23] the lower the encapsulation ratio, the lower the drug release. Moreover, the authors stated that the intensity of the burst release is mainly influenced by the amount of initial drug content in the NP. The authors mentioned also that in some cases, the drug release seemed to be controlled rather by the crystal dissolution than by the rate of diffusion above a certain drug concentration in the particle matrix. However, below this threshold concentration, the rate of diffusion is the second most important factor leading to an increased burst effect.

Since PCL and PLGA are biodegradable polyesters, and owing to the very low amounts of LMWH released from all formulations, esterase was added to the dissolution medium in order to evaluate its influence on the in vitro LMWH release. As observed in Fig. 2, which displays the release profiles of LMWH from (A) NP formulated with PCL and Eudragit RS used alone or in combination and (B) NP prepared with PLGA, Eudragit RS and RL alone or blended, the addition of esterase increased the amount of LMWH released from each formula-

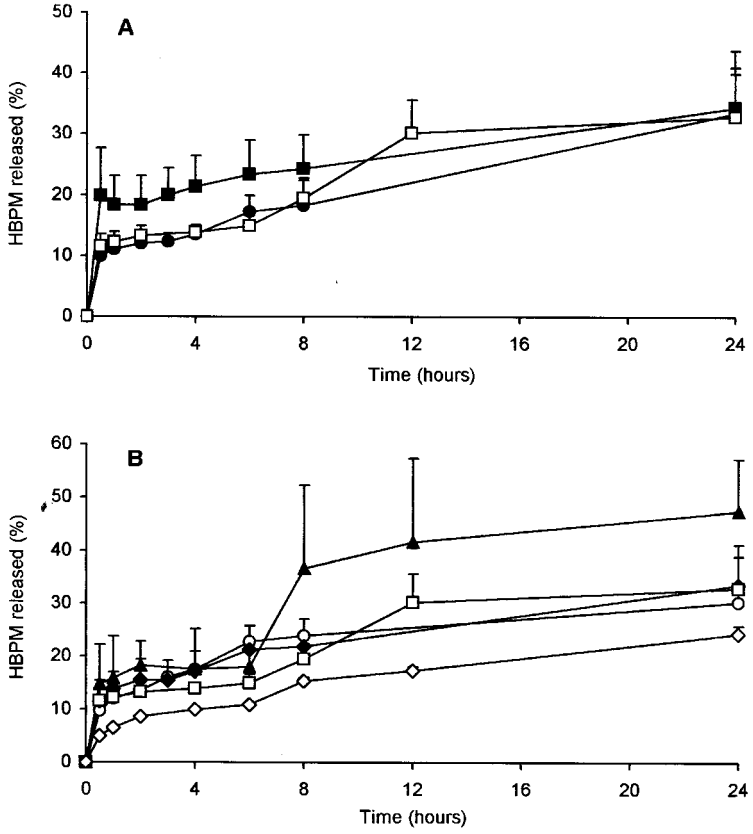

Figure 2. Release profiles of LMWH from polymeric nanoparticles prepared by the double emulsion technique with 5000 IU of LMWH in the internal aqueous phase (1 mL) and biodegradable or non biodegradable polymers used alone or in combination (250 mg, ratio 1/1). (A) PCL (■), Eudragit RS (□), RS/PCL (●). (B): Eudragit RS (□), Eudragit RL (◇)-PLGA (▲), RS/PLGA (◆), RS/RL/PLGA (○). Experiments were performed at 37°C and pH 7.4 in phosphate buffer containing esterase (50 units/mL added every 6 hr). Data shown as mean ± SD ($n = 3$).

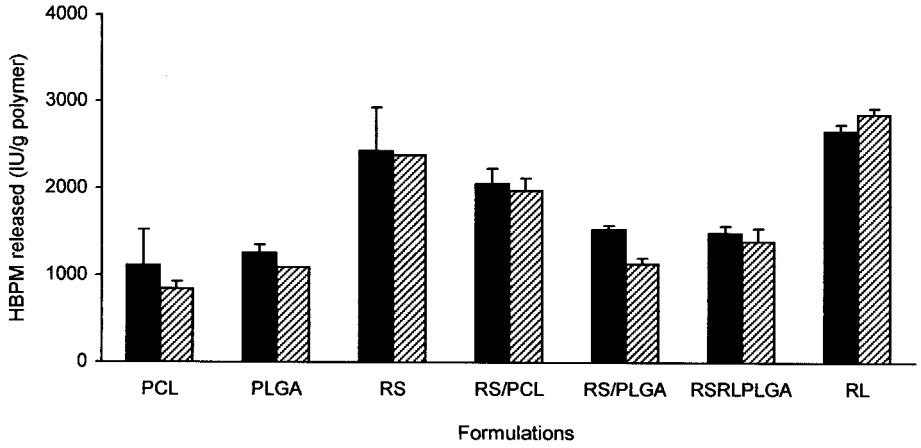

Figure 3. Comparison of the amount of LMWH released after 24 hr from various polymeric nanoparticles suspended in phosphate buffer containing esterase (50 units/mL added every 6 hr) and determined by nephelometry (black) and the biological activity based on the anti-factor Xa activity with a chromogenic substrate (hatched). Data shown as mean ± SD ($n = 3$).

tion (by two- to- threefold), except for PCL NP from which the release did not markedly change. Moreover, as observed without esterase, the release patterns were biphasic. However, the initial burst release seemed less marked, and was followed by a slow and continuous release of the drug up to 24 hr. Indeed, esterase increased the degradation of PCL and PLGA, the two biodegradable polyesters, involving an easier diffusion of LMWH and consequently an increase in its release. In addition, the degradation of the polymers may also be in favor of an increase in water uptake, which may promote the diffusion and release of the drug as well. However, owing to the more hydrophobic nature of PCL, the release of LMWH was almost similar to that obtained without esterase (30% vs. 25%). This may be explained by the semicrystalline state of PCL. In this case, the water uptake was probably reduced, involving a slow degradation for the 24 hr of the in vitro release test. On the contrary, the LMWH release was twofold for PLGA NP (40% vs. 20% without esterase). This may be explained by the less hydrophobic structure of PLGA and its totally amorphous state. Therefore water uptake inside the polymeric matrix, as well as matrix degradation, were favored. Although Eudragit polymers are not biodegradable, the increase in LMWH release in the presence of esterase may result from the breaking of the lateral ester bonds carrying the quaternary ammonium groups onto which the drug was adsorbed.

After the release of LMWH from polymeric NP, it was important to verify whether the biological activity of the drug was preserved. As illustrated in Fig. 3, the results showed that LMWH was unaltered by the encapsulation process and retained its biological activity determined by the anti-factor Xa activity. Furthermore, a good correlation was found between the amount of LMWH released from NP suspended for 24 hr in the dissolution medium containing esterase as determined by nephelometry and that determined by the biological method based on the anti-factor Xa activity.

CONCLUSION

It has been demonstrated that it was possible to manufacture LMWH NP with combination of biodegradable and non-biodegradable positively-charged polymers using the w/o/w double emulsion process with methylene chloride without loss of the biological activity of the drug. These NP were found to be attractive for oral administration, although the in vitro burst release may raise the problem of an early in vivo LMWH release prior to its transport via the intestine. However, previous results obtained with unfractionated heparin did not show any in vitro/in vivo correlation, since a signifi-

cant biological activity of heparin based on clotting time and anti-factor Xa activity was found after oral administration of polymeric micro- and nanoparticles to rabbits.[18,22] Further in vivo studies are now in progress to evaluate the potential of oral absorption of LMWH NP.

REFERENCES

1. Hirsh, J.; Warkentin, T.E.; Raschke, R.; Granger, C.; Ohman, E.M.; Dalen, J.E. Mechanisms of Action, Pharmacokinetics, Dosing Considerations, Monitoring, Efficacy and Safety. Chest **1998**, *114*, 489S–510S.
2. Carter, C.J.; Kelton, J.G.; Hirsh, J.; Cerskus, A.; Santos, A.V.; Gent, M. The Relationship Between the Hemorrhagic and Antithrombotic Properties of Low Molecular Weight Heparin in Rabbits. Blood **1982**, *59*, 1239–1245.
3. Sobel, M.; McNeill, P.M.; Carlson, P.L.; Kermode, J.C.; Adelman, B.; Conroy, R.; Marques, D. Heparin Inhibition of von Willebrand Factor-Dependent Platelet Function In Vitro and In Vivo. J. Clin. Invest. **1991**, *87*, 1787–1793.
4. Handeland, G.F.; Abildgaard, U.; Holm, H.A.; Arnesen, K.E. Dose Adjusted Heparin Treatment of Deep Venous Thrombosis: A Comparison of Unfractionated and Low Molecular Weight Heparin. Eur. J. Clin. Pharmacol. **1990**, *39*, 107–112.
5. Brzu, T.; Molho, P.; Tobelem, G.; Petitou, M.; Caen, J. Binding and Endocytosis of Heparin by Human Endothelial Cells in Culture. Biochim. Biophys. Acta **1985**, *845*, 196–203.
6. Ginsberg, J.S.; Kowalchuck, G.; Hirsh, J.; Brill-Edwards, P.; Burrows, R.; Coates, G.; Webber, C. Heparin Effect on Bone Density. Thromb. Haemost. **1990**, *64*, 286–289.
7. Morton, A.K.; Edwards, H.E.; Allen, J.C.; Phillips, G.O. An Evaluation of the Oral Administration of Commercial and Fractionated Heparin Samples in Rats. Int. J. Pharm. **1981**, *9*, 321–325.
8. Dal Pozzo, A.; Acquasaliente, M.; Geron, M.R. New Heparin Complexes Active by Intestinal Absorption. I. Multiple Ion Pairs with Basic Organic Compounds. Thromb. Res. **1989**, *56*, 119–124.
9. Tidball, C.S.; Lipman, R.I. Enhancement of Jejunal Absorption of Heparinoid Sodium Ethylenediaminetetraacetate in the Dog. Proc. Soc. Exp. Biol. Med. **1962**, *111*, 713–715.
10. Guarini, S.; Ferrari, W. Olive Oil-Provoked Bile Dependent Absorption of Heparin from the Gastrointestinal Tract in Rats. Pharm. Res. Commun. **1985**, *17*, 685–694.
11. Ueno, M.; Nakasaki, T.; Horikoshi, I.; Sakuragawa, N. Oral Administration of Liposomally-Entrapped Heparin to Beagle Dogs. Chem. Pharm. Bull. **1982**, *30*, 2245–2247.
12. Steiner, S.; Rosen, R. Delivery System for Pharmacological Agents Encapsulated with Proteinoids. U.S. Patent 4,925,673, 15 May 1990.
13. Leone-Bay, A.; Paton, D.; Variano, B.; Leipold, H.; Rivera, T.; Miura-Fraboni, J.; Baughman, R.A.; Santiago, N.J. Acylated Non-α-amino Acids as Novel Agents for the Oral Delivery of Heparin Sodium, USP. Control. Rel. **1998**, *50*, 41–49.
14. Gonze, M.D.; Salartash, K.; Sternbergh, W.C.; Baughman, R.A.; Leone-Bay, A.; Money, S.R. Orally Administered Unfractionated Heparin with Carrier Agent Is Therapeutic for Deep Vein Thrombosis. Circulation **2000**, *101*, 2658–2661.
15. Baughman, R.A.; Kapoor, S.C.; Agarwal, R.K.; Kisicki, J.; Catella-Lawson, F.; FitzGerald, G.A. Oral Delivery of Anticoagulant Doses of Heparin, a Randomized, Double Blind Controlled Study in Humans. Circulation **1998**, *98*, 1610–1615.
16. Money, S.R.; York, J.W. Development of Oral Heparin Therapy for Prophylaxis and Treatment of Deep Venous Thrombosis. Cardiovasc. Surg. **2001**, *9* (3), 211–218.
17. Jiao, Y.; Ubrich, N.; Marchand-Arvier, M.; Vigneron, C.; Hoffman, M.; Maincent, P. Preparation and In Vitro Evaluation of Heparin-Loaded Polymeric Nanoparticles. Drug Del. **2001**, *8*, 135–141.
18. Jiao, Y.; Ubrich, N.; Marchand-Arvier, M.; Vigneron, C.; Hoffman, M.; Lecompte, T.; Maincent, P. In Vitro and In Vivo Evaluation of Oral Heparin-Loaded Polymeric Nanoparticles in Rabbits. Circulation **2002**, *105*, 230–235.
19. Alex, R.; Bodmeier, R. Encapsulation of Water-Soluble Drugs by a Modified Solvent Evaporation Method. I. Effect of Process and Formulation Variables on Drug Entrapment. J. Microencaps. **1989**, *7*, 347–355.
20. Ardry, M. Dosage de l'Héparine: Étude Critique. Bull. Ordre **1967**, *135*, 699–708.
21. Teien, A.N.; Lie, M. Evaluation of an Amidolytic Heparin Assay Method: Increased Sensitivity by Adding Purified Antithrombin III. Thromb. Res. **1977**, *10*, 399–410.
22. Jiao, Y.; Ubrich, N.; Hoffart, V.; Marchand-Arvier, M.; Vigneron, C.; Hoffman, M.; Maincent, P. Preparation and Characterization of Heparin-Loaded Polymeric Microparticles. Drug Dev. Ind. Pharm. **2002**, *28*(8), 1033–1041.
23. Polakovic, M.; Görner, T.; Gref, R.; Dellacherie, E. Lidocaine Loaded Biodegradable Nanospheres. II. Modelling of Drug Release. J. Contr. Rel. **1999**, *60*, 169–177.